Hormokines:
A novel concept of plasticity in Neuro-Endo-Immunology

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1. ABBREVIATIONS

ADM:	adrenomedullin
BH4:	tetrahydrobiopterin
CGRP:	calcitonin gene related peptide
CH:	cycloheximide
CT:	calcitonin
CR:	CT receptor
CRLR:	CT receptor like receptor
GH:	growth hormone
GTPCH:	guanosine triphosphate cyclohydrolase
Isl-1:	Islet-1
IFNγ:	interferon gamma
IL-1β:	interleukin-1 beta
IL-6:	interleukin-6
LPS:	lipopolysaccharide, endotoxin
MISRE:	microbial infection-specific response-elements
MSC:	mesenchymal stem cells
NO:	nitric oxide
PPARγ:	peroxisome proliferator activated receptor-γ
RAMP:	receptor activity-modifying protein
PBMC:	peripheral blood mononuclear cells
ProCT:	procalcitonin
SIRS:	systemis inflammatory response synsrome
SRIF:	somatostatin
SSTR:	somatostatin receptor
TNFα:	tumor necrosis factor alpha
TZD:	thiazolidinediones

2. PREFACE

This thesis is based on the following publications. Asterics (*) indicate equal contributions by the authors.

I. ***In vitro* and *in vivo* calcitonin-I gene expression in parenchymal cells: a novel product of human adipose tissue**
Linscheid P, Seboek D, Nylen ES, Langer I, Schlatter M, Keller U, Becker KL, Muller B 2003 Endocrinology 144(12):5578-5584

II. **Expression and secretion of procalcitonin and calcitonin gene-related peptide by adherent monocytes and by macrophage-activated adipocytes**
Linscheid P, Seboek D, Schaer JD, Zulewski H, Keller U, Müller B 2004 Crit Care Med 32(8):1715-1721

III. **Autocrine/paracrine role of inflammation-mediated CGRP and ADM expression in human adipose tissue**
Linscheid P*, Seboek D*, Zulewski H, Keller U and Müller B 2005 Endocrinology 146(6):2699-2708

IV. **Somatostatin is expressed and secreted by human adipose tissue upon infection and inflammation**
Seboek D, Linscheid P, Zulewski H, Langer I, Christ-Crain M, Keller U, Muller B 2004 J Clin Endocrin Metab 89(10): 4833-4839

V. **Cytokine-induced metabolic effects in human adipocytes are independent of endogenous nitric oxide**
Linscheid P, Seboek D, Zulewski H, Scherberich A, Blau N, Keller U and Müller B 2005 Am J Physiol Endocrinol Metab- in press

VI. **Human bone marrow-derived mesenchymal stem cells differentiate into insulin, somatostatin and glucagon expressing cells**
Seboek D, Timper K, Eberhardt M, Linscheid P, Keller U, Müller B and Zulewski H (submitted)

VII. **Human adipose tissue-derived mesenchymal stem cells differentiate into insulin, somatostatin, and glucagon expressing cells**
Timper K*, Seboek D*, Eberhardt M, Linscheid P, Keller U, Müller B and Zulewski H 2006 Biochem Biophys Res Commun 341(4):1135-40

VIII. **Lentiviral vectors efficiently transduce human mesenchymal stem cell- and preadipocyte derived mature adipocytes**
Seboek D, Linscheid P, Firm C, Zulewski H, Salmon P, Russo AF, Keller U, Müller B (submitted)

3. SUMMARY

Hormones are produced by endocrine and neuroendocrine cells and mediate mainly systemic effects. Cytokines are produced by numerous cell types and mediate local effects. The production of calcitonin (CT) peptides follows either the classical hormonal expression which is believed important for calcium metabolism or cytokine-like expression which is induced by inflammatory stimuli. To describe this plasticity, the term “hormokine” was proposed.

The concept is based on the discovery of the ubiquitous expression of CT peptides (i.e., ProCT, CT gene-related peptide (CGRP) and adrenomedullin (ADM)) during sepsis. Using human mesenchymal stem cell (MSC) - and preadipocyte- derived mature adipocytes it was shown in this thesis study that different CT peptides are differentially regulated upon inflammatory stimuli and mediate distinct metabolic effects. Especially ProCT appeared to be a pivotal mediator during sepsis. Based on the findings, a trimodal expression pattern of the CALC I gene and a closely related biphasic behavior of infection-related ProCT secretion was postulated. The exact mode of actions of hormokines in the context of inflammation and infection remains enigmatic. Based on the structural homologies, different CT peptides have overlapping bioactivities, which they exert by binding to the same family of receptors. For example CGRP and ADM contribute to vasoregulation in inflammatory conditions and were found to modulate their own expression and bioactivities.

In a second phase of the thesis, the concept of hormokines was extended to the hypothesis, that other hormones can also be regulated and act as hormokines. In this context, an upregulation of somatostatin (SRIF) upon inflammatory stimulation was observed in human adipose tissue.

Because of the hormokine like behavior of SRIF, also an islet hormone, the idea arose that other islet hormones (e.g. insulin, glucagon) could be expressed ectopically. In this context, the plasticity of the neuro-endocrine phenotype was documented by experiments indicating that progenitor cells can adopt an islet-like phenotype upon differentiation and stimulation. Bone marrow derived MSCs and human adipose tissue derived preadipocytes were shown to be able to form islet-like clusters within days and recapitulate the sequential expression of key genes observed during endocrine pancreatic development.

To better investigate hormones and hormokines a procedure to transduce mature human adipocytes was established using lentiviral vectors.

4. BACKGROUND

a) The Calcitonin Peptides

Calcitonin (CT) was discovered 40 years ago, when it was assumed to be a hormone with a yet-to-be-determined role in human physiology (1). Since then, CT has been found to be only one entity among related circulating peptides which have pivotal roles in the metabolic and inflammatory host response to microbial infections (2). These peptides share marked structural homologies and include procalcitonin (ProCT), calcitonin gene-related peptide (CGRP) I and II, adrenomedullin (ADM), and amylin (Figure 1 and Figure 2)

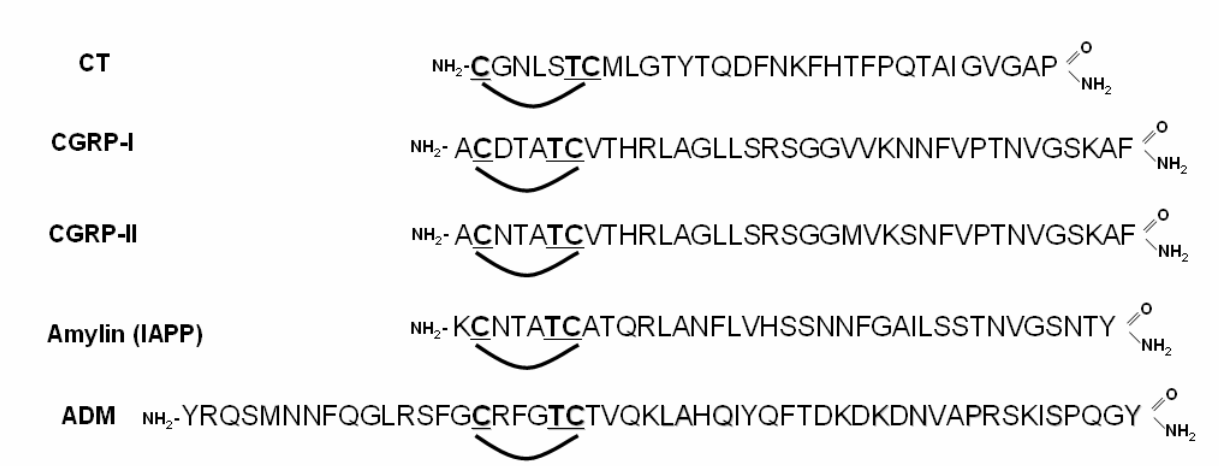

Figure 1: Amino acid sequences of human CT, CGRP I and II, amylin and ADM. The prohormone, ProCT, consists of 116 amino acids, in which the midportion consists of the 33-amino acid immature CT. Amylin is also referred to as islet amyloid polypeptide. The common marked amino acid homology within the calcitonin peptide superfamily suggests gene duplication of a common ancestral gene.

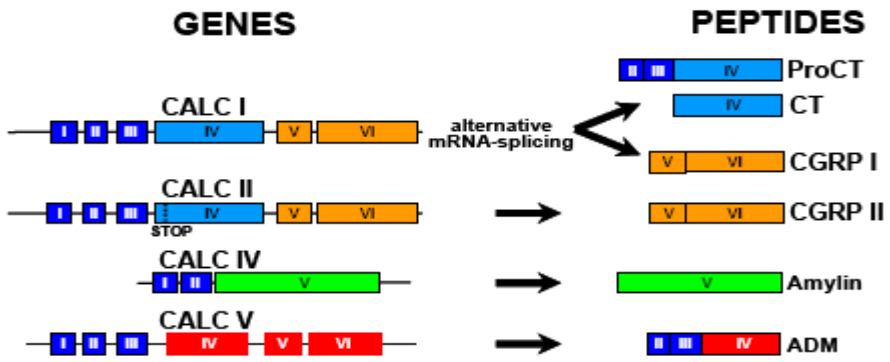

Figure 2: CALC genes and the CT peptides. The CALC I gene encodes three distinct peptides: ProCT, CT and CGRP I. ProCT and CT can not be expressed from the CALC II gene due to a stop codon in exon 4. CALC III (not shown) is a non-translated pseudogene. CALC IV encodes the peptide amylin. CALC V gene encodes ADM.

b) CT Peptides are Ubiquitously Expressed in Sepsis

The CALC I gene, by alternative processing of the primary RNA transcript, gives rise to two different so-called mature peptides: CT and CGRP I. In the absence of infection, the extra-thyroidal transcription of the CALC I gene is suppressed and is restricted to a selective expression in neuroendocrine cells found mainly in the thyroid and the lung. In these neuroendocrine cells, the mature CT-hormone is synthesized, stored in secretory granules and released after appropriate stimulation, such as hypercalcemia or pentagastrin (3).

Previous studies demonstrated that the inflammatory host response during a bacterial infection induces an ubiquitous increase of CT mRNA expression and immediate release of ProCT, the precursor of CT, from all tissues and cell types throughout the body (4). Thus, in sepsis, the entire body could be viewed as being an "endocrine gland". Interestingly, CGRP mRNA was also shown to be ubiquitously expressed upon bacterial infection (5). Hence, the presence of microbial infection-specific response-elements (MISRE) in the CALC I gene promoter were proposed, which, upon an inflammatory stimulus, could override the endocrine tissue-selective expression pattern (Figure 3).

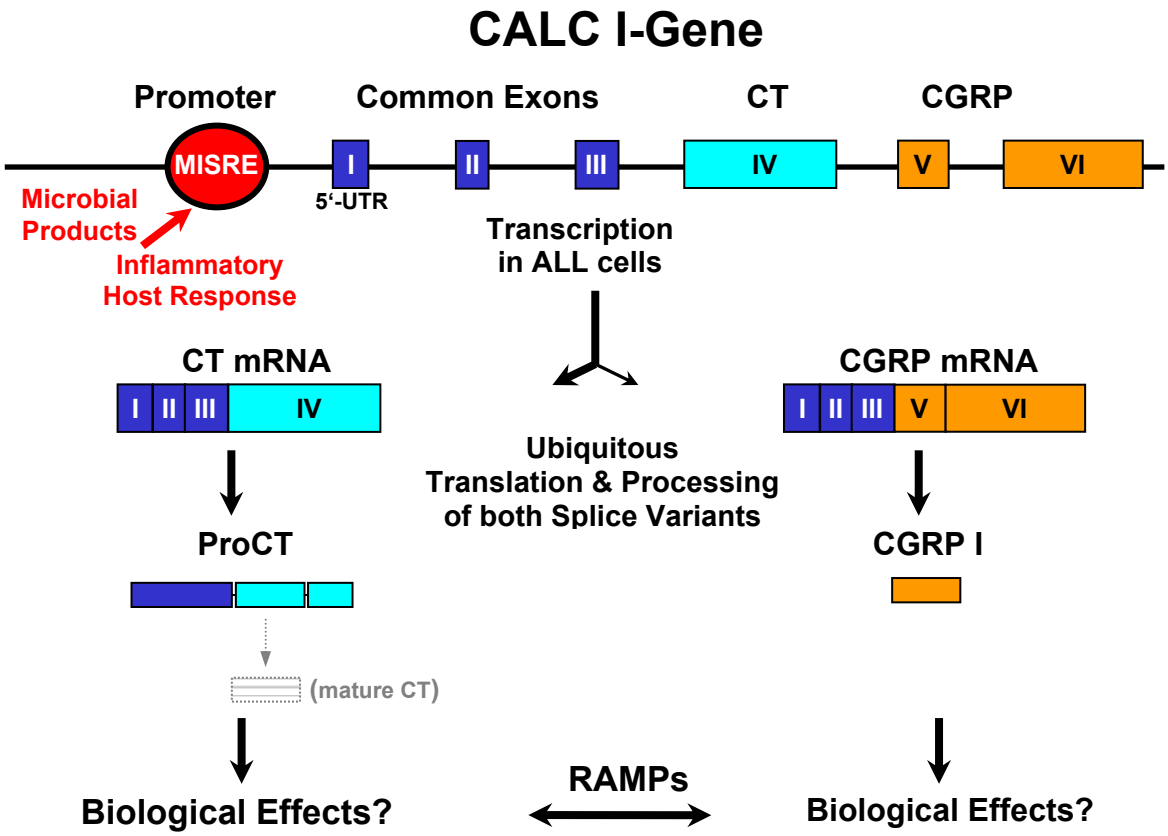

Figure 3: Model for the ubiquitous CALC I gene expression. Upon a stimulus released during the inflammatory host response, the CALC I gene expression is upregulated in all tissues, mediated through one or several MISRE within the promoter. Both splice variants are expressed in all cells: ProCT increases several thousand-fold and circulating CGRP levels are modestly elevated. Serum levels of mature CT are not increased. The CT peptides exert their bioeffects by binding to the same family of receptors, which can be modulated additionally by the actions of accessory proteins like receptor activity-modifying proteins (RAMPs).

It is of pertinence that CGRP II, amylin, and ADM are encoded on the CALC II, IV, and V genes, respectively. CGRP I, CGRP II and ADM, considered as auto- and/or paracrine factors in many tissues are very potent vasodilatory peptides. Interestingly, it was found that in sepsis, mRNAs for CGRP I, CGRP II and ADM also appear to be ubiquitously expressed (6). Accordingly, in humans, circulating levels of CGRPs and ADM have been found to be elevated during bacterial infections (7, 8). In sepsis, a competition arises between organs for reduced systemic blood pressure and blood

flow. Thus, in a teleologic perspective, due to their vasodilatory properties, the tissue-wide production of CGRPs and ADM assures blood supply to the individual tissues and thereby may oppose vasoconstrictory effects of other stress hormones released during sepsis (e.g. catecholamines, cortisol). The importance of ADM in the cardiovascular system has been demonstrated in ADM knock-out mice. ADM knock-out mice die at midgestation with severe cardiovascular abnormalities (9).

Amylin, the fifth member of the CT superfamily is expressed and co-secreted with insulin from the pancreatic beta cells. Insular deposits of amylin are characteristic in type 2 diabetes mellitus; hence the initial nomenclature of amylin as “islet amyloid polypeptide”. Interestingly, in sepsis the expression of amylin remains restricted to human islets and circulating levels have not been found in sepsis (6).

c) ProCT: a Pivotal Marker and Mediator in Sepsis and other Inflammatory Conditions

In bacterial infections, circulating levels of ProCT increase several-thousand-fold. In sepsis, the increase of ProCT levels correlates with the severity and mortality. ProCT has a superior diagnostic accuracy for the diagnose of sepsis as compared to other markers of inflammation (e.g., interleukin-6 (IL-6) or C-reactive protein (CRP)) (10-12). Administration of endotoxin to healthy human volunteers increased serum ProCT levels seen in sepsis and this increase persisted up to seven days (13). Several pro-inflammatory mediators have been shown to induce ProCT production *in vivo*, e.g. TNF-α, IL-2 and IL-6 (14). Whatever the initiating provocative insult may be, severe systemic inflammation *per se* may manifest increased serum levels of ProCT. In addition to the elevated ProCT levels observed during bacterial infections, increases of ProCT also occured in chemical pneumonitis (14), in burns (15, 16), in heat stroke (17), in mechanical trauma (18), and following surgery (19).

Several properties of ProCT favour this molecule as a therapeutic target in sepsis. In contrast to the transiently increased classical cytokines, for which immunoneutralization trials in humans have shown disappointing results, the massive increase of circulating ProCT persists for several days (13). ProCT is nearly always increased in overt sepsis; its onset is early (within 3 hrs), it is a stable peptide in serum samples and it can be easily measured. The excellent diagnostic accuracy should greatly improve patient selection for any study of ultimate therapeutic efficacy,

i.e., of ProCT immunoneutralization in humans. Importantly, administration of ProCT to septic hamsters with peritonitis doubled their death rate. Conversely, treatment with ProCT-reactive antiserum increased the survival in septic hamsters and pigs (20, 21). Recent experiments have demonstrated that such immunoneutralization is effective even when the ProCT- reactive antiserum is administered after the pigs became moribund (22). In addition, the biological activity of ProCT also appears to depend on the inflammatory status of the organism: in the aforementioned hamster model, only the injection of human ProCT to septic animals worsened the outcome (20), while in healthy animals the administration of similar doses of ProCT did not show any detrimental effects. Thus, the toxic effects of ProCT are restricted to an inflamed organism.

d) Receptors for CT Peptides: A Functional Entity

Based on the structural homologies, different CT peptides have overlapping bioactivities, which they exert by binding to the same family of receptors (3). There are two subgroups of these G protein-coupled receptors with seven transmembrane domains: CT receptors (CR) and CT receptor-like receptors (CRLR).

Three accessory proteins, which are called receptor-activity-modifying proteins (RAMPs 1-3), act upon these receptors, thereby altering their specific responsiveness and ligand affinity. The binding affinities of the different CT peptides have been demonstrated to be modulated by the three RAMPs (Table 1). These observations appear to correlate with the diverse physiologic effects of the individual CT peptides (23). For example, association of CRLR with RAMP 1 on the cell surface results in a CGRP I or II responsive cell. The co-expression of RAMP 2 with CRLR on the cell surface results in an ADM responsiveness of the cells (Figure 4). RAMP-assisted modulation of the ligand specificity of CR and CRLR is an exciting new principle; however, its exact physiological role remains to be determined. It is tempting to speculate that the extraordinary increase of circulating ProCT in sepsis prevents CGRP and ADM from exerting their actions, and thus acts as a competitive antagonist. Therefore, an outline of the pathogenic factors of the systemic inflammatory response with special emphasis on the role of ProCT can be investigated.

Table 1: RAMPs define ligand specificity of CR/CRLR

2 Receptors	*3 RAMPs*	*4 Ligands*
CR	none	**CT**
	+ RAMP-**1**	**CGRP I or –II, Amylin** >> CT
	+ RAMP-**2**	**CT** >> CGRP I or -II, Amylin
	+ RAMP-**3**	**Amylin** >> CT / CGRP I and -II
CRLR	+ RAMP-**1**	**CGRP I and -II**
	+ RAMP-**2**	**ADM**
	+ RAMP-**3**	**ADM**

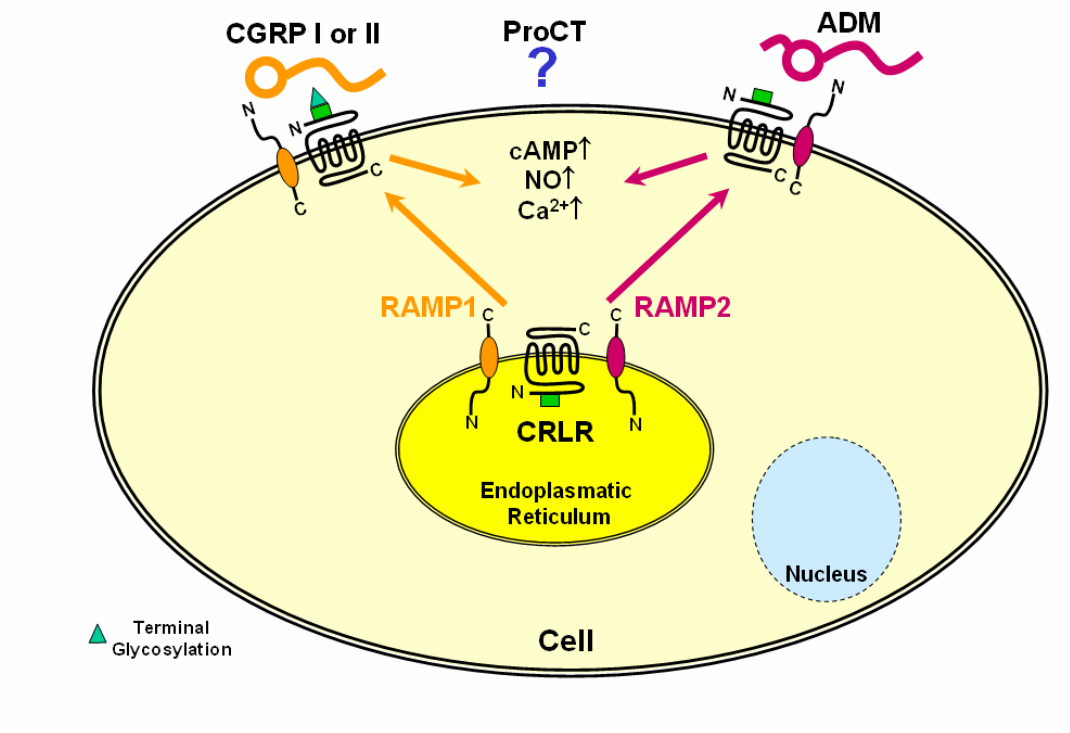

Figure 4: The role of RAMP 1 and 2 and CRLR in generating selective CGRP and ADM receptors. The vasodilatory actions of CGRP and ADM are brought about through two signaling mechanisms, direct cAMP mediated relaxation of vascular smooth muscle cells and the stimulation of phospholipase C in endothelial cells, resulting in an increase of $[Ca^{2+}]i$ and NO/cGMP-mediated vasodilatation.

e) Nitric Oxide (NO) in Inflammation: Relation with CT Peptides

Severe illness induces a uniform systemic inflammatory response syndrome (SIRS) independently of an infection. SIRS is clinically defined by white blood count higher than 12,000 or lower than 4,000 cells/µl; a heart rate higher than 90 beats/min; a respiratory rate higher than 20 breaths/min; and a body temperature higher than 38 ºC or lower than 36 ºC. When SIRS is present, and infection is proven or suspected and the term sepsis is used. Metabolically, SIRS, with or without infection, is characterized by a catabolic state, with insulin resistance as a key feature.

Insulin resistance, with or without hyperglycemia, is common in critically ill patients, even in those without preexisting diabetes (24). Hyperglycemia or relative insulin deficiency (or both) during critical illness, and especially sepsis, confer a predisposition to complications, and death (25, 26). *In vitro*, the responsiveness of leukocytes stimulated by inflammatory mediators is inversely correlated with glycemic control (27). Importantly, aggressive insulin infusion therapy to counter the insulin resistance and improve glycemic control reduced mortality by 50% in surgical patients requiring prolonged intensive care (28). Notably, this beneficial effect occurred regardless of whether there was a history of diabetes or hyperglycemia, and the greatest reduction of mortality was found in septic patients.

During sepsis, large amounts of NO are produced (29, 30). NO is an important second messenger molecule implicated in a wide variety of physiological functions, including vascular relaxation, cytotoxicity and immune response (31). The conversion of L-arginine to L-citrulline and NO is catalyzed by enzymes of the nitric oxide synthase (NOS) family. The mandatory cofactor tetrahydrobiopterin (BH4) is rate-liming on NO synthesis in most cell types. Its synthesis is co-induced to inducible NOS (iNOS) by enhanced expression of guanosine triphosphate cyclohydrolase (GTPCH). BH4 was proposed as a possible regulator of iNOS mRNA stability. Two of the three known NOS isoforms, the neuronal and endothelial NOS (nNOS and eNOS) are Ca^{2+}-dependent. The inducible, Ca^{2+}-independent iNOS is implicated in host defense and vasodilation. iNOS is expressed in numerous cell types including macrophages, liver, vascular smooth muscle and adipose tissue following induction by cytokines or bacterial lipopolysacharide (LPS). Interestingly, targeted disruption of iNOS protects against obesity-linked insulin resistance in mouse muscle (32). Exaggerated NO production following iNOS induction causes insulin resistance by interfering with insulin action on glucose transport into muscle cells (33). In contrast,

a number of studies, mostly performed with muscle cells, suggest that NO contributes to insulin-mediated glucose uptake (34, 35). The role of NO in adipose tissue mediated insulin resistance in humans is largely unknown.

Several CT peptides have been found to induce insulin resistance, to decrease peripheral glucose clearance, and to increase hepatic glucose output (36, 37). CGRPs and ADM reduced the glucose-stimulated insulin secretion in the pancreas (38). Early reports indicated that both human pancreatic amylin and rat CGRP I are potent inhibitors of both basal and insulin-stimulated glucose uptake and glycogen synthesis in the skeletal muscle and in liver (36, 39). Furthermore, amylin is known to inhibit stimulated insulin secretion through a paracrine effect in islet cells (38). Hence, it is tempting to speculate that in inflammatory conditions CT peptides could modulate this insulin resistance.

Both CGRP and ADM have anti-inflammatory, metabolic and vascular actions which are beneficial in inflammation and sepsis (3). The vasodilatory actions of CGRP and ADM are mostly the result of NO-mediated relaxation of vascular smooth muscle cells (30, 40, 41). Conversely, the endotoxin triggered release of CGRP is NO- and prostaglandin-dependent (42). Thus, NO mediates both the production and the action of CT peptides. Importantly, recombinant human ProCT is a potent suppressor of cytokine-induced iNOS expression and NO synthesis in rat vascular smooth muscle cells in a dose-dependent manner, with a maximal effect at ProCT levels of 100ng/ml (43). However, at the highest concentration utilized (5000ng/ml), ProCT did not inhibit iNOS expression or NO production. Furthermore, ProCT had no effect on unstimulated cells. It was hypothesized that in inflammatory conditions, and especially in sepsis, CT peptides modulate insulin resistance in a NO-dependent manner.

f) Somatostatin and its receptors

Somatostatin (SRIF) was initially described as a secreted product of the hypothalamus acting as a potent inhibitor of growth hormone (GH) secretion (44). Subsequently, high densities of SRIF-producing neuroendocrine cells have been localized throughout the central and peripheral nervous systems, in the endocrine pancreas and in the gut, and to a lower extent in the thyroid, adrenals, submandibular glands, kidneys, prostate and placenta (45-47). SRIF expression and

secretion was also described in murine inflammatory and immune cells upon activation (45). In the gastrointestinal tract SRIF inhibits the secretion of numerous peptide hormones (insulin, glucagon, gastrin and cholecystokinin), gastric emptying, gallbladder contraction and exocrine gut secretion (46, 48). Induction of SRIF by inflammatory cytokines IL-1β, TNF-α and IL-6 was demonstrated *in vitro* in rat diencephalic cells (46, 49, 50). Increased SRIF-mRNA expression has been described in murine macrophage cell lines upon cytokine stimulation (51). Accordingly, increased plasma SRIF levels were measured in jugular and portal veins in endotoxin-injected sheep and in septic pigs (52, 53). Five distinct receptors mediating SRIF activity are widely expressed in many tissues. As a neurotransmitter, SRIF inhibits the release of GH, dopamine, norepinephrine, thyreotropin-releasing-hormone (TRH) and corticotropin-releasing-hormone (CRH). Further modulatory roles have been ascribed to SRIF in inflammatory conditions and lymphocyte function (54-56).

g) Adipose Tissue

Adipose tissue is capable of producing hormones, cytokines and other proteins involved in inflammation and insulin resistance and thereby rendering it the body's largest "endocrine organ". Obesity is a major reason for insulin resistance (57). The prevalence of obesity is increasing worldwide, not only in industrialized but also in developing countries. The obesity related, so-called "metabolic syndrome" with its cluster of disorders such as diabetes, dyslipidemia and arterial hypertension, leads to an array of inflammatory complications related to atherosclerosis and characterized by a high morbidity and mortality. Importantly, critically ill obese patients have been found to be at increased risk of morbidity and mortality compared to the non-obese patients. Overall mortality was 30% for the morbidly obese patients and 17% for the non-obese group, mostly due to pulmonary infections and sepsis (58). It was previously reported that with the exception of amylin, all members of the CT peptide superfamily were expressed on the mRNA level in adipose tissue *in vivo* during the systemic inflammatory host response to an infection in an animal model (4, 6). Improvement of the understanding of the physiological function of adipocytes is a prerequisite for understanding the molecular mechanisms that underpin these diseases (59).

5. AIM OF THE THESIS

The aim of the thesis was:

- to study the hormokine properties of ProCT in humans with emphasis on its gene expression regulation and physiological effects
- to establish if the other members of the CALC gene family display hormokine properties in humans
- to investigate if other classical hormones display hormokine properties
- to unravel if the hormokine phenomenon extends to a cell biological mechanism of tissue plasticity that provides tissues with the capacity to adapt and respond to physiological perturbations (e.g. inflammation)

These aims were primarily investigated using a multi-disciplinary approach that combined a novel human adipocyte cell culture model, with gene expression profiling upon inflammatory stimuli, gene expression profiling following treatment with CT peptides to delinaete paracrine properties, and biochemical assays to underpin the funtional roles of hormokines in sepsis.

6. MAIN FINDINGS AND CONCLUSION

Here is presented a summary of the major findings and conclusions of the thesis study. The results are based on the papers presented in the subsequent chapters.

The hormokine properties of ProCT were initially built on findings with rodents subjected to sepsis (4). Since then, observations from patients with sepsis indicated that the hormokine behavior of ProCT is a common principle that extend from rodents to humans (60). A novel cell culture system utilising human adipocytes was developed to allow detailed studies of the molecular mechanisms controlling the expression of ProCT and other CT peptides, and their biological activities during sepsis.

In human adipocyte primary cultures and in adipose tissue samples from infected and non-infected patients with different levels of serum ProCT inflammation-mediated CALC I gene expression was analyzed. In *ex vivo* differentiated adipocytes, expression of CT mRNA increased 24-fold ($p<0.05$) by the administration of LPS and 37-fold ($p<0.05$) by interleukin-1β (IL-1β) after 6 hours. ProCT secretion into culture supernatant increased 13.5-fold ($p<0.01$) with LPS treatment and 15.2-fold ($p<0.01$) by IL-1β after 48 hours. In co-culture experiments, adipocyte CT mRNA expression was evoked by E. coli activated macrophages, in which CT mRNA was undetectable. The marked IL-1β-mediated-ProCT release was inhibited by 89% during co-administration with IFNγ. In patients with infection and markedly increased serum ProCT, CT mRNA was detected in adipose tissue biopsies. Hence, the experiments carried out to answer the hypothesis lead to the conclusion that ProCT, which is suspected to mediate deleterious effects in sepsis and inflammation, is a novel product of adipose tissue secretion. The inhibiting effect of IFNγ on IL-1β-induced CT mRNA expression and on ProCT secretion might explain previous observations that serum ProCT concentrations increase less in systemic viral as compared to bacterial infections. Figure 5 presents a schematic diagram of the different expression pattern of ProCT as a hormokine and CT as classical hormone. *(Linscheid P, Seboek D, Nylen ES, Langer I, Schlatter M, Keller U, Becker KL, Muller B 2003: In vitro and in vivo calcitonin-I gene expression in parenchymal cells: a novel product of human adipose tissue Endocrinology 144(12):5578-5584)*

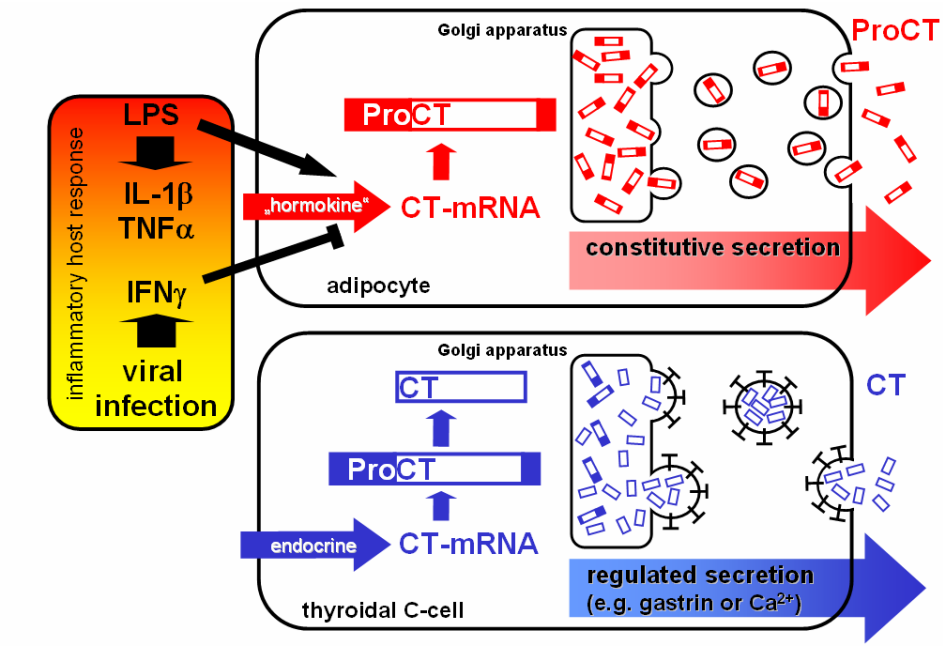

Figure 5: Schematic diagram of CALC I expression in adipocytes as hormokines and thyroidal C cells as hormones. In the classical neuroendocrine paradigm, the expression of CT mRNA is restricted to neuroendocrine cells, mainly C cells of the thyroid. Initially, the 116-amino acid prohormone ProCT is synthesized and subsequently processed to the considerably smaller mature CT. In sepsis and inflammation, proinflammatory mediators induce CT mRNA. In contrast to thyroidal cells, adipocytes lack secretory granules, and hence, unprocessed ProCT is released in a nonregulated, constitutive manner.

These observations led to the hypothesis, that ProCT guidance could limit antibiotic overuse in mostly viral acute respiratory tract infections (61). Clinical investigations showed, that ProCT guided treatment reduced the use of antibiotics, without jeopardazing clinical outcome (60). Knowing the clinical usefulness of ProCT, the interest in understanding the regulation and biological role grew. Therefore, we investigated the crosstalk of classical immune cells (i.e., monocytes and macrophages) with adipocytes during inflammation and bacterial infection *in vitro* (62). Interestingly, no CT mRNA was found in leukocytes from septic patients and in peripheral blood mononuclear cell (PBMC) -derived macrophages after incubation with *E. coli,* LPS, IL-1β or TNFα. Conversely, in co-culture experiments, stimulated human macrophages were able to induce ProCT and CGRP I induction in adipocytes. In monocytes only a transient expression of CT mRNA during the initial 18 h during attachment was observed. It could be concluded that the adhesion-

induced, transient expression and secretion of ProCT and CGRP I *in vitro* may play an important role during monocyte adhesion and migration *in vivo*. PBMC-derived macrophages may contribute to the marked increase in circulating ProCT by recruiting parenchymal cells within the infected tissue, as exemplified with adipocytes (Figure 6). *(Linscheid P, Seboek D, Schaer JD, Zulewski H, Keller U, Müller B 2004: Expression and secretion of procalcitonin and calcitonin gene-related peptide by adherent monocytes and by macrophage-activated adipocytes Crit Care Med 32(8):1715-1721)*

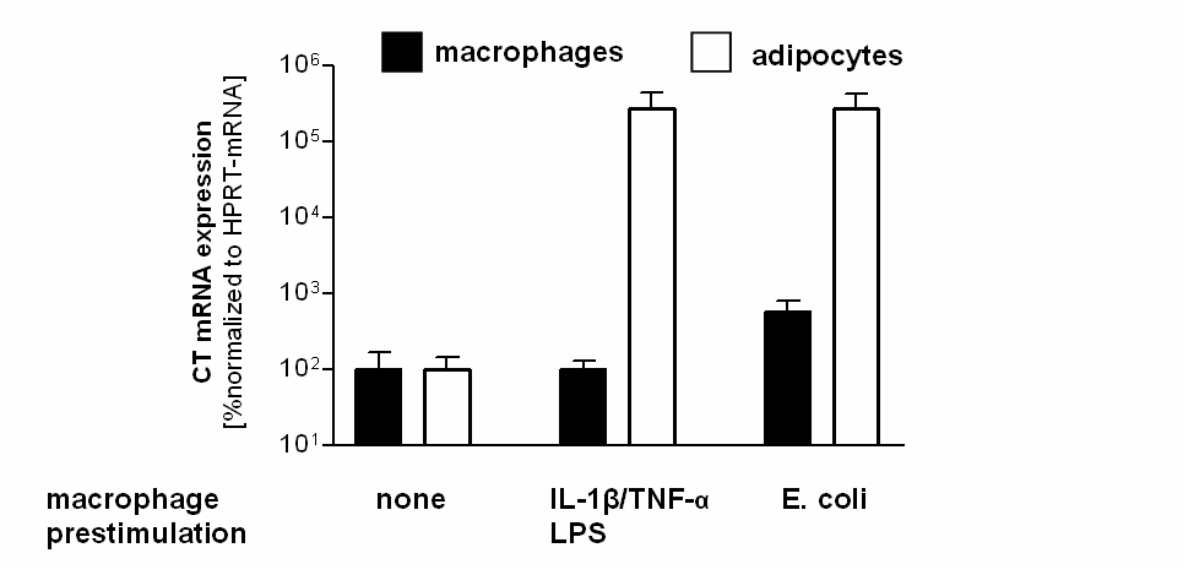

Figure 6: Macrophage-mediated CT mRNA expression in human adipocytes. PBMC-derived macrophages in cell culture inserts with 0.4-um pores were prestimulated for 2 hrs with cytokines/LPS or *E. coli*. Inserts were thoroughly washed and added to nonstimulated adipocytes. After 24 hrs in co-culture, RNAs of macrophages and adipocytes were separately isolated.

Based on the first two publications, a trimodal expression pattern of the CALC I gene and a closely related biphasic behavior of infection-related ProCT secretion was postulated (Table 2).

Table 2: Trimodal expression pattern of the CALC I gene

	Thyroid	Adipose tissue	Monocytes
mRNA	constitutive	regulated sustained by cytokines and LPS detectable > 6 hrs	regulated rapid, transient upon adhesion detectable > 2 hrs
Peptide	regulated e.g. by Ca^{2+} or gastrin	constitutive detectable after> 10 hrs persistent (>24 hrs)	constitutive detected > 4 hrs transient (< 18 hrs)
Main function	endocrine	sepsis-associated ProCT increase	local vasodilation
Tissue mass	low	high	low

For further investigations a steadier source of human adipocytes than surgical explants was needed. To this aim, human MSCs were differentiated into an adipocyte phenotype. The MSC-derived adipocytes were comparable to adipocytes derived from preadipocytes as assessed by light microscopy, fat accumulation, lipolysis and glucose uptake (62, 63). Having this models established the next aim of the thesis was to explore the expression, interactions and potential roles of adipocyte-derived CT peptide production. Expression of CT peptide-specific transcripts was analyzed by RT-PCR and quantitative real-time PCR in human adipose tissue biopsies and in three different inflammation-challenged human adipocyte models. ProCT, CGRP and ADM secretions were assessed by immunological methods. Adipocyte transcriptional activity, glycerol release and insulin-mediated glucose transport were studied after exogenous CGRP and ADM exposure. With the exception of amylin, CT peptides were expressed in adipose tissue biopsies from septic patients, inflammation-activated mature explanted adipocytes and macrophage-activated preadipocyte-derived adipocytes. ProCT and

CGRP productions were significantly augmented in IL-1β and LPS-challenged MSC-derived adipocytes, but not in undifferentiated MSC. In contrast, ADM expression occurred before and after adipogenic differentiation. IFNγ co-administration inhibited IL-1β-mediated ProCT and CGRP secretion by 78% and 34%, respectively, but augmented IL-1β-mediated ADM secretion by 50%. Exogenous CGRP and ADM administration induced CT, CGRP I and CGRP II mRNAs and dose-dependently (10^{-10} and 10^{-6} M) enhanced glycerol release. In contrast, no CGRP- and ADM-mediated effects were noted on ADM, TNFα and IL-1β mRNA abundances.

In summary, CGRP and ADM are two differentially regulated novel adipose tissue secretion factors exerting autocrine/paracrine roles. Their lipolytic effect (glycerol release) suggests a metabolic role in adipocytes during inflammation. The induction of CALC I and II mRNAs with exogenous CGRP and ADM, suggests a positive autocrine feedback loop. This feedback loop was markedly enhanced by incubation with cycloheximide, an unspecific inhibitor of protein synthesis (Figure 7). This "superinduction" suggests the presence of one or several short-lived proteins, which suppresses CALC gene expression in non-inflamed non-neuroendcrine cells, suggesting a hormokine silencing factor. *(Linscheid P*, Seboek D*, Zulewski H, Keller U and Müller B 2005: Autocrine/paracrine role of inflammation-mediated CGRP and ADM expression in human adipose tissue Endocrinology 146(6):2699-2708).*

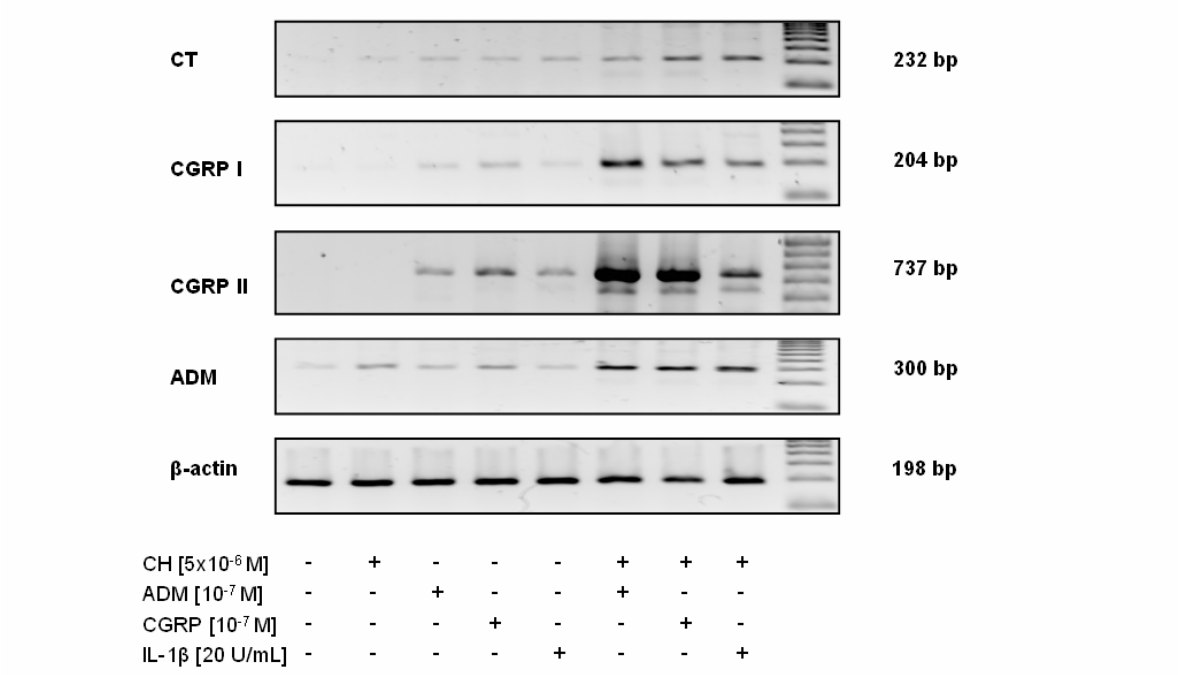

Figure 7: CALC-gene mRNA induction in MSC derived adipocytes: autoregulation and superinduction. ADM and CGRP lead to a similar induction of CALC I and II gene as compared to pro-inflammatory IL-1β. Incubation with cycloheximide (CH) markedly potentiated this effect, resulting in a superinduction of CALC gene mRNAs after 6 hours.

After investigating adipose tissue as a source of CT peptides and discover it also as a target of CT peptides, the aim of the thesis was to invesitgate the hypothesis if there is an influence of CT peptides on NO synthesis in inflammed adipocytes. eNOS mRNA was highly expressed in omental and to a lesser extent in human subcutaneous adipose tissue biopsies, but not in purified adipocytes, in MSC- and in preadipocyte-derived adipocytes, respectively. Trace amounts of iNOS mRNA was detected in adipose tissue samples of donors with abdominal infection, as opposed to non-infected subjects. IFNγ in combination with IL-1β or LPS, evoked a transient (4 h < t < 24 h) iNOS mRNA expression in human MSC- and preadipocyte-derived adipocytes, respectively. This induction was preceded by cytokine-specific mRNAs. In addition, it was accompanied by an activation of the tetrahydrobiopterin (BH4) synthesis pathway and by inhibition of peroxisome proliferator-activated receptor-γ 2 (PPARγ2). In contrast to murine 3T3-L1-derived adipocytes, iNOS protein and NO oxidation products remained undetectable in iNOS mRNA positive human adipocytes. Accordingly, co-administration of NOS inhibitors (i.e. L-NAME, L-NMMA, 1400W) had no effects on insulin-mediated glucose uptake and lipolysis,

respectively. It was concluded that in human adipocytes endogenous NO is not involved in metabolic regulation during both basal and cytokine-activated conditions (Figure 8). *(Linscheid P, Seboek D, Zulewski H, Scherberich A, Blau N, Keller U and Müller B 2005: Cytokine-induced metabolic effects in human adipocytes are independent of endogenous nitric oxide Am J Physiol Endocrinol Metab- in press)*

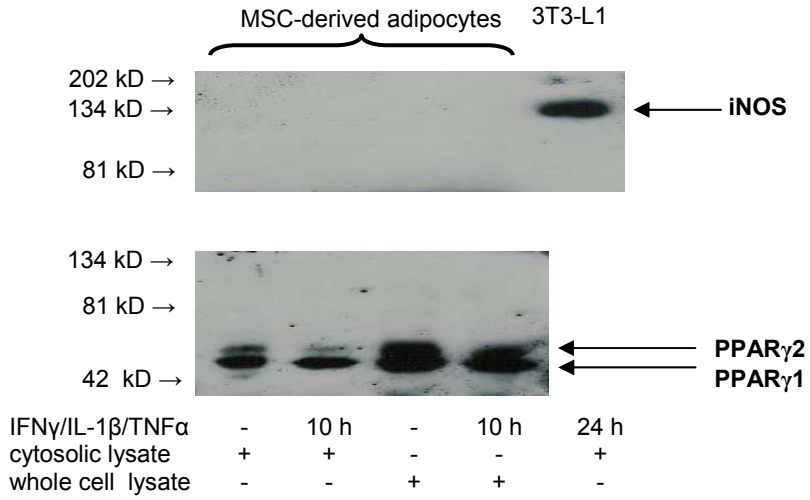

Figure 8: iNOS and PPARγ protein expression in MSC-derived adipocytes
Adipocytes were subjected to iNOS mRNA inducing treatments as indicated. Whole cell lysates (or cytosolic lysates where indicated) containing 40 µg (MSC-derived adipocytes) or 10 µg (3T3-L1) total protein were subjected to iNOS and PPARγ2 detection by Western blot. Adipocytes were treated for 10 h with IFNγ/IL-1β/TNFα. Results are representatives from four two separate experiments.

The results of the above mentioned investigations show, that in contrast to all other CALC genes, the CALC IV gene amylin was never induced by inflammatory stimulation. However, amylin is co-secreted with insulin from differentiated β cells of the endocrine pancreas. During the thesis the hypothesis arose, if other hormones,

e.g. pancreatic hormones could also act as hormokines. For evaluation of this hypothesis expression and secretion of SRIF and its receptors were investigated in human adipose tissue upon inflammatory stimulation *in vitro* and in tissues from patients with septic disease.

Preadipocyte-derived adipocytes, MSC-derived adipocytes and mature explanted adipocytes expressed SRIF mRNA upon LPS or IL-1β treatments. LPS- and IL-1β-mediated SRIF mRNA induction was blocked by pretreatment with dexamethasone. Using co-cultures and quantitative real-time PCR we demonstrate adipocyte SRIF induction by secretion factors from activated PBMC-derived macrophages. In contrast to basal adipocytes, SRIF protein was detected in culture supernatants of LPS- and of combined TNFα/IL-1β/LPS-treated adipocytes. SRIF protein was visualized by immunohistochemistry in explanted minced adipose tissue upon overnight incubation in culture medium supplemented with combined IL-1β and LPS. In septic patients expression of SRIF-mRNA and SRIF protein was found in visceral but not in subcutaneous adipose tissue. Adipocyte mRNA abundance of SRIF rezeptors (SSTR) 1-5 were differentially regulated by inflammatory treatments.

In summary, human visceral adipose tissue secretes SRIF during inflammation and sepsis and expresses several SSTR. It is tempting to speculate that visceral adipose tissue-derived SRIF plays a modulatory role in the immunological and metabolic response to inflammation, e.g., relative insulin hyposecretion and hyperglycemia. With this study a novel hormokine, e.g., SRIF was described (Figure 9). *(Seboek D, Linscheid P, Zulewski H, Langer I, Christ-Crain M, Keller U, Muller B 2004: Somatostatin is expressed and secreted by human adipose tissue upon infection and inflammation J Clin Endocrin Metab 89(10): 4833-4839)*

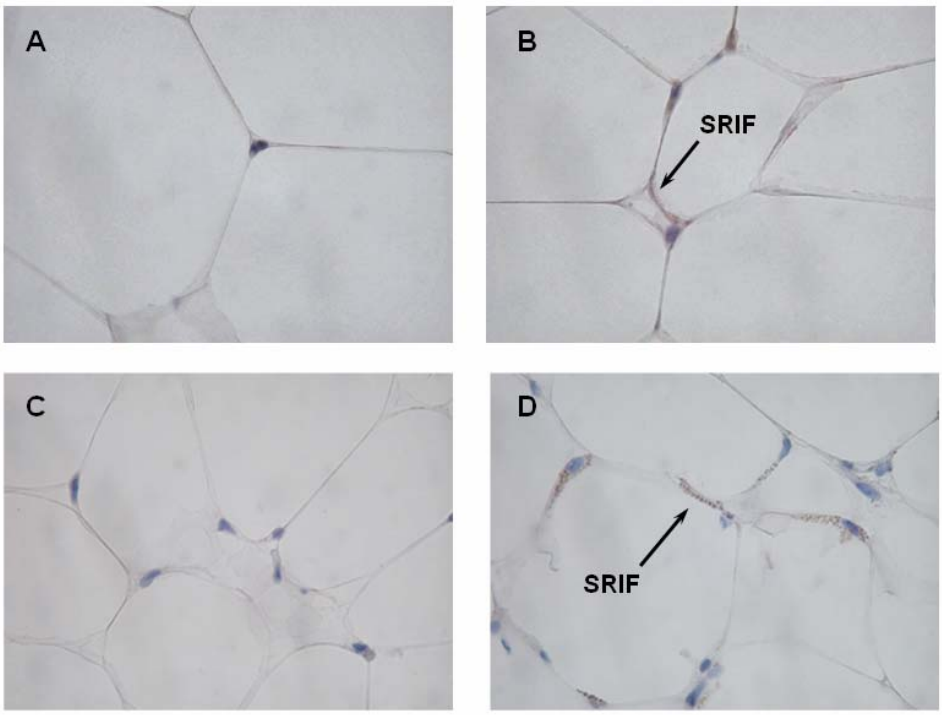

Figure 9: SRIF immunohistochemical staining in human adipose tissue. Adipose tissue sections were immunohistochemicaly stained to determine the presence of SRIF. A) Subcutaneous adipose tissue biopsies obtained from non-infected individuals were taken as control tissue. B) Subcutaneous adipose tissue biopsies obtained from non-infected individuals were stimulated with LPS and IL-1β over night in cell culture medium. C) Subcutaneous and D) visceral adipose tissue biopsies were obtained intra-operatively from a septic patient. All magnifications 1:400.

Thereafter, I became interested in the plasticity of hormokines and. Because of the hormokine like behavior of somatostatin, the hypothesis arose that other islet hormones, namely insulin or glucagon could be expressed ectopically. The idea was that non-endocrine cells might produce islet derived hormones upon adequate stimulation and/or differentiation, comparable to the ubiquitous expression of CT peptides during infections. Multipotential stem cells form various organs appear to share this particular property of CT peptide secreting cells as they are able to induce the expression of gene products that were initially not produced by their respective tissue of origin. Insulin expressing cells were generated form stem cells originated from the pancreas, bone marrow, liver and embryonic stem cells. Extrapanctreatic insulin expression was seen in bone marrow as well as liver and adipose tissue in

response to hyperglycemia in mice. Human islet derived stem cells were shown to adopt a hepatic phenoptype *in vitro* (64) and *in vivo* (65).

The study presented herein shows that bone marrow derived human MSC and human adipose tissue derived preadipocytes express the stem cell markers nestin and ABCG2 as well as the transcription factor islet-1 (Isl-1). Upon induction of differentiation with defined culture conditions these cells adopt a pancreatic endocrine phenotype with up-regulation of the crucial pancreatic transcription factors Isl-1, Ipf-1, Ngn3, Pax-4, Pax-6, Nkx-2.2 and Nkx-6.1. In parallel an up-regulation of the islet proteins insulin, glucagon, somatostatin and the glucose transporter glut-2 was observed. Similar gene expression profile was found in MSC of type 1 diabetic patients without residual insulin secretion. The ability of human MSC to adopt a pancreatic endocrine phenotype indicates a developmental potential of these cells that may help to develop new stem cell based therapies for type 1 diabetes using an autologous transplantation approach (Figure 10). (*Seboek D, Timper K, Eberhardt M, Linscheid P, Keller U, Müller B and Zulewski H: Human bone marrow-derived mesenchymal stem cells differentiate into insulin, somatostatin and glucagon expressing cells (submitted)* and *(Timper K*, Seboek D*, Eberhardt M, Linscheid P, Keller U, Müller B and Zulewski H 2006: Human adipose tissue-derived mesenchymal stem cells differentiate into insulin, somatostatin, and glucagon expressing cells Biochem Biophys Res Commun 341(4)1135-40).*

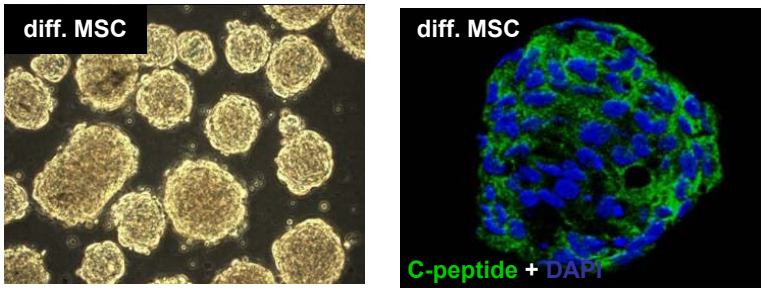

Figure 10: Islet like clusters generated from MSC. Phase contrast image of islet like clusters after differentiation of MSC. For immunocytochemistry islet-like clusters were collected after 3 days in differentiation medium. Staining of differentiated clusters for c-peptide. Nuclear staining in blue with DAPI.

The findings might have important implications. Therefore, promoter studies with hormokine promoters were planned to be carried out. However, human mature adipocytes are difficult to transduce or transfect. The final aim of the thesis was to establish a procedure for introducing genetic information into MSC- and preadipocyte-derived adipocytes. Expression of the GFP reporter gene was assessed after exposure to lentiviruses. Preserved adipocyte function was evaluated by insulin-induced glucose uptake.

Adipocytes expressed GFP after 5 days post-transduction. Up to 60% of mature adipocytes were GFP positive. Transduction of undifferentiated bone marrow-derived MSCs and preadipocytes did not affect their capacity to adopt an adipocyte phenotype upon differentiation. Insulin-induced glucose uptake was not affected in transduced mature adipocytes. Transfection efficiency by adenoviral-mediated gene transfer into both differentiated and undifferentiated cells was similar to lentiviral infection, as evaluated by cell counts. However, this procedure was associated with over 50% cell death within the first 5 days.

Lentiviral vectors provide an effective gene transfer techniques for the genetic modification of MSC- and preadipocyte-derived human adipocytes without apparent loss of cell-specific functions. This technique may provide a basis for further studies on the biology of adipose tissue and has the potential to become a target for gene therapy in obesity-related disorders (Figure 11). *(Seboek D, Linscheid P, Firm C, Zulewski H, Salmon P, Russo AF, Keller U, Müller B: Lentiviral vectors efficiently transduce human mesenchymal stem cell- and preadipocyte derived mature adipocytes (submitted))*

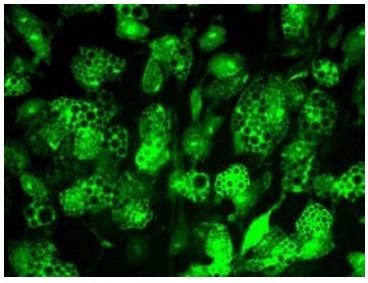

Figure 11: Transduction of human MSC- derived adipocytes. Mature MSC derived adipocytes were exposed to lentiviral vectors containing GFP under a PGK promoter (53RPA PGK GFP). Picture was taken 4 days after transduction with the Olympus IX 50 fluorescent microscope.

Taking the data together, the ubiquitous expression of CT peptides upon sepsis and showing expression of pancreatic endocrine genes in stem cells of different origin, it can be suggested that a mechanism may be present in both cases that unlocks the tissue specific boundary.

This work anticipates that the interdisciplinary approach will clarify shared mechanisms of metabolic dysfunctions (i.e. insulin deficiency and resistance, respectively) of allegedly distinct diseases, namely obesity, type 2 diabetes and sepsis with their ensuring complications characterized by a high morbidity and mortality.

7. FUTURE PERSPECTIVES

In spite of these important findings, it is as yet unclear how ProCT and the other CT peptides may influence the complex events occurring in systemic inflammation. The ubiquitous expression of CT peptides and other hormones upon sepsis suggest that a mechanism may be present that unlocks the tissue specific boundary. Detailed intracellular mechanisms underlying the regulation, function and plasticity of hormokines using human adipocytes can be achieved with the virus techniques.

Furthermore, to understand the phenomenon of trans-differentiation may become very useful in the development of innovative strategies for generation of insulin expressing cells from non-pancreatic tissues, like bone marrow and adipose tissue. Excited by the plasticity of hormokines in human cells, it is tempting to speculate that mesenchymal stem cells and fat precursor cells can be turned into islets. The concept of hormokines implies unforseen possibilities in the regulation of the insulin gene and for the treatment of diabetes. Stem cells could be isolated from tissue biopsies of diabetic patients e.g. bone marrow or adipose tissue, expanded *ex vivo* and re-transplanted into the donor/recipient, curing his diabetes without the need for immunosuppressive therapy.

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9. PAPERS

***In vitro* and *in vivo* calcitonin-I gene expression in parenchymal cells: a novel product of human adipose tissue**
Linscheid P, Seboek D, Nylen ES, Langer I, Schlatter M, Keller U, Becker KL, Muller B 2003 Endocrinology 144(12):5578-5584

Expression and secretion of procalcitonin and calcitonin gene-related peptide by adherent monocytes and by macrophage-activated adipocytes
Linscheid P, Seboek D, Schaer JD, Zulewski H, Keller U, Müller B 2004 Crit Care Med 32(8):1715-1721

Autocrine/paracrine role of inflammation-mediated CGRP and ADM expression in human adipose tissue
Linscheid P*, Seboek D*, Zulewski H, Keller U and Müller B 2005 Endocrinology 146(6):2699-2708

Somatostatin is expressed and secreted by human adipose tissue upon infection and inflammation
Seboek D, Linscheid P, Zulewski H, Langer I, Christ-Crain M, Keller U, Muller B 2004 J Clin Endocrin Metab 89(10): 4833-4839

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Linscheid P, Seboek D, Zulewski H, Scherberich A, Blau N, Keller U and Müller B 2005 Am J Physiol Endocrinol Metab- in press

Human bone marrow-derived mesenchymal stem cells differentiate into insulin, somatostatin and glucagon expressing cells
Seboek D, Timper K, Eberhardt M, Linscheid P, Keller U, Müller B and Zulewski H (submitted)

Human adipose tissue-derived mesenchymal stem cells differentiate into insulin, somatostatin, and glucagon expressing cells
Timper K*, Seboek D*, Eberhardt M, Linscheid P, Keller U, Müller B and Zulewski H 2006 Biochem Biophys Res Commun 341(4):1135-40

Lentiviral vectors efficiently transduce human mesenchymal stem cell- and preadipocyte derived mature adipocytes
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In Vitro and *in Vivo* Calcitonin I Gene Expression in Parenchymal Cells: A Novel Product of Human Adipose Tissue

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Circulating levels of calcitonin precursors (CTpr), including procalcitonin (ProCT), increase up to several thousand-fold in human sepsis, and immunoneutralization improves survival in two animal models of this disease. Herein, we analyzed inflammation-mediated calcitonin I gene (CALC I) expression in human adipocyte primary cultures and in adipose tissue samples from infected and noninfected patients with different levels of serum ProCT. In *ex vivo* differentiated adipocytes, the expression of CT mRNA increased 24-fold ($P < 0.05$) after the administration of *Escherichia coli* endotoxin (lipopolysaccharide) and 37-fold ($P < 0.05$) after IL-1β administration by 6 h. ProCT protein secretion into culture supernatant increased 13.5-fold ($P < 0.01$) with lipopolysaccharide treatment and 15.2-fold ($P < 0.01$) with IL-1β after 48 h. In coculture experiments, adipocyte CT mRNA expression was evoked by *E. coli*-activated macrophages in which CT mRNA was undetectable. The marked IL-1β-mediated ProCT release was inhibited by 89% during coadministration with interferon-γ (IFNγ). In patients with infection and markedly increased serum ProCT, CT mRNA was detected in adipose tissue biopsies. Hence, we demonstrate that ProCT, which is suspected to mediate deleterious effects in sepsis and inflammation, is a novel product of adipose tissue secretion. The inhibiting effect of IFNγ on IL-1β-induced CT mRNA expression and on ProCT secretion might explain previous observations that serum ProCT concentrations increase less in systemic viral compared with bacterial infections. (*Endocrinology* 144: 5578–5584, 2003)

ADIPOSE TISSUE IS increasingly recognized as a major endocrine organ in humans. The numerous peptide hormones released by adipocytes have been proposed to affect energy homeostasis, glucose and lipid metabolism, immune response, and reproduction (1). Most of these signaling molecules appear to be deregulated when mass is markedly altered, being increased in the obese state or decreased in lipoatrophy.

In systemic microbial infections, circulating levels of calcitonin (CT) precursors (CTpr), including procalcitonin (ProCT), increase up to several thousand-fold, and this increase correlates with the severity of the illness and with mortality (2–4). Furthermore, CTpr may contribute to the deleterious effects of systemic infection as shown in experimental animals (5–7).

CTpr originate from the calcitonin I (CALC I) gene on chromosome 11. Similar to many peptide hormones, mature CT is initially biosynthesized as a larger prohormone, ProCT, which is subsequently processed into smaller peptides, including CT (8, 9). The classical neuroendocrine paradigm limits the expression of CALC I exclusively to neuroendocrine cells, mainly the C cells of the thyroid. However, increased plasma ProCT levels have been reported in thyroidectomized patients with inflammation (10, 11). We recently documented the generalized, tissue-wide, nonneuroendocrine expression of CT-mRNA in animal models of sepsis (12, 13). To elucidate the source of ProCT in human sepsis, we studied the effects of cytokines and lipopolysaccharide (LPS) on CALC I induction and ProCT secretion in human adipocytes. In addition, we examined CT-mRNA expression in adipose tissue samples obtained from infected and noninfected patients.

Abbreviations: CT, Calcitonin; CTpr, calcitonin precursors; HPRT, hypoxanthine-guanine phosphoribosyltransferase; IFNγ, interferon-γ; IMDM, Iscove's modified Dulbecco's medium; LPS, lipopolysaccharide; ProCT, procalcitonin.

Materials and Methods

Adipocyte cultures

For *ex vivo* stimulation, after informed consent was granted, 50–500 g adipose tissue were obtained from noninfected patients undergoing plastic surgery. Primary cultures of human adipocytes were performed as previously described (14, 15) with modifications. Briefly, adipose tissue was minced, digested in 1 mg/ml collagenase 2 (Worthington Biochemical Corp., Freehold, NJ), filtered (150-μm pore size nylon mesh) and centrifuged at 200 $\times$ *g*. The cell pellet was resuspended twice in erythrocyte lysis buffer, washed, and seeded at a density of approximately 33,000 cells/cm^2 in 6- or 12-well plates. After 18-h incubation in DMEM/Ham's F-12 with 10% fetal calf serum allowing attachment, cells were washed in PBS and cultured in serum-free medium supplemented with agents (isobutylmethylxanthine, dexamethasone, insulin, transferrin, and T_3) that induce differentiation of preadipocytes to adipocytes. During the first 2 d, 1 μM rosiglitazone (provided by GlaxoSmithKline, Worthing, UK) was also present. Triglyceride-storing adipocytes, representing 40–80% of cultured cells, are visible within 5–10 d. Differentiation was confirmed by RT-PCR analysis for adipocyte-specific peroxisome proliferator-activated receptor γ2 expression (16). Adipocytes were maintained for an additional 4 d in DMEM/Ham's F-12 with 10% FCS before experiments.

In addition, floating mature adipocytes obtained after the centrifugation step were washed, inoculated into 50-ml flasks (BD Biosciences, Franklin Lakes, NJ) completely filled with medium (DMEM/Ham's F-12 with 10% FCS), and allowed to attach to the upper surface for 72 h at 37 C (17, 18). Flasks were subsequently turned around, and the attached purified, triglyceride-storing adipocytes were cultured in 5 ml medium for experiments.

Adipocytes were stimulated for time periods ranging from 2–60 h with the following agents: 1 μg/ml lipopolysaccharide (LPS), 100 U/ml interferon-γ (IFNγ), 10 ng/ml TNFα, and 20 U/ml IL-1β. Human-specific cytokines were purchased from PeproTech (London, UK), and LPS (*Escherichia coli* 026:B6) was obtained from Sigma-Chemie (Buchs, Switzerland).

The viability of adipocytes after stimulation was assessed via trypan blue staining: viable cells exclude trypan blue; dead cells stain blue.

Adipocytes and macrophages in cocultures

White blood cells were isolated by Ficoll-Plaque Plus (Amersham Pharmacia Biotech, Uppsala, Sweden) and washed four times with Hanks' buffered salt solution (Invitrogen, Basel, Switzerland) supplemented with 0.5% human albumin (Blutspendedienst SRK, Bern, Switzerland). Cells were resuspended in Iscove's Modified Dulbecco's Medium (IMDM) with 20% human serum and seeded in cell culture inserts with 0.4-μm pore size (BD Biosciences). After 1-h incubation to allow attachment of monocytes, inserts were washed four times with Hanks' buffered salt solution supplemented with 0.5% human albumin. Fresh IMDM with 20% human serum was supplied, and monocytes were cultured for 5 d, allowing differentiation to macrophages. For experiments, inserts with macrophages were added to wells containing *ex vivo* differentiated adipocytes, which were previously kept for 2 d in IMDM with 20% human serum. Upper chambers containing macrophages were supplemented for 2 h with live *E. coli* and kept in coculture with adipocytes for an additional 22 h.

RT-PCR

Total RNA from homogenized tissues or adipocyte cultures was extracted by the single step guanidinium isothiocyanate method with a commercial reagent (Tri-Reagent, Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's protocol. Extracted RNA was quantified spectrophotometrically, and the quality was assessed by gel electrophoresis. Equal amounts of RNA per tissue or *in vitro* treatment were subjected to RT (Omniscript RT kit, Qiagen, Basel, Switzerland). PCR was performed on a conventional thermal cycler (TGradient, Biometra, Gottingen, Germany) using the PCR *Taq* core kit (Qiagen) and the following intron border-spanning oligonucleotides: CT (232-bp product; GenBank accession no. X00356), 5′-TGAGCTGGAGCAGGAG-CAAG-3′ (sense) and 5′-GTTGGCATTCTGGGGCATGCTAA-3′ (antisense); IL-6 (284-bp product; GenBank accession no. NM_000600), 5′-GCAAAGAGGCACTGGCAGAAA-3′ (sense) and 5′-CAGGCTG-GCATTTGTGGTTG-3′ (antisense); TNFα (310-bp product; GenBank accession no. M10988), 5′-GGCCCAGGCAGTCAGATCAT-3′ (sense) and 5′-GGGGCTCTTGATGGCAGAGA-3′ (antisense); and β-actin (198-bp product; GenBank accession no. AF076191), 5′-TTCTGACCCATGC-CCACCAT-3′ (sense) and 5′-ATGGATGATGATATCGCCGCGCTC-3′ (antisense). The annealing temperature was 65 C, except for CT (67 C). Thirty-five cycles of PCR were used for CT and TNFα detection. Cycles were reduced to 28 for IL-6 and β-actin to stop the reaction in the linear phase of amplification. IL-6 and TNFα were used as controls of inflammatory stimulation, and β-actin was used to verify equal quantities of RNA loading in each reaction. PCR products were separated and visualized on 1.5% agarose gels containing 0.5 μg/ml ethidium bromide. PCR product identity was confirmed by direct nucleotide sequencing of the PCR products by dye deoxy terminator cycle sequencing.

Quantitative analyses of CT-mRNA expression

cDNA obtained as described above was subjected to quantitative real-time PCR analysis using the ABI 7000 sequence detection system (PerkinElmer, Branchburg, NJ). Specific primers yielding short PCR products suitable for SYBR-Green detection were designed using Primer Express software (version 1.0, PE Applied Biosystems, Foster City, CA). Sequences of primers were as follows: CT (92-bp product; GenBank accession no. X00356), 3′-GTGCAGATGAAGGCCAGTGA-5′ (sense) and 3′-TCAGATTACCACACCGCTTAGATC-5′ (antisense); and hypoxanthine-guanine phosphoribosyltransferase (HPRT; 85-bp product; GenBank accession no. M26434), 3′-TCAGGCAGTATAATCCAAA-GATGGT-5′ (sense) and 3′-AGTCTGGCTTATATCCAACACTTCG-5′ (antisense). The reaction volume was 22 μl, and the conditions were set as suggested by the manufacturer. Each cDNA sample tested for quantitative CT mRNA expression was also subjected to HPRT mRNA analysis. Results were expressed as the ratio of the respective CT mRNA and HPRT mRNA threshold values. Product identity was confirmed by sequence analysis and electrophoresis on a 2.5% agarose gel containing ethidium bromide.

CT precursor concentrations

ProCT concentrations were determined in supernatants by an ultrasensitive chemiluminometric assay with a functional sensitivity of 6 pg/ml (ProCa-S Assay, B.R.A.H.M.S. GmbH, Hennigsdorf-Berlin, Germany).

Patients

Adipose tissue samples were obtained from four infected patients with elevated serum ProCT requiring laparotomy (mean age, 44 yr; range, 19–65 yr). The septicemia was due to peritonitis because of perforated sigmoid diverticulitis, perforated appendicitis, ischemic colitis of the sigmoid colon, and necrotizing proctocolitis with perforation of the rectum and descending colon. Also, adipose tissue was collected from noninfected patients requiring elective surgery (mean age, 53 yr; range, 29–71 yr). Informed consent was obtained. Harvested tissues were immediately incubated in RNA-later (Ambion, Inc., Austin, TX) to prevent RNA degradation. The samples were snap-frozen and stored at

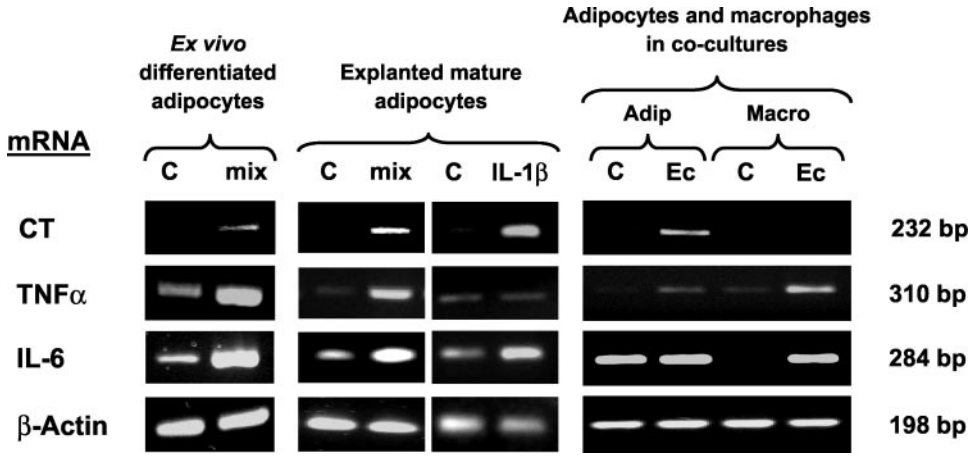

FIG. 1. Induction of the CALC I gene in cultured adipocytes. RT-PCR analysis was performed with RNA obtained from *ex vivo* differentiated adipocytes and from mature adipocytes kept in so-called ceiling cultures after 6-h stimulation with or without cytokines (IFNγ, 100 U/ml; TNFα, 10 ng/ml; IL-1β, 20 U/ml) and LPS (1 μg/ml), denoted as mix. Alternatively, *ex vivo* differentiated adipocytes were kept in cocultures with macrophages activated with *E. coli* (Ec). Presented data are one representative from at least five independent experiments.

−70 C. Tissues were powdered under liquid nitrogen before RNA extraction using Tri-Reagent.

Statistical analysis

Results are presented as the mean ± SEM. Groups of experiments were compared statistically using *t* tests. In addition, two group comparisons corrected for multiple testing, *i.e.* one-way ANOVA with *post hoc* analysis for least square difference, were performed.

Results

CALC I gene induction in vitro

We first analyzed the effects of inflammatory mediators on CT mRNA expression in adipose tissue-derived cells. In *ex vivo* differentiated adipocytes and in mature explanted adipocytes obtained from noninfected patients undergoing plastic surgery, CT mRNA was not detected by conventional RT-PCR analysis using 35 amplification cycles (Fig. 1). After 45 cycles of real-time PCR using specifically designed primers, trace amounts of CT mRNA were detected on a 2.5% agarose gel (Fig. 2A). Accordingly, analysis of ProCT content in supernatants of *ex vivo* differentiated unstimulated, control adipocytes was below or around the detection limit of 5 pg/ml (Fig. 2B). After 6-h exposure to a combination of LPS and inflammatory cytokines (IFNγ, TNFα, and IL-1β), both the *ex vivo* differentiated as well as the mature adipocytes revealed induced CT mRNA expression (Fig. 1). CT mRNA induction was also observed in adipocytes kept in coculture with *E. coli* activated macrophages (Fig. 1). In contrast, CT mRNA induction was not observed in macrophages stimulated with *E. coli*.

cDNAs obtained from adipocytes treated with LPS or single cytokines were subjected to quantitative real-time PCR analysis. Threshold values of CT mRNA were normalized using the HPRT mRNA value obtained from the respective cDNA preparation during the same PCR run. IFNγ alone had no effect on CT mRNA induction (Fig. 2A), whereas TNFα provoked a 13.2-fold increase compared with nonstimulated control adipocytes. After treatments with mixed cytokines or LPS alone, the increases in CT mRNA induction were 23.2-

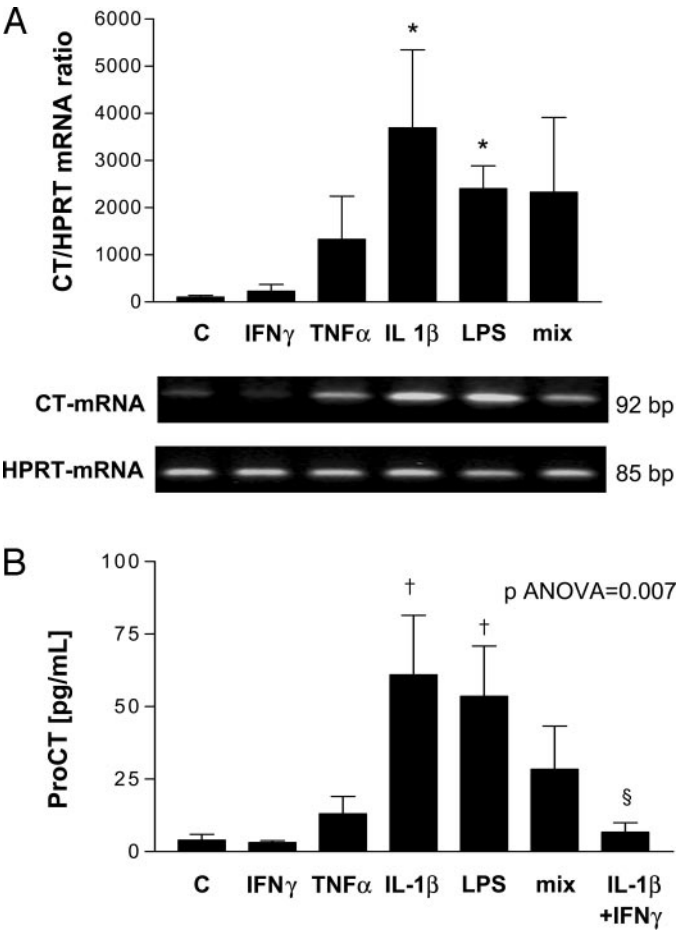

FIG. 2. Quantitative analysis of CT mRNA expression and ProCT release by *ex vivo* differentiated adipocytes. A, Quantitative real-time PCR analysis was performed with cDNA obtained from *ex vivo* differentiated adipocytes using SYBR-Green detection. Incubation time was 6 h, and the following concentrations were used: IFNγ, 100 U/ml; TNFα, 10 ng/ml; IL-1β, 20 U/ml; and LPS, 1 μg/ml. CT mRNA threshold values were normalized with HPRT mRNA values and amplification products were visualized on 2.5% agarose gels. B, After 48-h cytokine treatments, supernatants of *ex vivo* differentiated adipocytes were subjected to chemiluminometric ProCT protein analysis. Random values between 0 and 5 were generated for measurements below the detection limit of 5 pg/ml. The data shown are the mean ± SEM from three (A) or four (B) independent experiments. *, $P < 0.05$ *vs.* control. †, $P < 0.01$ for the comparison of LPS and IL-1β *vs.* control, respectively. §, $P < 0.01$ for the comparison of IL-1β plus IFNγ *vs.* IL-1β alone.

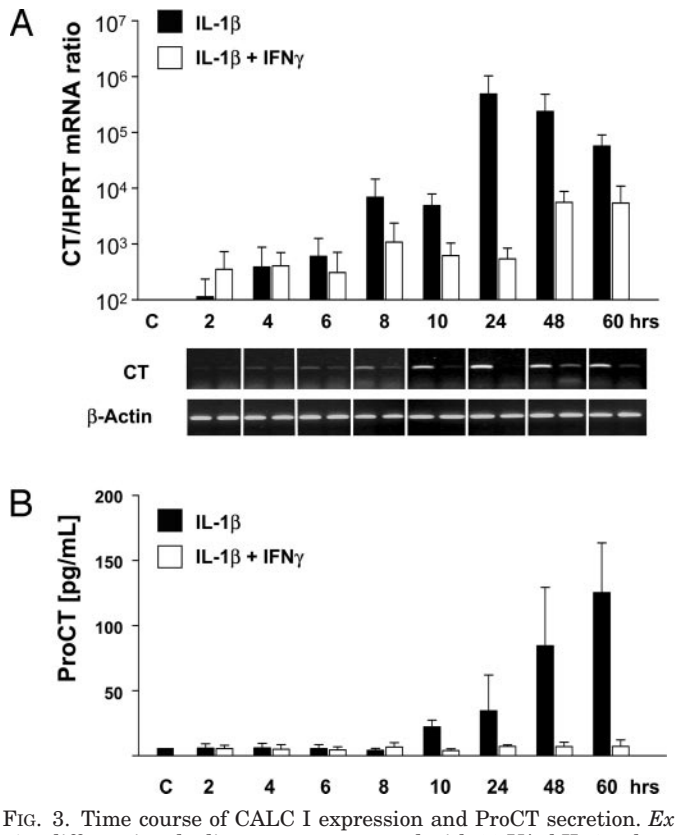

FIG. 3. Time course of CALC I expression and ProCT secretion. *Ex vivo* differentiated adipocytes were treated with 20 U/ml IL-1β alone or together with 100 U/ml IFNγ. After 2-, 4-, 6-, 8-, 10-, 24-, 48-, or 60-h incubation periods, total RNA was extracted and analyzed for CT mRNA abundance with quantitative real-time PCR and conventional RT-PCR (A). ProCT secretion into culture supernatant was analyzed by chemiluminometric assay (B). Results are presented as the mean ± SD of two independent experiments.

and 24-fold ($P < 0.01$), respectively. Interestingly, the strongest induction of CT mRNA was observed after treatment with IL-1β, resulting in a 37-fold ($P < 0.01$) increase. The marked induction of CT mRNA by IL-1β was confirmed in explanted mature adipocytes (Fig. 1).

Subjecting undifferentiated preadipocytes to cytokine treatment did not result in CT mRNA expression (not shown)

ProCT secretion in vitro

In supernatants of control or IFNγ-treated adipocytes, the ProCT protein concentration was below or at the detection limit of 5 pg/ml after 48-h incubation (Fig. 2B). In contrast, ProCT protein secretion was increased to 13 ± 6.0 pg/ml in supernatants of TNFα-treated cells (Fig. 2B). Administration of LPS or IL-1β alone or of combined cytokines led to average ProCT protein concentrations of 53.6 ± 17.3 pg/ml ($P < 0.01$), 61.0 ± 20.5 pg/ml ($P < 0.01$), and 28.4 ± 14.9 pg/ml, respectively (n = 4 for each agent). IL-1β induced ProCT secretion at concentrations as low as 0.2 U/ml (not shown). The viability of adipocytes after 48-h exposure to LPS, IFNγ, TNFα, and IL-1β, alone or in combination, was unchanged as assessed by trypan blue staining (not shown).

Interestingly, in *ex vivo* differentiated adipocytes, antagonistic effects of IFNγ on IL-1β activity were noted. Administration of 100 U/ml IFNγ for 48 h reduced IL-1β-mediated ProCT secretion by 89% (Fig. 2B). Accordingly, mRNA analysis by both conventional RT-PCR and quantitative real-time PCR revealed a strong transcriptional inhibition of CT mRNA expression by IFNγ over time periods ranging up to 60 h (Fig. 3A). ProCT release, which was measurable starting from 10 h of stimulation, was strongly inhibited in the presence of IFNγ at all time points (Fig. 3B).

CT mRNA expression in adipose tissue obtained from septic and nonseptic humans

In several patients with infection and elevated serum ProCT we found CT mRNA expression in sc and omental fat depots (Fig. 4). Control experiments confirmed that, as expected, the CALC I gene was not expressed in adipose tissues from noninfected control patients. Average CT mRNA expression in adipose tissue was enhanced 1962-fold in RNAs obtained from infected *vs.* noninfected patients, as assessed by real-time PCR.

Discussion

The present studies are the first demonstration of ProCT production and secretion by human adipocytes in the presence of inflammatory mediators. It also confirms the extrathyroidal production of human ProCT previously shown in animal studies (12). Our initial adipocyte model consisted of *ex vivo* differentiated preadipocytes, in which other cell types (*e.g.* endothelial cells) were potentially present in the cultures; these could provide a nonadipose source of CT mRNA expression and ProCT release. Hence, the experiments were successfully repeated using mature and purified adipose cells cultured in adipocyte-selecting ceiling cultures (17, 18). The density of adherent adipocytes obtained by this technique is relatively low, but suitable for RT-PCR analysis. The experiments using adipocytes and macrophages in coculture

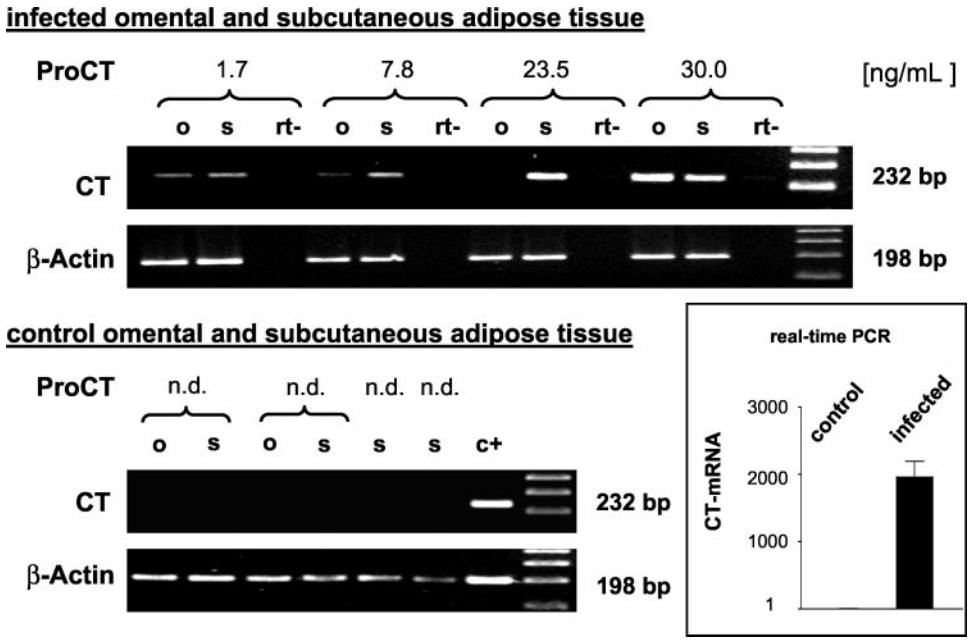

FIG. 4. Extrathyroidal nonneuroendocrine expression of CT mRNA in humans with infection. Subcutaneous (s) and omental (o) adipose tissue biopsies were obtained intraoperatively from patients with infection and markedly increased circulating ProCT levels as indicated. Noninfected tissues were obtained from patients with normal levels of serum ProCT as controls. Total RNA extractions were subjected to CT mRNA analysis by RT-PCR. Amplification products were visualized on agarose gels containing 0.5 μg/ml ethidium bromide. Verification of mRNA as the source of amplification template was obtained by omitting the RT in reactions for pooled infected samples (rt−), resulting in no bands after PCR. RNA extracted from a medullary thyroid carcinoma cell line was taken as a positive control (c+). The results of quantitative real-time PCR analysis are presented in arbitrary units as the mean ± SEM. The data shown are from four infected and four control patients, respectively. n.d., ProCT concentration less than 0.5 ng/ml.

demonstrated that molecules of endogenous origin have the capacity to induce the CALC I gene in adipocytes. Interestingly, CT mRNA was not detectable in activated macrophages. Furthermore, CT mRNA could not be induced in nondifferentiated preadipocytes and in numerous human cell lines. This suggests that infection-mediated CALC I gene expression is limited to differentiated parenchymal cells, here exemplified by adipocytes.

Among the inflammatory cytokines tested in the present report, IL-1β acted as a potent stimulator of CT mRNA expression and ProCT synthesis. TNFα moderately stimulated CT mRNA expression and ProCT release. Both IL-1β and TNFα have been ascribed significant roles in the cytokine mediation of sepsis and septic shock (19). Interestingly, parenterally administered recombinant TNFα was reported to increase serum ProCT levels into the septic range in noninfected humans, and ProCT could be measured in supernatants from TNFα and IL-6 stimulated liver slices, tissue which is composed of various cell types (20). However, in these studies the cellular source and mechanisms could not be determined, because no further molecular analyses had been performed. Presumably, the increase in CT mRNA gene transcription is mediated by one or several microbial-specific response elements in the CALC I gene promoter (21). During bacterial infections, a combined stimulation by microbial products (*e.g.* LPS) and of proinflammatory mediators of the host response (*e.g.* TNFα and IL-1β) results in a generalized tissue-wide induction of CT mRNA and a consequent secretion of CTpr, including ProCT. LPS treatment alone also strongly induced ProCT synthesis. Hence, infection-related CALC I gene expression in adipocytes appears not to depend on inflammatory mediators from other cell types. This is in accordance with CD14 expression in human adipocytes (22) as well as LPS activity mediated via Toll-like receptors in murine adipocytes (23).

In several adipose tissue biopsies from infected subjects with high circulating ProCT we demonstrated *in vivo* extrathyroidal expression of CT mRNA. As expected, in fat samples from noninfected control patients CT mRNA was not present. Due to the large mass of adipose tissue in the human

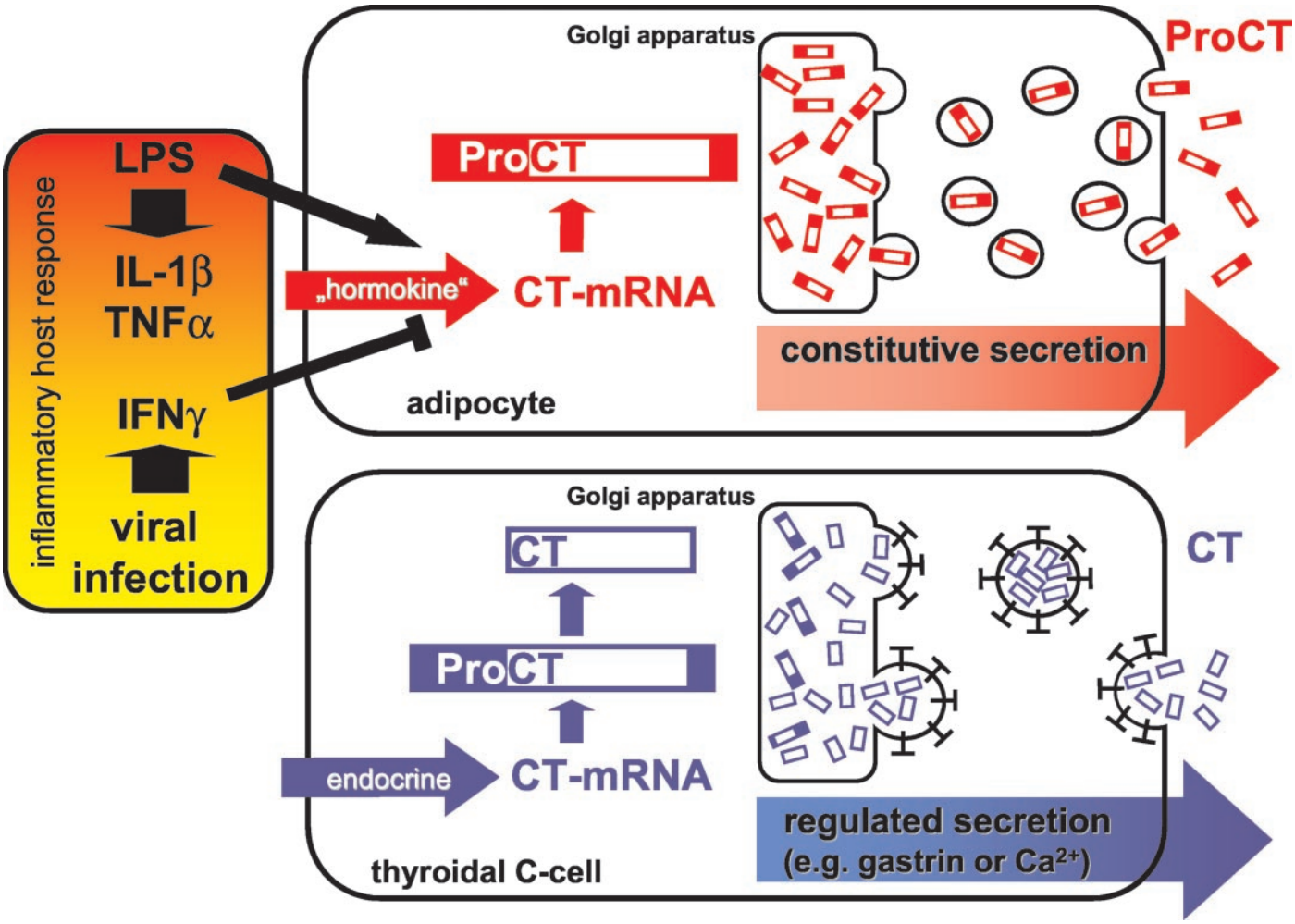

FIG. 5. Schematic diagram of CALC I expression in adipocytes and thyroidal C cells. In the classical neuroendocrine paradigm, the expression of CT mRNA is restricted to neuroendocrine cells, mainly C cells of the thyroid. Initially, the 116-amino acid prohormone ProCT is synthesized and subsequently processed to the considerably smaller mature CT. In sepsis and inflammation, proinflammatory mediators induce CT mRNA. In contrast to thyroidal cells, adipocytes and other parenchymal cells lack secretory granules, and hence, unprocessed ProCT is released in a nonregulated, constitutive manner.

organism, we postulate that adipocytes contribute substantially to the systemic elevation of circulating ProCT in infected patients. Increased morbidity and mortality were recently reported in critically ill morbidly obese patients compared with nonobese patients (24). It is tempting to speculate that adipose tissue-derived ProCT contributes to the complications reported in obese intensive care units patients. In this context it is notable that the administration of human ProCT worsened the outcome, whereas immunoneutralization of endogenous ProCT improved survival in septic hamsters (5). In septic pigs, iv immunoneutralization of ProCT reduced mortality and improved physiologic and metabolic parameters even when administered after the animals were moribund (6). However, at present in humans no clinical data are available on this issue. Mortality rates in sepsis are very dependent on multiple host- and pathogen-related factors (*e.g.* comorbidities of the patient, virulence of the bacteria, among others). Hence, a presumed harmful effect of the additional ProCT secretion by the abundant adipose tissue mass in obese patients might be masked by these powerful infection-related factors. Possible roles of other cell types and tissues in humans are currently under investigation in our laboratory.

The present finding of CT mRNA in stimulated adipocytes and adipose tissue from septic patients is in contrast to the conventional endocrine concept of a tissue-specific CALC I expression restricted mostly to thyroidal C cells. Previously, the hypothesis was advanced, that CALC I gene products are a prototype of hormokine mediators (12). As such, they may follow either a classical hormonal expression or, alternatively, a cytokine-like expression pathway (Fig. 5). In sepsis, the predominance of serum ProCT as opposed to serum mature CT is indicative of a constitutive pathway within cells lacking secretory granules and, hence, a bypassing of much of the classic neuroendocrine enzymatic processing. Consequently, as is the case for most cytokines, in sepsis there is little to no intracellular storage of ProCT within nonneuroendocrine cells (12).

Our findings provide a molecular basis for the utility of circulating ProCT in the clinical diagnosis of systemic bacterial infections. Costimulation experiments using the strongest CT mRNA inducer, IL-1β, revealed that IFNγ acts as a potent inhibitor of IL-1β-mediated CALC I gene induction. IFNs, including IFNγ, play a pivotal role in early antiviral defense mechanisms (25). In contrast to bacterial infection, viral infections usually induce only a modest increase in circulating ProCT (2, 10). This phenomenon is clinically relevant; for example, one can expedite the diagnosis and treatment of a meningitis of bacterial origin and distinguish it from viral meningitis (26, 27). Our finding of an antagonistic effect of IL-1β and IFNγ on CT-mRNA induction and ProCT release might explain this clinically important phenomenon. During the host response to a viral infection, IFNγ secretion might inhibit CT mRNA induction, thereby reducing the increase in ProCT levels in response to an inflammatory stimulus (Fig. 5). Other studies previously described opposing effects of IL-1β and IFNγ (28). Activation of nuclear factor-κB plays a central role in inflammatory signaling (29), but cytokine-specific activation of upstream factors, including signal transducers and activators of transcription and Janus kinases, are possible mediators of the antagonistic activities described herein.

In conclusion, the present report is the first demonstration of human extrathyroidal CT mRNA expression and ProCT release from parenchymal tissue. Furthermore, we have demonstrated that adipose tissue-derived cell cultures are useful for the investigation of inflammatory CT mRNA expression and ProCT release. It is hoped that this new experimental model will provide a tool to further study mechanisms and action of sepsis-related extrathyroidal ProCT production.

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II

Expression and secretion of procalcitonin and calcitonin gene-related peptide by adherent monocytes and by macrophage-activated adipocytes

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Crit Care Med 32(8):1715-1721

Expression and secretion of procalcitonin and calcitonin gene-related peptide by adherent monocytes and by macrophage-activated adipocytes*

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Objective: **To explore the roles of peripheral blood mononuclear cells (PBMCs) and PBMC-derived macrophages in sepsis-related increased procalcitonin and calcitonin gene-related peptide (CGRP) I production.**

Design: **Prospective, *in vitro* primary human cell culture study and human tissue samples gene expression analysis.**

Setting: **University hospital research laboratories.**

Patients: **Cells from healthy donors and septic patients.**

Interventions: **PBMCs were obtained from healthy donors. Isolation of pure monocyte cultures was performed by magnetic depletion of nonmonocyte cells from PBMCs. Adipose tissue biopsies and circulating leukocytes were collected from septic patients. Expressions of calcitonin messenger RNA and CGRP I messenger RNA were analyzed using reverse transcriptase-polymerase chain reaction and quantitative real-time polymerase chain reaction. Supernatant procalcitonin and CGRP protein content were determined by ultrasensitive chemiluminometric and radioimmunoassays, respectively.**

Measurements and Main Results: **PBMCs expressed and secreted procalcitonin and CGRP within 3–5 hrs after adherence to endothelial cells or plastic surfaces. This induction was transient, as it was not detectable after 18 hrs. No calcitonin or CGRP I messenger RNA was observed in leukocytes obtained from septic patients with markedly increased serum procalcitonin concentrations. Stimulation with cytokines, endotoxin, or *Escherichia coli* did not induce expression of calcitonin and CGRP I messenger RNA in PBMC-derived macrophages. However, inflammatory factors released from activated macrophages induced a marked expression of procalcitonin and CGRP in co-cultured human adipocytes.**

Conclusions: **The adhesion-induced, transient expression and secretion of procalcitonin and CGRP *in vitro* may play an important role during monocyte adhesion and migration *in vivo*. PBMC-derived macrophages may contribute to the marked increase in circulating procalcitonin by recruiting parenchymal cells within the infected tissue, as exemplified with adipocytes. (Crit Care Med 2004; 32:1715–1721)**

KEY WORDS: sepsis; procalcitonin; adipocytes; peripheral blood mononuclear cell; calcitonin gene-related peptide; macrophages

Serum concentrations of calcitonin-I (CALC I) gene products including procalcitonin (ProCT) and, to a lesser extent, the splice variant calcitonin gene-related peptide (CGRP) are increased during systemic inflammatory diseases (1–5). Recent findings suggest ProCT to be a modulator of the inflammatory reaction (6). Accordingly, immunoneutralization of ProCT markedly improved survival in septic hamsters and pigs (7–9).

The classic neuroendocrine paradigm limits the expression of CALC I and CALC II genes to neuroendocrine cells (10). The CALC II gene, a gene duplication of the CALC I gene, contains a stop codon in exon IV and thus expresses exclusively CGRP II protein. In thyroidal C-cells, mature calcitonin (CT) is initially biosynthesized as a precursor protein (i.e., ProCT) that is subsequently processed into smaller peptides and finally amidated into mature CT (10). Upon infection, circulating ProCT concentrations are also increased in thyroidectomized patients, suggesting nonthyroidal sources of inflammation and sepsis-mediated ProCT (1, 11). Accordingly, we recently reported extrathyroidal expression of CALC I in septic humans and the release of ProCT by human adipocytes upon inflammatory stimulation (12).

CGRP is mainly detected in perivascular nerve terminals (13). Being the most potent endogenous vasodilator identified thus far (14, 15), CGRP is thought to be a critical mediator of neuromicrovascular regulation in models of neurogenic inflammation (16). Proposed modes of vasodilating action include endothelium and nitric oxide-dependent (17) as well as endothelium-independent mechanisms (18).

Peripheral blood mononuclear cells (PBMCs) and PBMC-derived macrophages orchestrate the inflammatory reaction by secretion of immunomodulatory mediators and are thus important candidate sources of inflammation- and sepsis-related nonneuroendocrine ProCT production. Expression of CALC I gene

*See also p. 1801.

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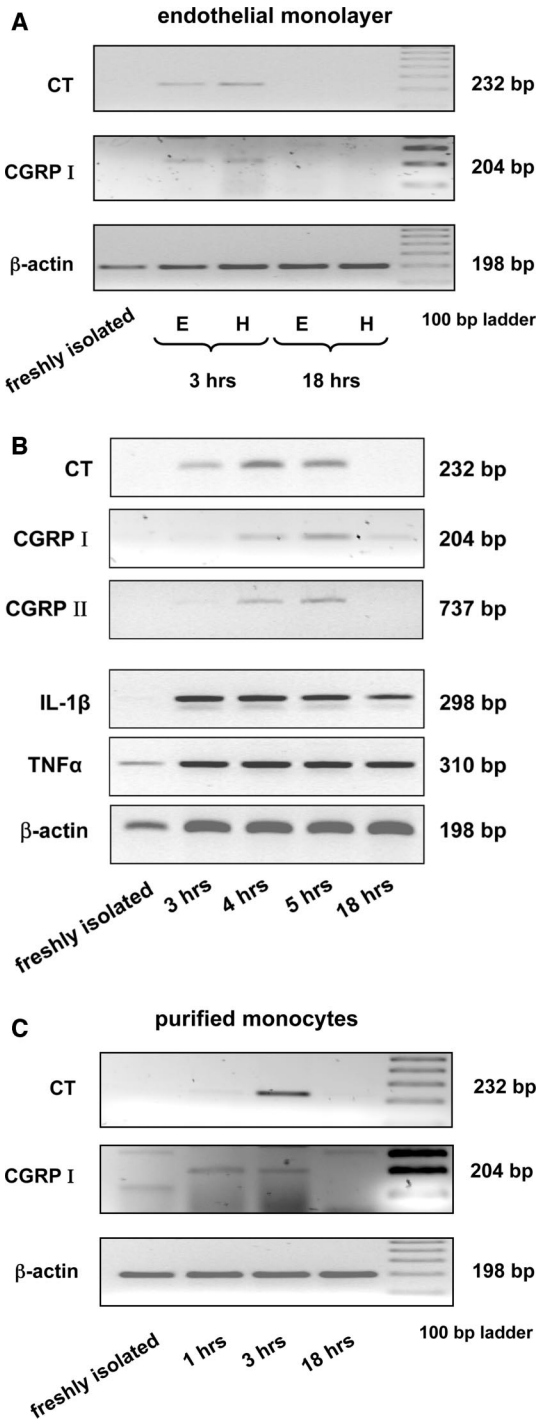

Figure 1. Transient expression of calcitonin (*CT*)-I (CALC-I) and CT-II (CALC-II) genes in monocytes upon adhesion to plastic or activated endothelial monolayer. Peripheral blood mononuclear cells (PBMCs) obtained from healthy subjects by Ficoll separation were seeded either on previously tumor necrosis factor (*TNF*)-α-activated ECV304 (*E*) or on umbilical vein endothelial cells (*H*), endothelial monolayers (*A*), or plastic surfaces (*B*). Alternatively, pure monocytes were obtained from PBMCs by magnetic depletion sorting of T cells, NK cells, B cells, dendritic cells, and basophils before seeding on tissue culture dishes (*C*). Total RNA was extracted from cells after incubation periods as indicated. Specific messenger RNAs relevant to procalcitonin and calcitonin gene-related peptide expression were analyzed by reverse transcriptase-polymerase chain reaction, and amplicons were visualized on 1.5% agarose gels containing ethidium bromide. To confirm activation of monocytes, TNF-α and interleukin (*IL*)-1β messenger RNAs were also analyzed. Shown data are one representative of at least two (*A*), five (*B*), or three (*C*) independent experiments. *CGRP*, calcitonin gene-related peptide; *bp*, base pairs.

products in PBMCs has been reported by some authors (19–21). However, no ProCT was detected in blood cells subjected to endotoxin and cytokines in a whole blood model (22). In an attempt to approach this problem, we studied ProCT and CGRP expression in adherent PBMCs, PBMC-derived macrophages, and macrophage-activated adipocytes. In addition, CT and CGRP I messenger RNA (mRNA) were analyzed in white blood cells from patients with septic disease and increased serum ProCT.

MATERIALS AND METHODS

Isolation and Culture of Human PBMCs. Buffy coats were obtained from healthy donors. PBMCs were isolated by Ficoll gradient separation and washed three times in cold Hanks' buffered salt solution (Gibco BRL, Basel, Switzerland) supplemented with 0.2% albumin (Blutspendedienst SRK, Bern, Switzerland). Cells were plated in six-well plates (BD Falcon, Bedford, MA) after resuspension in Iscove's-modified Dulbecco's medium (Gibco BRL) containing 20% human pooled serum (PAA laboratories, Linz, Austria) or 10% fetal bovine serum (FBS; Gibco BRL), respectively. In some experiments, 20 units/mL human-specific interleukin (IL)-1β (Peprotech, London, UK) and 1 μg/mL lipopolysaccharide (LPS; Sigma, Buchs, Switzerland) were added. After 1 hr, adherent cells were washed three times with Hanks' buffered salt solution and further kept in culture medium.

Purification and Culture of Monocytes. PBMCs obtained as described previously were subjected to magnetic cell sorting using MACS Monocyte Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Purified monocytes were seeded in six-well plates, repeatedly washed after 1 hr, and used for experiments as described for adherent PBMCs.

Adhesion Experiments on Vascular Endothelial Monolayer. ECV304 endothelial cells (23) and human umbilical vein endothelial cells were activated with 10 ng/mL human-specific tumor necrosis factor (TNF)-α (Peprotech, London, UK) for 5 hrs. Fresh culture medium was supplied, and PBMCs isolated as described previously were subsequently added and allowed to attach for 1 hr. Nonadherent cells were removed by thoroughly washing with Hanks' buffered salt solution. Total RNA was harvested after incubation periods ranging up to 18 hrs.

Macrophage and Adipocyte Co-Culture Experiments. Preadipocytes were isolated from adipose tissue samples and differentiated to adipocytes as previously described (12). Five-day-old PBMC-derived macrophages cultured in cell culture inserts with 0.4-μm pore size (BD Falcon, Bedford, MA) were treated for 2 hrs with combined cytokines/LPS or live *Escherichia coli*. Thereafter, cell culture in-

serts containing the activated macrophages were washed three times and added to six-well plates with adipocytes for 24 hrs. Macrophage and adipocyte total RNAs were isolated separately and subjected to reverse transcriptase-polymerase chain reaction analysis.

Collection of Leukocytes and Adipose Tissue Samples. Leukocytes were isolated by Ficoll gradient separation from infected patients with elevated serum ProCT requiring laparotomy (mean age, 44 yrs; range, 19–65 yrs). In addition, adipose tissue biopsies were obtained from the same patients. The septicemias were due to peritonitis because of perforated sigmoid diverticulitis, ischemic colitis of the sigmoid colon, and necrotizing proctocolitis with perforation of the rectum and descending colon, respectively. As controls, leukocytes and adipose tissue samples were collected from noninfected patients requiring elective surgery (mean age, 53 yrs; range, 29–71 yrs). The procedure was approved by the institutional ethical committee, and informed consent was obtained. Harvested tissues were immediately incubated in RNAlater (Ambion, Austin, TX) to prevent RNA degradation. The samples were snap frozen and stored at −70°C. Tissue ali-

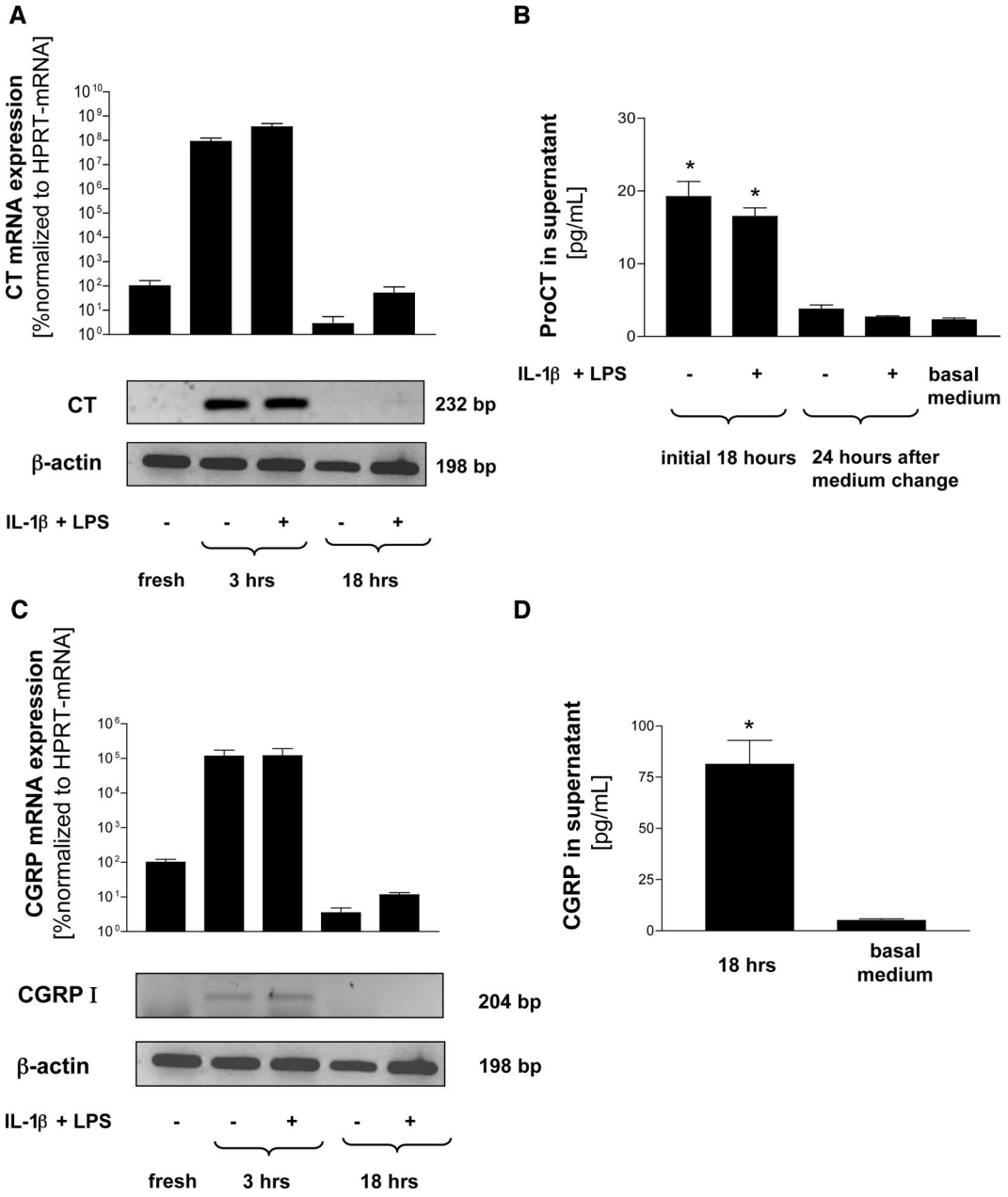

Figure 2. Quantitative analysis of transient calcitonin (*CT*)-I (CALC-I) gene expression in adherent monocytes. Peripheral blood mononuclear cells (PBMCs) were seeded in the presence or absence of combined 20 units/mL interleukin (*IL*)-1β and 1 μg/mL lipopolysaccharide (*LPS*). After 1 hr, nonadherent cells were removed by repeated washing. Fresh medium with supplements was provided. Total RNA was extracted from freshly isolated PBMCs and from adherent monocytes after 3 and 18 hrs of incubation, respectively. Complementary DNA was generated and subjected to calcitonin (*CT*; *A*) and calcitonin gene-related peptide (*CGRP*) I messenger RNA (*mRNA*; *C*) analysis by quantitative real-time polymerase chain reaction using specifically designed primers yielding 92 base pair (*bp*) and 83 bp products, respectively. Results were confirmed by conventional linear range reverse transcriptase-polymerase chain reaction yielding 232 bp and 204 products, respectively, visualized on ethidium bromide agarose gel. Procalcitonin (*ProCT*) secretion into culture supernatants containing human serum was analyzed after 18 hrs of incubation and during the subsequent 24 hrs into fresh medium (*B*). CGRP was analyzed in culture supernatants after 18 hrs of incubation (*D*). Data are mean ± SEM (n = 4). *$p < .05$ compared with basal medium. *HPRT*, hypoxanthine-guanine phosphoribosyltransferase.

quots were powdered under liquid nitrogen before RNA extraction using TRI Reagent (Molecular Research Center, Cincinnati, OH).

RT-PCR. Total RNA was isolated using a commercial reagent TRI Reagent (Molecular Research Center) according to the manufacturer's protocol. Aliquots of 1 μg of RNA were subjected to reverse transcription (Omniscript RT kit, Qiagen, Basel, Switzerland). PCR was performed on a conventional thermal cycler (Whatman Biometra Tgradient, Göttingen, Germany) using the following intron border-spanning oligonucleotides: CT (232 base pair [bp] product, GenBank ×00356): 5'-TGAGCTGGAGCAGGAGCAAG-3' (sense) and 5'-GTTGGCATTCTGGGGCATGCTAA-3' (antisense); CGRP I (204 bp product, GenBank ×02330): 5'-TGAGCTGGAGCAGGAGCAAG-3' (sense) and 5'TCAGCTGCTCAGGCTTGAAGG-3' (antisense); CGRP II (737 bp product, GenBank NM_000728): 5'-CGGCCGCTCGCGCTGCCCTG-3' (sense) and 5'-GGTGGAGCTGCATGATCAAC-3' (antisense); IL-1β (298 bp product, GenBank BC008678): 5'-TTCCCTGCCCACAGACCTTC −3' (sense) and 5'- AGGCCCAAGGCCACAGGTAT-3' (antisense); TNF-α (310 bp product, GenBank ×01394): 5'- GGCCCAGGCAGTCAGATCAT −3' (sense) and 5'- GGGGCTCTTGATGGCAGAGA-3' (antisense); guanosine triphosphate cyclohydrolase (226 bp product, GenBank U19523): 5'- TTGGTTATCTTCCTAACAAG −3' (sense) and 5'-GTGCTGGTCACAGTTTTGCT-3' (antisense); CD-163 (295 bp product, GenBank NM004344): 5'-ACATAGATCATGCATCTGTCATTTG −3' (sense) and 5'- CATTCTCCTTGGAATCTCACT TCTA −3' (antisense); β-actin (198 bp product, GenBank AF076191): 5'-TTCTGACCCATGCCCACCAT-3' (sense) and 5'-ATGGATGATGATATCGCCGCGCTC −3' (antisense). Annealing temperature was 65°C, except for CT (67°C), CGRP I (touchdown 68.8–65°C), and guanosine triphosphate cyclohydrolase (55°C). Thirty-five cycles of PCR were used, except for CGRP I (38 cycles). Cycles were reduced to 28 for β-actin to stop the reaction in the linear phase of amplification. PCR products were separated and visualized on 1.5% agarose gels containing 0.5 μg/mL ethidium bromide. PCR product identity was confirmed by direct nucleotide sequencing of the PCR products by dye deoxy terminator cycle sequencing.

Quantitative Analyses of CT and CGRP I mRNA Expression. Complementary DNA obtained as described previously was subjected to quantitative real-time PCR analysis using the ABI 7000 Sequence detection system (Perkin Elmer, Boston, MA). Specific primers yielding short PCR products suitable for Sybr-Green detection were designed using Primer Express software (version 1.0, PE Applied Biosystems). Sequences of primers were as follows: CT (92 bp product, GenBank ×00356): 3'-GTGCAGATGAAGGCCAGTGA-5' (sense) and 3'-TCAGATTACCACACCGCTTAGATC-5' (antisense); CGRP I (83 bp product, GenBank ×02330): 3'-CCCAGAAGAGAGCCTGTGACA-5' (sense) and 3'-CTTCACCACACCCCCTGATC-5' (antisense); hypoxanthine-guanine phosphoribosyltransferase (HPRT; 85 bp product, GenBank M26434): 3'-TCAGGCAGTATAATCCAAAGATGGT −5' (sense) and 3'-AGTCTGGCTTATATCCAACACTTCG- 5' (antisense). Reaction volume was 22 μL, and the conditions were set as suggested by the manufacturer. Each complementary DNA sample tested for quantitative CT mRNA and CGRP I mRNA was also subjected to HPRT mRNA analysis. Results were expressed as the ratio of CT or CGRP I mRNA with HPRT mRNA threshold values, respectively. Product identity was confirmed by sequence analysis and electrophoresis on a 2.0% agarose gel containing ethidium bromide.

Peptide Measurements. ProCT concentrations were determined in supernatants by an ultrasensitive chemiluminometric assay (ProCa-S Assay, B.R.A.H.M.S. AG, lowest standard 5 pg/mL). Two monoclonal antibodies bind at sites within two peptides: CT and CT carboxy peptide I (previously referred to as katacalcin).

CGRP concentrations were determined in supernatants using CGRP radioimmunoassay Kit (Phoenix Pharmaceuticals, Belmont, CA).

Statistical Analysis. Quantitative data were analyzed using Graph Pad Prism software. Groups of experiments were compared statistically using Mann-Whitney U tests. In addition, multiple comparisons were performed using Kruskal-Wallis test.

RESULTS

CALC I gene was never expressed in freshly isolated PBMCs from healthy, noninfected individuals (Figs. 1 and 2). Upon adhesion to TNF-α-activated ECV304 endothelial cells and umbilical vein endothelial cells, respectively, CT mRNA and CGRP I mRNA were faintly detectable after 3 hrs but not after prolonged (18-hr) incubation (Fig. 1*A*). This transient phenomenon was not specific to adherence on endothelial monolayers as both CALC I and CALC II transcripts were present in PBMCs attached directly to tissue culture dishes for 3–5 hrs but not after 18-hr incubation periods (Fig. 1*B*). Induction of IL-1β and TNF-α mRNA confirmed the activated state of adherent PBMCs between 3 and 18 hrs of culture. In contrast to adherent PBMCs, only trace amounts of CT mRNA were detected in the nonadherent lymphocytic fraction of PBMC (not shown). PBMCs attached to culture dishes are most likely monocytes; thus, the observed transient adhesion-induced expression of CALC genes is most likely a property of monocytes. To confirm this notion, we next evaluated isolated pure monocyte cultures and observed the same pattern of transient adhesion-activated expression of CT and CGRP mRNAs (Fig. 1*C*).

After 3 hrs of incubation, the CT and CGRP I mRNA threshold values normalized to HPRT mRNA obtained by quantitative real-time PCR increased approximately 10^6-fold and 10^3-fold, respectively,

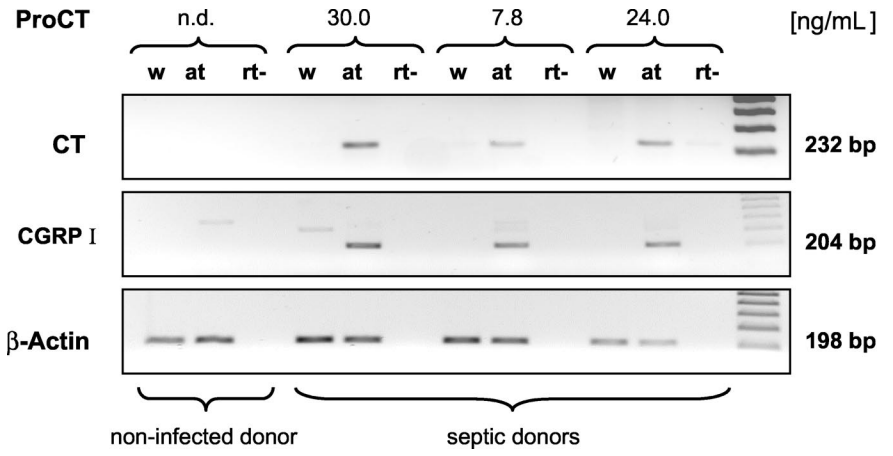

Figure 3. Calcitonin (*CT*) and calcitonin gene-related peptide (*CGRP*) I messenger RNA expression in white blood cells and adipose tissue biopsies from septic patients. CT and CGRP I messenger RNAs were analyzed by reverse transcriptase-polymerase chain reaction in white blood cells (*w*) and adipose tissue (*at*) samples obtained from septic patients with markedly increased serum procalcitonin (*ProCT*) as indicated. Verification of messenger RNA as the source of amplification template was obtained by omitting the reverse transcriptase in reactions for adipose tissue, resulting in no bands after polymerase chain reaction (*rt-*). Amplicons were visualized on 1.5% agarose gel containing ethidium bromide. Serum procalcitonin (*ProCT*) was as indicated in respective patients. *n.d.*, ProCT <0.5 ng/mL. Shown data are one representative out of five noninfected patients and three infected patients with elevated serum procalcitonin. *bp*, base pairs.

compared with freshly isolated PBMCs (Fig. 2, *A* and *C*). CT and CGRP I mRNA concentrations returned to preculture concentrations following 18 hrs of incubation. Accordingly, immunoreactive ProCT accumulated in supernatants only during the first 18 hrs of monocyte culture (Fig. 2*B*), and almost no secretion was observed into fresh culture medium during the subsequent 24-hr incubation. Immunoreactive CGRP accumulated in FBS-containing culture medium during the initial 18-hr culture period (Fig. 2*D*). Due to high background concentrations, culture medium containing human serum did not allow measurement of CGRP concentrations. Culture medium supplemented with FBS does not allow prolonged culturing of human PBMCs. Consequently, CGRP secretion could not be determined in subsequent culture period.

No effects were noted on CT and CGRP mRNA expression and ProCT secretion in the presence of exogenous IL-1β and LPS. Similar results were obtained when experiments were performed in FBS-containing medium instead of human serum (not shown).

In patients with bacterial sepsis and markedly increased serum ProCT concentration, CT and CGRP I mRNAs were not detectable in white blood cells. In contrast, CT and CGRP I mRNA were expressed in adipose tissue biopsies of these patients (Fig. 3).

In contrast to adhesion-activated monocytes, neither CT nor CGRP I mRNA expression could be detected in mature PBMC-derived macrophages (day 5) under basal conditions or after stimulation with various inflammatory mediators (Fig. 4*A*). Expression of CD 163 mRNA confirmed macrophage differentiation (24), and guanosine triphosphate cyclohydrolase I mRNA induction served as a positive control for the inflammatory activation of macrophages (25). Although CT and CGRP I mRNA was not detectable in *E. coli* or cytokines/LPS-activated macrophages themselves, the phagocytes were able to induce a marked expression of both mRNAs in adipocytes. This is demonstrated by co-culture experiments using cell culture inserts with 0.4-μm pores, which allow diffusion of soluble factors (Fig. 4*B*). A $\geq 10^3$-fold increase in CT-mRNA abundance was detected by quantitative real-time PCR in adipocytes kept in co-culture with activated macrophages. CGRP I mRNA was expressed in parallel to CT mRNA in adipocytes. In some experiments, trace amounts of CT and CGRP I mRNA were detected in the macrophages after *E. coli* treatment.

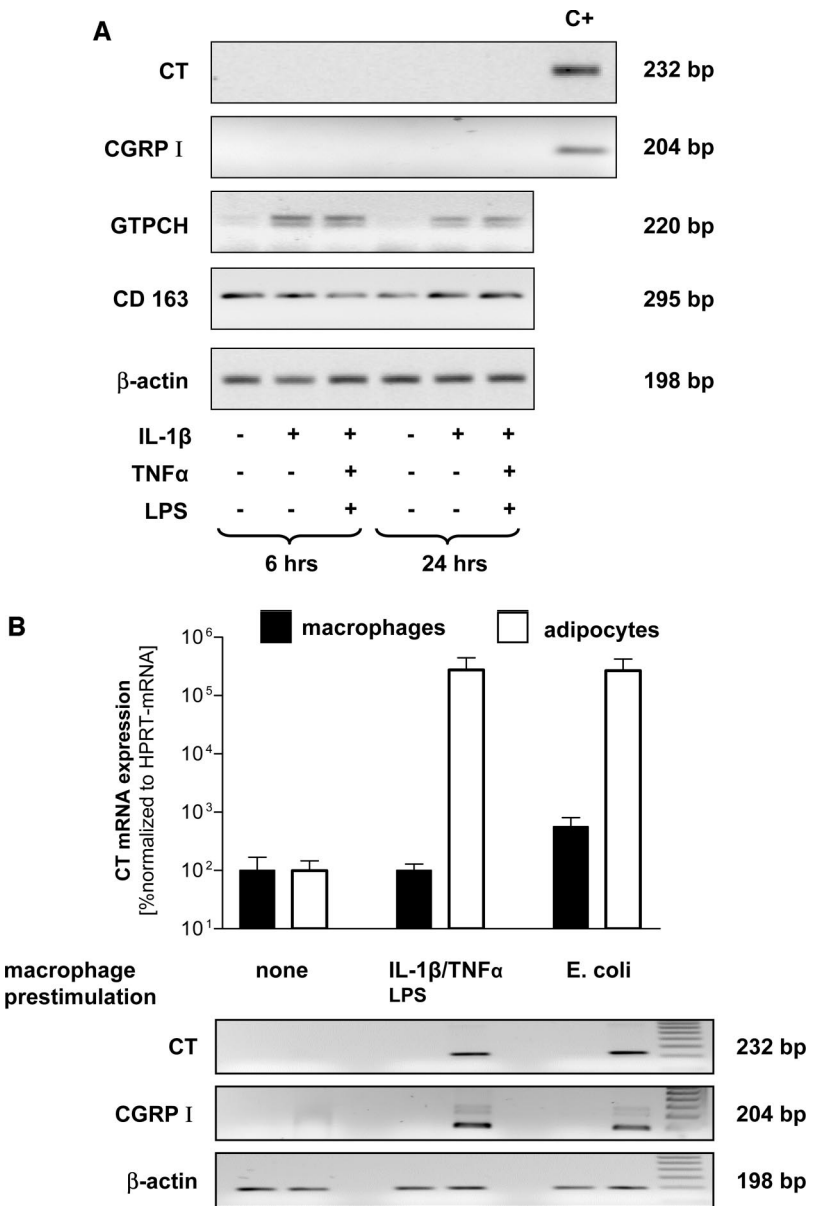

Figure 4. Macrophage-mediated calcitonin (*CT*)-I (CALC-I) gene induction in adipocytes. Five-day-old peripheral blood mononuclear cell-derived macrophages in cell culture inserts with 0.4-μm pores were prestimulated for 2 hrs with cytokines/lipopolysaccharide (*LPS*) or *Escherichia coli* as indicated. Inserts were thoroughly washed and added to nonstimulated adipocytes in six-well plates. After 24 hrs in co-culture, RNAs of macrophages and adipocytes were separately isolated. Complementary DNA obtained by reverse transcription was subjected to CT and calcitonin gene-related peptide (*CGRP*) I messenger RNA (*mRNA*) analysis by reverse transcriptase-polymerase chain reaction. In addition, hypoxanthine-guanine phosphoribosyltransferase (*HPRT*)-normalized quantitative real-time polymerase chain reaction analysis was performed for CT mRNA. Shown data are one representative of six similar experiments (conventional polymerase chain reaction) and mean ± SEM (n = 4) (real-time polymerase chain reaction). *GTPCH*, guanosine triphosphate cyclohydrolase; *IL*, interleukin; *TNF*, tumor necrosis factor; *bp*, base pairs.

DISCUSSION

Monocytes and macrophages represent a relevant source of acute phase mediators. However, the potential role of these cells as sources of sepsis- and inflammation-induced CALC I gene products is controversial (19–22). Herein, we demonstrate that ProCT and CGRP are indeed PBMC-derived secretion factors. However, this phenomenon is transient and occurs exclusively during a short period following attachment of cells. Since

we obtained virtually identical results upon PBMC adhesion to both endothelial monolayers and plastic surfaces, the transient ProCT and CGRP expression described herein does not depend on specific PBMC-endothelial interaction but rather on adhesion-induced activation. The spontaneous expression of CT and CGRP I mRNA in cultured PBMCs was previously reported, and a significant role in sepsis-related increased ProCT and CGRP was proposed (20, 21). However, these reports do not provide data on PBMCs kept in culture for time periods >4 hrs, and questions regarding specific roles of adherent and nonadherent PBMCs were not addressed. Our data clearly demonstrate that purified monocytes transiently express CALC I gene, although contributions from other cell types cannot be ruled out. Detachment of activated monocytes might explain why in some experiments trace amounts of CT mRNA were detected in the nonadherent lymphocytic fraction of PBMCs. In contrast to previous reports (20, 21), we were unable to observe amplifying effects mediated by the addition of exogenous IL-1β and LPS on ProCT and CGRP expressions. Neutralization of TNF-α results in lower ProCT, as measured by flow cytometry in PBMCs (19). Thus, we hypothesize that inflammatory cytokines play a role in the herein demonstrated transient expression and that in our hands, maximal stimulation was already achieved by endogenous cytokine production (e.g., IL-1β, TNF-α). Initially, we performed experiments using 20% human serum allowing prolonged (>24 hrs) culture of adherent PBMCs and to obtain PBMC-derived macrophages. As FBS-supplemented media were used in the previously mentioned PBMC studies, experiments were repeated using this serum. However, no significant differences between human and bovine serum were noted on transient CT and CGRP mRNA expressions in adherent PBMCs during the initial 18-hr incubation period.

PBMCs represent a leukocyte subpopulation and circulate in the vascular bed before potential adherence to an endothelial wall. Thus, we analyzed CALC I gene expression in these cells in humans with septic disease. In accordance with previous reports on septic animals (26–28) and humans (12), the septic patients participating in the present study had markedly elevated serum ProCT, and both CT and CGRP I mRNA were detected in adipose tissue biopsies. Despite the fact that in human sepsis leukocytes are exposed to numerous inflammatory mediators, we found neither CT nor CGRP I mRNA in these cells. This is in accordance with the lack of LPS-stimulated leukocyte ProCT production *in vitro*, as reported in a whole blood model (22). The absence of CT mRNA in white blood cells is in striking contrast to the high expression of classic cytokines (e.g., TNF-α) within these cells during sepsis. Moreover, evidence from case reports of septic patients with high serum concentrations of ProCT, even after near-complete eradication of the leukocyte population by chemotherapy, suggests that white blood cells are not a major source of the increased ProCT concentrations found in human sepsis (29, 30).

PBMC-derived macrophages play important roles in the host defense and are a relevant source of inflammatory mediators. Even though CALC I gene is transiently expressed during the initial phase of differentiation, inflammatory activation of macrophages on day 5 did not induce CT or CGRP I mRNA. In contrast, we demonstrate that in the activated state, macrophages secrete inflammatory mediators that subsequently induce CALC I gene expression in adipocytes kept in co-culture. Thus, macrophages appear to play a role in sepsis-related increased serum ProCT by recruiting and stimulating surrounding parenchyme tissue, as herein exemplified by adipocytes. We recently identified IL-1β and TNF-α as potent inducers and interferon-γ as an effective attenuator of CT mRNA and ProCT in human primary adipocytes (12). Being well-known secretion products of activated macrophages, roles of IL-1β and TNF-α in the previously mentioned process appear likely.

ProCT was proposed to amplify inflammation-mediated nitric oxide synthesis (6) and to promote monocyte chemoattraction (31). CGRP, a well-known neuroendocrine peptide released from perivascular nerve terminals (13), was recognized as the most potent endogenous vasodilating molecule (14, 15). Thus, the PBMC-derived release of immunoreactive ProCT and CGRP described herein suggests a thus far not recognized role of adherent PBMCs in mediating vasodilation and consecutive migration of mononuclear cells into the infected tissue.

The conflicting results of previous studies (19–22) regarding expression of ProCT and CGRP in leukocytes can be explained by the herein described dynamics of CALC I gene expression in PBMCs. Compared with adipose tissue, PBMCs represent a small tissue mass. In addition, the short-lived adhesion-induced CALC I gene expression in PBMCs is in contrast to the sustained ProCT release by IL-1β- and LPS-challenged adipocytes (12). Hence, even though local effects of PBMC-derived ProCT and CGRP can be hypothesized, a significant role of these

Table 1. Trimodal pattern of calcitonin-I gene expression occurring in neuroendocrine cells, parenchymal cells (e.g., adipocytes), and monocytes

	Neuroendocrine	Adipose Tissue	Monocytes
Transcription of mRNA	Constitutive	Regulated; protracted, sustained by cytokines and LPS detectable after >6 hrs	Regulated; rapid, transient upon adhesion detectable after >2 hrs
Splicing of mRNA	Specific; CT mRNA in thyroidal C-cells; CGRP I mRNA in sensory nerves	Nonspecific	Nonspecific
Secretion of protein	Regulated; CT by S-Ca^{2+} or gastrin in thyroid; CGRP by bradykinin, capsain, or heat in sensory nerves	Constitutive; detectable after >10 hrs	Constitutive; detectable after >4 hrs
		Persistent (>24 hrs)	Transient (<18 hrs)
Tissue mass	Low	Very high	Low
Main function	S-Ca^{2+} ↓ (CT); neurogenic inflammation (CGRP)	Sepsis-associated ProCT increase	Local vasodilation (CGRP, ProCT?)

mRNA, messenger RNA; LPS, lipopolysaccharide; CT, calcitonin; CGRP, calcitonin gene-related peptide; ProCT, procalcitonin.

The adhesion-induced, transient expression and secretion of procalcitonin and calcitonin gene-related peptide in vitro *may play an important role during monocyte adhesion and migration* in vivo.

cells as sources of sepsis-related circulating ProCT and CGRP appears unlikely. A major, although indirect, role of macrophages lies in the induction of ProCT and CGRP expression in nearby parenchyme cells, as exemplified by adipocytes. Together with our previously published findings (12, 26, 28), we propose a trimodal pattern of CALC I gene expression occurring in neuroendocrine cells, parenchymal cells (e.g., adipocytes), and monocytes (Table 1). The herein outlined description of the distinct expression pattern of the CALC I gene is helpful in the further understanding of the concept of "hormokines" (10).

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III

Autocrine/paracrine role of inflammation-mediated CGRP and ADM expression in human adipose tissue

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Autocrine/Paracrine Role of Inflammation-Mediated Calcitonin Gene-Related Peptide and Adrenomedullin Expression in Human Adipose Tissue

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Human adipose tissue is a contributor to inflammation- and sepsis-induced elevation of serum procalcitonin (ProCT). Several calcitonin (CT) peptides, including ProCT, CT gene-related peptide (CGRP), and adrenomedullin (ADM) are suspected mediators in human inflammatory diseases. Therefore, we aimed to explore the expression, interactions, and potential roles of adipocyte-derived CT peptide production. Expression of CT peptide-specific transcripts was analyzed by RT-PCR and quantitative real-time PCR in human adipose tissue biopsies and three different inflammation-challenged human adipocyte models. ProCT, CGRP, and ADM secretions were assessed by immunological methods. Adipocyte transcriptional activity, glycerol release, and insulin-mediated glucose transport were studied after exogenous CGRP and ADM exposure. With the exception of amylin, CT peptides were expressed in adipose tissue biopsies from septic patients, inflammation-activated mature explanted adipocytes, and macrophage-activated preadipocyte-derived adipocytes. ProCT and CGRP productions were significantly augmented in IL-1β and lipopolysaccharide-challenged mesenchymal stem cell-derived adipocytes but not in undifferentiated mesenchymal stem cells. In contrast, ADM expression occurred before and after adipogenic differentiation. Interferon-γ coadministration inhibited IL-1β-mediated ProCT and CGRP secretion by 78 and 34%, respectively but augmented IL-1β-mediated ADM secretion by 50%. Exogenous CGRP and ADM administration induced CT, CGRP I, and CGRP II mRNAs and dose-dependently (10^{-10} and 10^{-6} M) enhanced glycerol release. In contrast, no CGRP- and ADM-mediated effects were noted on ADM, TNFα, and IL-1β mRNA abundances. In summary, CGRP and ADM are two differentially regulated novel adipose tissue secretion factors exerting autocrine/paracrine roles. Their lipolytic effect (glycerol release) suggests a metabolic role in adipocytes during inflammation. (*Endocrinology* 146: 2699–2708, 2005)

THE CALCITONIN (CT) gene family presumably evolved from a common ancestor gene because calcitonin peptides share amino acid homologies and display related receptor binding and biological activities (1). The calcitonin I (CALC I) gene gives rise to two distinct peptides by tissue-specific alternative splicing of the pre-mRNA: CT encoded in exon 4 in thyroidal C-cells and calcitonin gene-related peptide (CGRP) I encoded in exons 5 and 6 in sensory nerves (2). CALC II produces CGRP II but not CT because exon 4 contains a stop codon (1). CALC III is a pseudogene (3). Adrenomedullin (ADM) is encoded by CALC IV and amylin by CALC V genes (1).

CT was named after its postulated role in calcium homeostasis (4). CGRP I and II are almost identical neurotransmitters with potent vasodilatory properties (5). ADM, another vasoactive peptide, was originally discovered in human pheochromocytoma (6). Amylin is a 37-amino acid peptide hormone that is cosecreted with insulin by the pancreatic β-cells in response to a nutrient stimulus (7). CT-, CGRP-, ADM-, and amylin-mediated signaling occurs via calcitonin receptor (CTR) and CR-like receptor (CRLR) with receptor activity-modifying proteins (RAMPs) 1–3 determining specific binding (8).

Serum concentrations of CT peptides, including procalcitonin (ProCT), CGRP, and ADM are markedly elevated in severe inflammation, systemic infections, sepsis, and sepsis-like conditions (9–11). Importantly, recent data suggest deleterious sepsis-related effects of ProCT, CGRP, and ADM (12–16). In contrast to the classic neuroendocrine paradigm, CT and CGRP I mRNA are ubiquitously expressed in septic hamsters and baboons (17–19). In humans, we recently demonstrated nonneuroendocrine CALC I expression and ProCT secretion in adipose tissue of sepsis patients and cytokine-challenged primary adipocytes (20, 21). Herein we sought to further elucidate the dynamics and metabolic effects of inflammation- and sepsis-related CGRP and ADM production in human adipose tissue. Because primary adipocyte availability is often scarce, mesenchymal stem cell (MSC)-derived adipocytes were also used as a model of functional adipocytes (22). Specifically, we aimed to clarify whether different CT peptides are differentially expressed and secreted on inflammatory stimulation and whether their production is dependent on the differentiation status. In addition, we studied transcriptional activity, glycerol release, and insulin-mediated glucose transport in adipocytes after exposure to exogenous CGRP and ADM.

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Abbreviations: ADM, Adrenomedullin; CALC I, calcitonin I; CGRP, CT gene-related peptide; CRLR, CR-like receptor; CT, calcitonin; CTR, calcitonin receptor; FBS, fetal bovine serum; IFN, interferon; LPS, lipopolysaccharide; MSC, mesenchymal stem cell; NEP, neutral endopeptidase; PBMC, peripheral blood mononuclear cell; PPAR, peroxisomal proliferator-activated receptor; ProCT, procalcitonin; RAMP, receptor activity-modifying protein.

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Materials and Methods

Adipocyte cultures

Human mature explanted adipocytes and preadipocyte-derived adipocytes were obtained as previously described (20).

Human MSCs between passages 4 and 10 (Cambrex Bio Science Verviers, S.p.r.l., Verviers, Belgium) were seeded in 6-well plates and grown in MSC basal medium (Cambrex) until confluent. Adipogenic differentiation was induced by incubating the cells in DMEM/F12 (Invitrogen, Basel, Switzerland) containing 3% fetal bovine serum (FBS, Invitrogen) and the following supplements: 250 μM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, 0.2 nM 3,3,5-triiodo-L-thyronine, 5 μM transferrin (all from Sigma, Buchs, Switzerland), 100 nM insulin (Novo Nordisk, Küsnacht, Switzerland), 1 μM rosiglitazone (GlaxoSmithKline, Worthing, UK). After 15–18 d, supplements were removed by washing three times with warm PBS, and experiments were started 2–4 d after completing differentiation. MSC-derived adipocytes had visible lipid droplets and expressed adipocyte-specific mRNAs, *e.g.* peroxisomal proliferator-activated receptor (PPAR)γ2, leptin, adiponectin, or GLUT4. IL-1β and lipopolysaccharide (LPS) administration induced glycerol release and glucose uptake was stimulated by insulin addition.

Adipocytes were subjected to treatments using the following agents: 100 U/ml interferon (IFN)-γ, 10 ng/ml TNFα, 20 U/ml IL-1β (all human-specific cytokines from PeproTech, London, UK). CGRP, CGRP(8–37), ADM, and ADM(22–52) were purchased from Phoenix Europe GmbH (Karlsruhe, Germany), LPS (*Escherichia coli* 026:B6), and cycloheximide (ready made) were from Sigma.

Viability of adipocytes after stimulation was assessed with trypan blue staining: viable cells excluding trypan blue; dead cells staining blue.

Adipocytes and macrophages in coculture

White blood cells were isolated by Ficoll-Paque PLUS (Amersham, Uppsala, Sweden) and used for adipocyte macrophages coculture experiments as previously described (21).

RT-PCR

Total RNA from homogenized tissues or adipocyte cultures was extracted by the single-step guanidinium-isothiocyanate method with a commercial reagent (Tri Reagent; Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's protocol. Extracted RNA was quantified spectrophotometrically at 260 nm (BioPhotometer; Vaudaux-Eppendorf AG, Schönenbuch, Switzerland). Ratio of extinction at 260 and 280 nm was between 1.5 and 2.0, and the quality was assessed by gel electrophoresis. One microgram total RNA was subjected to reverse transcription (Omniscript reverse transcription kit; QIAGEN, Basel, Switzerland). PCR was performed on a conventional thermal cycler (Tgradient; Biometra, Göttingen, Germany) using PCR Taq core kit (QIAGEN). Human gene-specific primers and conditions were as indicated in Table 1. Amplification products were separated and visualized on 2% agarose gels containing 0.5 μg/ml ethidium bromide. A 100-bp molecular ruler (Bio-Rad Laboratories, Reinach, Switzerland) was run as reference marker. PCR product identity was confirmed by direct nucleotide sequencing of the PCR products by dye deoxy terminator cycle sequencing.

Quantitative analysis of CT, CGRP, and ADM mRNA expression

cDNA obtained as described above was subjected to quantitative real-time PCR analysis using the ABI 7000 sequence detection system (PerkinElmer, Norwalk, CT). Specific primers yielding short PCR products suitable for Sybr-Green detection were designed using Primer Express software (version 1.0; Applied Biosystems, Rotkreuz, Switzerland). Sequences of primers were as indicated in Table 1. Reaction volume was 22 μl, and the conditions were set as suggested by the manufacturer. For quantitative mRNA estimations, results were expressed as the ratio of the respective CT mRNA, CGRP mRNA, and ADM mRNA, respectively, to hypoxanthine-guanine phosphoribosyltransferase mRNA threshold values. Product identity was confirmed by sequence analysis and electrophoresis on a 2.5% agarose gel containing ethidium bromide.

Protein measurements

MSC-derived adipocytes were incubated for 48 h with treatments. Supernatant aliquots were kept at −70 C until analyzed.

ProCT concentrations were determined in supernatants by an ultrasensitive chemiluminometric assay with a functional sensitivity of 6 pg/ml (ProCa-S assay; B.R.A.H.M.S. GmbH, Hennigsdorf-Berlin, Germany).

ADM and CGRP concentrations were determined in supernatants by human-specific RIAs (Phoenix Europe). The CGRP kit (IC_{50} 10–20 pg/

TABLE 1. List of human gene-specific primers in RT-PCR analysis

mRNA	Sense primer	Antisense primer	Size	GeneBank no.	Annealing temp (C)	No. of cycles
		Conventional PCR				
CT	TGAGCTGGAGCAGGAGCAAG	GTTGGCATTCTGGGGCATGCTAA	232	X00356	67	35
CGRP I	TGAGCTGGAGCAGGAGCAAG	TCAGCTGCTCAGGCTTGAAGG	204	X02330	65	35
CGRP II	CGGCCGCTCGCGCTGCCCTG	GGTGGAGCTGCATGATCAAC	737	NM_000728	65	35
ADM	CGGATGTCCAGCAGCTACCC	GTAGCCCTGGGGGCTGATCT	300	NM_001124	65	28
Amylin	TGAGAAGCAATGGGCATCCTG	GGGGCAAGTAATTCAGTGGCTCT	273	X68830	65	35
PPARγ2	GCGATTCCTTCACTGATAC	GCATTATGAGACATCCCCAC	580	NM_015869	55	28
IL-6	GCAAAGAGGCACTGGCAGAAA	CAGGCTGGCATTTGTGGTTG	284	M54894	60	25
TNFα	GGCCCAGGCAGTCAGATCAT	GGGGCTCTTGATGGCAGAGA	310	M10988	65	35
IL-1β	TTCCCTGCCCACAGACCTTC	AGGCCCAAGGCCACAGGTAT	298	BC008678	65	35
CTR	TGACCGAATGCAGCAGTTAC	TTCAACCAGGTGGATGATGA	492	NM_001742	65	35
CRLR	ACCAGGCCTTAGTAGCCACA	ACAAATTGGGCCATGGATAA	296	NM_005795	65	35
RAMP1	CTGCCAGGAGGCTAACTACG	GACCACGATGAAGGGGTAGA	298	NM_005855	65	30
RAMP2	GGGGGACGGTGAAGAACTAT	GTTGGCAAAGTGGATCTGGT	227	NM_005854	65	30
RAMP3	AACTTCTCCCGTTGCTGCT	GACGGGTATAACGATCAGCG	165	NM_005856	65	35
NEP	TTGTAGGTTCGGCTGAGGCT	TGTGGCCAGATTGATTCGTC	410	NM_007289	65	35
β-actin	TTCTGACCCATGCCCACCAT	ATGGATGATGATATCGCCGCGCTC	198	NM_001101	65	28
		Quantitative real-time PCR				
CT	GTGCAGATGAAGGCCAGTGA	TCAGATTACCACACCGCTTAGATC	92	X00356	60	45
CGRP I	CCCAGAAGAGAGCCTGTGACA	CTTCACCACACCCCCTGATC	83	X02330	60	45
ADM	ACTTGGCAGATCACTCTCTTAGCA	ATCAGGGCGACGGAAACC	72	NM_001124	60	45
HPRT	TCAGGCAGTATAATCCAAAGATGGT	AGTCTGGCTTATATCCAACACTTCG	85	M26434	60	45

HPRT, Hypoxanthine-guanine phosphoribosyltransferase.

tube) cross-reacts 0.09% with amylin and 0.004% with ADM. The ADM kit (IC_{50} 6–20 pg/tube) cross-reacts 0.032% with CGRP I as indicated by the manufacturer.

Glycerol release into culture medium

On d 1 differentiation medium was removed from MSC-derived adipocytes. Cells were washed three times in warm PBS and kept in phenol red-free DMEM:F12 containing 3% FBS. Medium was changed on d 2 and supplements were added. On d 3, 800-μl supernatant aliquots were collected and kept frozen at −20 C until used for glycerol measurement (glycerol UV-method, Roche Molecular Biochemicals/R-Biopharm, Darmstadt, Germany).

Insulin-mediated glucose uptake

On d 1 differentiation medium was removed from MSC-derived adipocytes. Cells were washed three times in warm PBS and kept in DMEM/F12 containing 5 mM glucose and 3% FBS. Supplements were added on d 2. On d 3 at t = 0 min, 100 nM insulin were added to some wells. At t = 20 min, 1 μC deoxy-D-glucose, 2-[^{3}H(G)] [PerkinElmer (Schweiz) AG, Schwerzenbach, Switzerland] was added to all wells. After 15 min cells were washed three times in ice-cold PBS and lysed in 0.1% sodium dodecyl sulfate. Radioactivity was measured in a scintillation counter.

Patients

Adipose tissue biopsies were obtained from healthy patients undergoing abdominal plastic surgery. Adipose tissue samples were also obtained from four infected patients, as documented with elevated serum ProCT (23), requiring laparotomy (mean age 44 yr, range 19–65 yr). The septicemia were due to peritonitis because of perforated sigmoid diverticulitis, perforated appendicitis, ischemic colitis of the sigmoid colon, and necrotizing proctocolitis with perforation of the rectum and descending colon. In addition, adipose tissue was collected from noninfected patients requiring elective surgery (mean age 53 yr, range 29–71 yr). Informed consent was obtained. Harvested tissues were immediately incubated in RNA-later (Ambion, Inc., Austin, TX) to prevent RNA degradation. The samples were snap frozen and stored at −70 C. Tissues were powdered under liquid nitrogen before RNA extraction.

All patients gave written informed consent to participate in the study. The study protocol was reviewed and approved by the Human Ethics Committee of Basel.

Statistical analysis

Results are presented as means ± SEM. Groups of experiments were compared statistically using Student's *t* tests. In addition, two group comparisons corrected for multiple testing, *i.e.* one-way ANOVA with *post hoc* analysis for least-square differences, were performed.

Results

Sepsis-induced expressions of CT mRNAs in adipose tissue

CGRP I, CGRP II, and ADM mRNAs were detected in adipose tissue biopsies obtained from four septic patients with elevated serum ProCT (Fig. 1A). In contrast, the respective transcripts were not detected in normal, noninfected subjects (Fig. 1B). Amylin mRNA was found in neither control nor septic adipose tissue biopsies.

Inflammation-induced expressions of CT mRNAs in primary adipocytes

Expressions of CGRP I, CGRP II, and ADM mRNAs were analyzed by RT-PCR in explanted mature adipocytes kept in ceiling cultures (20). Endothelial cells represent an important fraction of nonadipose cells in adipose tissue (24). Previously published PCR primers for endothelial nitric oxide synthase mRNA (25) showed expression in whole adipose tissue but not adipocytes (not shown). This verified the absence of contaminating stromal cells in explanted mature adipocyte preparations. Compared with basal conditions, CGRP I and ADM mRNA expression were markedly induced after 24 h of exposure to TNFα, IL-1β, and LPS treatments, respectively (Fig. 2). In some experiments a weak inflammatory CGRP II mRNA induction was also observed. In contrast, amylin mRNA was never detected in mature adipocytes.

Next, we studied CT peptide gene induction in preadipocyte-derived adipocytes cocultured with peripheral blood mononuclear cell (PBMC)-derived macrophages. In some experiments coincubation with unstimulated macrophages resulted in weak induction of CGRP I, CGRP II, and ADM mRNAs in adipocytes. Conversely, adipocyte CGRP I, CGRP II, and ADM mRNA expressions were markedly increased when macrophages were activated with live *E. coli* before coculture with adipocytes. ADM mRNA was detected in macrophages but not CGRP I and CGRP II mRNAs. Amylin mRNA was detected in neither adipocytes nor macrophages.

Inflammatory CT peptide expression and secretion in MSC-derived adipocytes

Production of CT peptides was analyzed in MSCs before and after adipogenic differentiation. PPARγ2 mRNA, an adipocyte-specific marker, was present in MSC-derived adipocytes but not in undifferentiated MSCs (Fig. 3). ADM mRNA was induced by IL-1β, LPS, or combined IL-1β/TNFα/LPS treatments in undifferentiated MSCs but not the mRNAs of CT, CGRP I, and CGRP II. In contrast, cytokine treatment of MSC-derived adipocytes induced CT, CGRP I, and CGRP II as well as ADM mRNA expression. Amylin mRNA was detected in neither MSCs nor MSC-derived adipocytes.

In addition to conventional PCR, abundance of CT, CGRP I, and ADM mRNAs on inflammatory treatments was analyzed in MSC-derived adipocytes by quantitative real-time PCR. Compared with controls, CT mRNA was increased 79-fold ($P < 0.05$) on IL-1β treatment, 111-fold ($P < 0.05$) on LPS treatment, and 61-fold ($P < 0.05$) on combined IL-1β/TNFα/LPS treatment (Fig. 4A). CGRP I mRNA was increased 1.4-fold on IL-1β treatment, 2.4-fold ($P < 0.05$) on LPS treatment, and 3.2-fold ($P < 0.05$) on combined IL-1β/TNFα/LPS treatment. ADM mRNA was increased 8.4-fold on IL-1β-treatment, 18-fold on LPS treatment, and 80-fold ($P < 0.05$) on combined IL-1β/TNFα/LPS treatment.

Concentrations of ProCT, CGRP, and ADM in basal culture supernatants after 48 h were 9.7 ± 1.4, 11.1 ± 0.8, and 221 ± 31.8 pg/ml, respectively (Fig. 4B). ProCT secretion was increased 4.8-fold to 47.4 ± 8.0 pg/ml ($P < 0.05$) on IL-1β treatment, 5.0-fold to 48.2 ± 3.3 pg/ml ($P < 0.05$) on LPS treatment, and 4.5-fold to 43.9 ± 2.6 pg/ml ($P < 0.05$) on combined IL-1β/TNFα/LPS treatment. CGRP protein secretion was increased 2.5-fold to 28.7 ± 1.8 pg/ml ($P < 0.05$) on IL-1β treatment, 3.2-fold to 35.3 ± 2.2 pg/ml ($P < 0.05$) on LPS treatment, and 4.1-fold 45 ± 1.0 pg/ml ($P < 0.05$) on combined IL-1β/TNFα/LPS treatment. ADM protein secretion was increased 3.1-fold to 683 ± 40.9 pg/ml ($P < 0.05$) on IL-1β treatment, 3.7-fold to 815 ± 50.1 pg/ml ($P < 0.05$) on

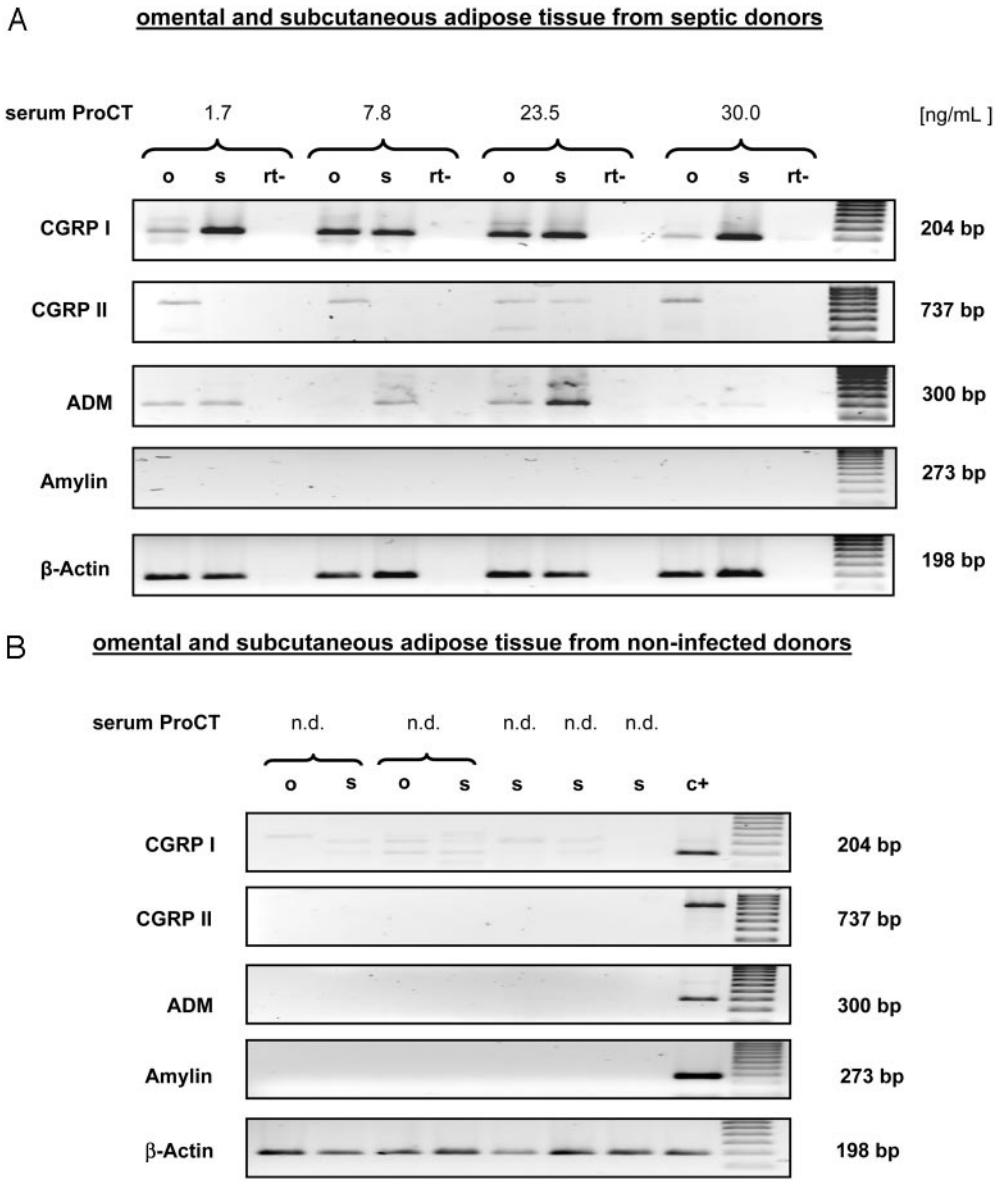

FIG. 1. CGRP I, CGRP II, and ADM mRNA expressions in adipose tissue biopsies of septic patients. Omental (o) and sc (s) adipose tissue biopsies were obtained from four septic patients and subjected to RT-PCR analysis for CT peptide transcripts (A). Serum ProCT level of each donor is indicated on top. Samples from noninfected donors were used as controls (B). n.d., Not detected; rt−, no reverse transcriptase controls to exclude genomic DNA contamination; c+, positive control.

LPS treatment, and 6.8-fold to 1495 ± 75.1 pg/ml ($P < 0.05$) on combined IL-1β/TNFα/LPS treatment.

IFNγ administration alone had no influence on CT peptide expression and secretion (Figs. 3 and 4). IL-1β-induced CT, CGRP, and CGRP II mRNAs were potently blocked when IFNγ was coadministered. Accordingly, IL-1β-mediated ProCT and CGRP release was inhibited by 78% to 10.1 ± 1.5 pg/ml ($P < 0.05$) and 34% to 18.6 ± 4.2 pg/ml, respectively, by IFNγ coadministration (Fig. 4B). In contrast, combined IFNγ and IL-1β administration resulted in 4.0-fold increased ADM mRNA abundance, compared with IL-1β alone. Accordingly, IL-1β-mediated ADM secretion into culture supernatants was increased 2.0-fold to 1347 ± 64.2 pg/ml ($P < 0.05$) in the presence of IFNγ.

Combined IL-1β/TNFα/LPS treatment evoked maximal CGRP I and ADM induction and was therefore chosen for short-term induction analysis. CT, CGRP I, and CGRP II mRNAs were present after 2-h incubations (Fig. 4C). Low-level ADM mRNA was markedly augmented after 1 h.

Distinct CGRP- and ADM-mediated autoregulatory and metabolic effects in adipocytes

mRNAs of CTR, CRLR, RAMP1, RAMP2, and RAMP3 as well as neutral endopeptidase (NEP) were detected by RT-PCR in basal MSC-derived adipocytes (Fig. 5A). Exogenous CGRP and ADM were administered to MSC-derived adipocytes in concentrations ranging from 10^{-10} to 10^{-6} M. After 24 h, induction of CT, CGRP I, and CGRP II mRNA was observed, even at low concentrations (Fig. 5B). In contrast,

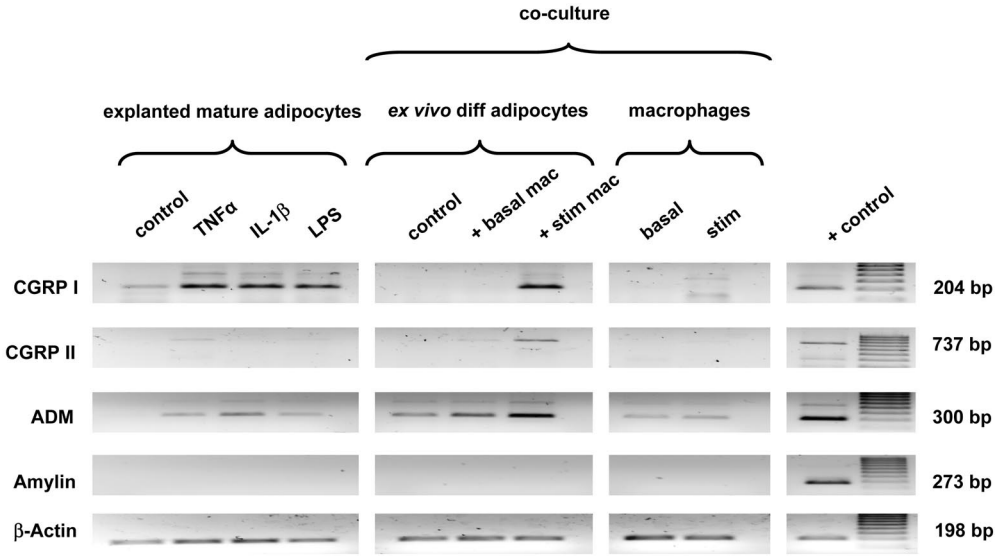

FIG. 2. Inflammatory CGRP I, CGRP II, and ADM mRNA inductions in human primary adipocytes. Human explanted adipocytes were subjected to 24 h TNFα, IL-1β, and LPS treatments, respectively. Preadipocyte-derived adipocytes were kept in coculture with activated PBMC-derived macrophages for 24 h. Total RNA was separately isolated and induction of CT peptide transcripts was analyzed by RT-PCR analysis. mac, Macrophages. Shown results are representatives of three independent experiments.

the same mRNAs were not induced in adipocytes exposed to truncated peptides CGRP(8–37) and ADM(22–52). One-nanomolar ADM-mediated mRNA inductions were blocked by 1 h preadministration of 1 μM CGRP(8–37) and ADM(22–52), alone or in combination (Fig. 5C). One-micromolar ADM(22–52) did not antagonize mRNA inductions when 10 nM ADM was added instead of 1 nM (not shown). Ten-nanomolar CGRP-mediated mRNA inductions were blocked by 1 μM CGRP(8–37) but not by 1 μM ADM(22–52). IL-6 mRNA was induced by ADM at high concentrations but not by CGRP (Fig. 5B). mRNAs of ADM, IL-1β, and TNFα mRNAs were induced by 1 ng/ml LPS but not by 10^{-6} M CGRP or ADM (Fig. 5D).

In preliminary experiments, 6 h had been determined as the minimal incubation time needed for detecting CT peptide mRNA induction in MSC-derived adipocytes treated with IL-1β alone. Therefore, possible effects of cycloheximide-mediated protein synthesis inhibition on CT peptide mRNA induction were studied after 6-h incubation periods. Five micromalar cycloheximide alone had no effect on CT mRNA induction (Fig. 5E). When coadministered to 100 nM ADM, 100 nM CGRP, or 20 U/ml IL-1β, a

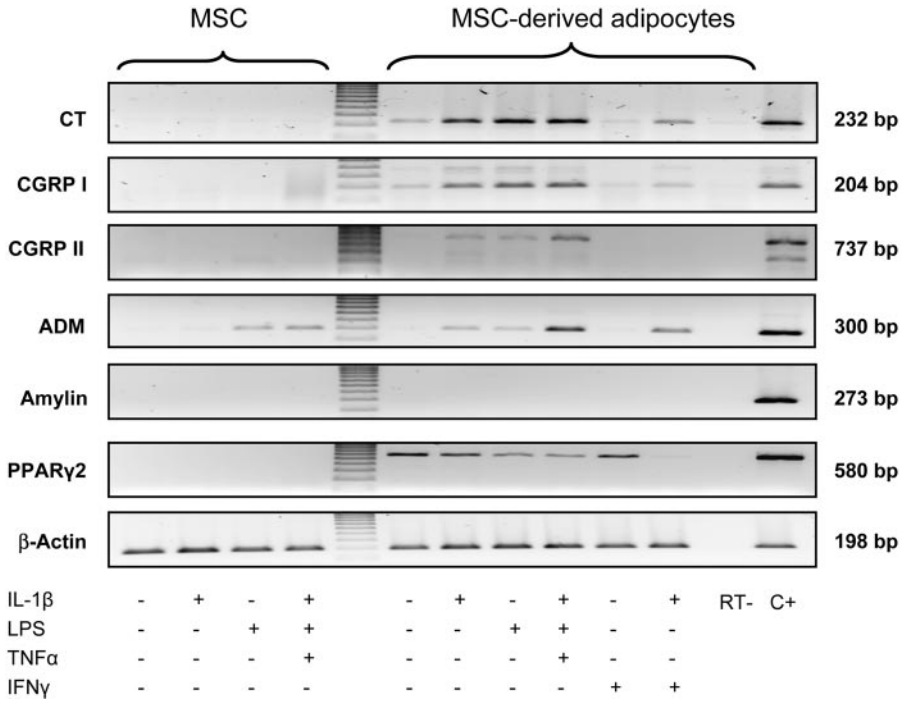

FIG. 3. CT peptide mRNA inductions in MSCs and MSC-derived adipocytes. Total RNA was isolated from MSCs and MSC-derived adipocytes on 24-h inflammatory treatments as indicated and reverse-transcribed. cDNA was subjected to RT-PCR analysis. Shown results are representatives of four independent experiments. RT−, Negative control lacking reverse transcriptase; C+, positive control.

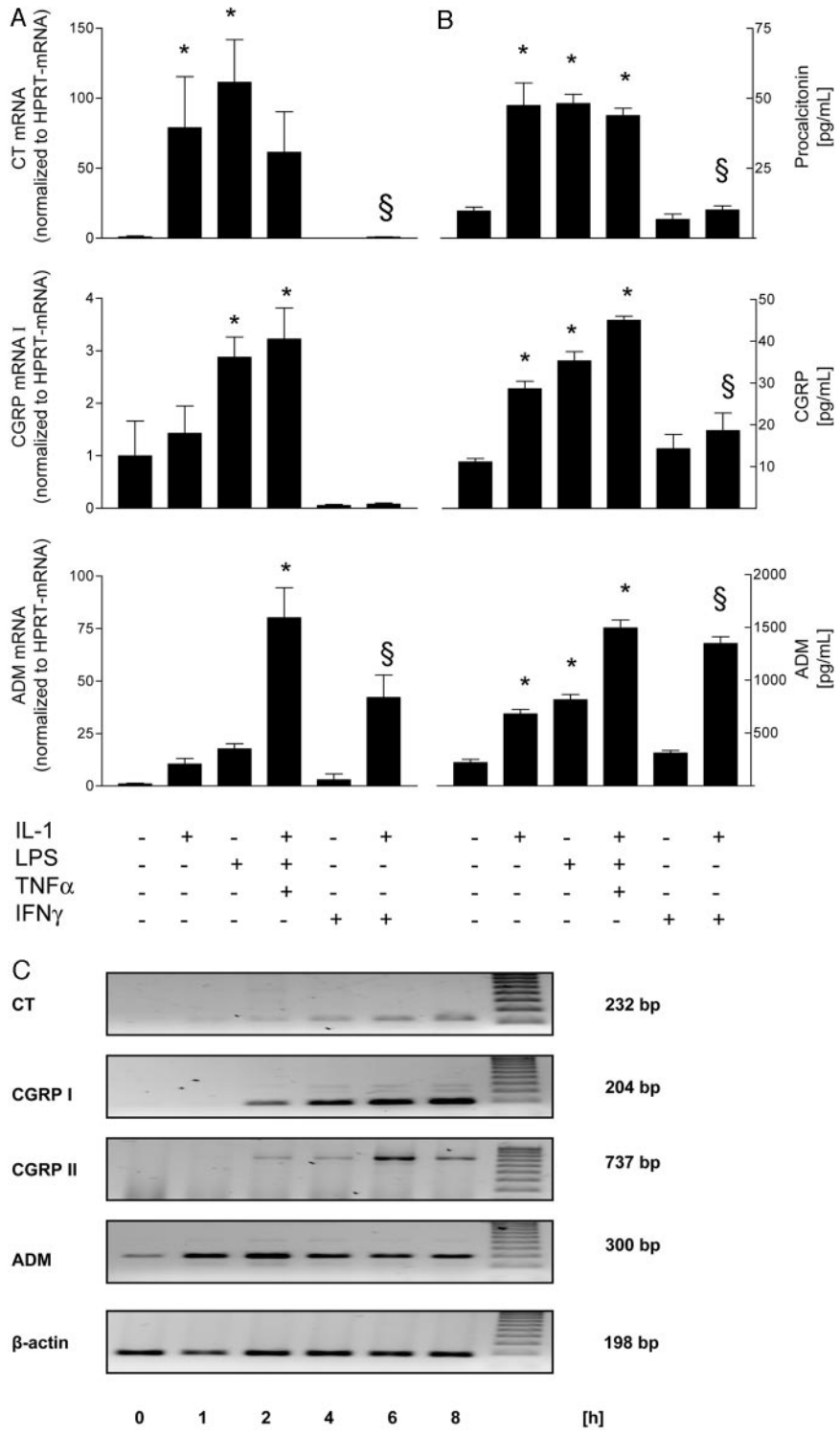

FIG. 4. Quantitative analysis of inflammation-induced ProCT, CGRP, and ADM productions. MSC-derived adipocytes were subjected to treatments as indicated. Abundance of CT, CGRP I, and ADM mRNAs was analyzed after 48 h by quantitative real-time PCR using primers specifically designed for sybr green detection (A). Release of corresponding peptides was determined by supersensitive chemiluminometric assay (ProCT) or RIA (CGRP and ADM) (B). CT peptide mRNA inductions by combined IL-1β/TNFα/LPS were analyzed by RT-PCR after short incubations up to 8 h. Shown results are means ± SEM from at least three independent experiments (A, B) or representatives from four independent observations (C). *, $P < 0.05$, compared with controls; §, $P < 0.05$ compared with IL-1β alone.

potentiating effect was noted on CT, CGRP I, CGRP II, and ADM mRNAs.

Lipolytic activity was assessed by measuring glycerol release into culture medium. Addition of CGRP and ADM between 1 nM and 1 μM evoked a dose-dependent activation of lipolysis. In contrast, addition of truncated peptides CGRP(8–37) or ADM(22–52) did not induce lipolysis (Fig. 6). However, preadministration of 1 μM CGRP(8–37) or ADM(22–52) alone or in combination did not antagonize ADM and CGRP effects on lipolysis.

Potential effects of CGRP and ADM on insulin sensitivity were studied by comparing basal and insulin-mediated glucose uptake after their administration. Adipocytes were relatively insulin resistant on 24 h TNFα exposure (Fig. 7). Coadministered CGRP or ADM did not further impair insulin action. In some experiments, presence of CGRP and

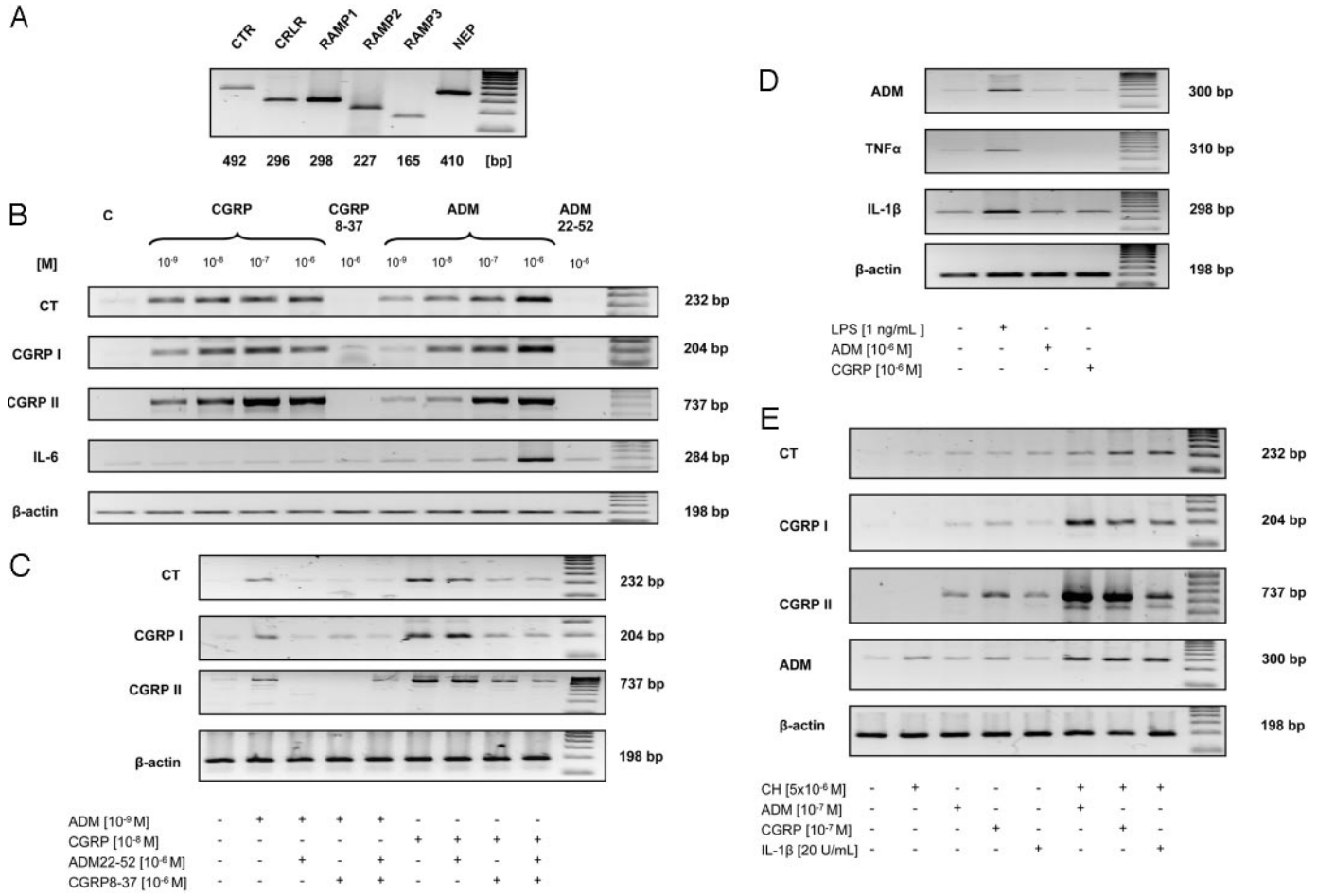

FIG. 5. CGRP- and ADM-mediated effects in MSC-derived adipocytes. MSC-derived adipocytes were subjected to RT-PCR analysis of genes known to mediate CT peptide-signaling (A). CT peptide mRNAs were analyzed by RT-PCR in MSC-derived adipocytes after 24 h exposure to indicated concentrations of exogenous CGRP and ADM (B). To test specificity of observations, truncated peptides CGRP(8–37) and ADM(22–52) were administered alone (B) or in combination with ADM and CGRP (C). Effect of high CGRP and ADM concentrations, respectively, were analyzed on ADM, TNFα, and IL-1β mRNAs (D). One nanogram per milliliter LPS was administered alone as a positive stimulation control. Protein synthesis was blocked by cycloheximide (CH) administration in CGRP-, ADM-, and IL-1β-treated cells, and RT-PCR analysis was performed after 6-h incubations (E). Shown results are representative of at least three separate experiments.

ADM alone resulted in reduced insulin-mediated glucose uptake. However, statistical significance was not reached.

Discussion

Herein we demonstrate the sepsis-related production and potential functions of the two novel human adipose tissue secretion products CGRP and ADM. Lethal outcome of sepsis correlates with high serum levels of CT peptides including ProCT, CGRP, and ADM (15, 16, 23). Septic shock, a major cause of mortality and morbidity in intensive care units, is mainly characterized by hypotension and multiple organ failure, and the underlying cellular and molecular mechanisms are still poorly understood (26). Recent *in vivo* data suggest that high ProCT concentrations mediate deleterious effects: ProCT immunoneutralization increased survival rate in septic hamsters and pigs, whereas exogenous ProCT administration worsened the outcome (12–14). Immunomodulating properties of ProCT might be partially responsible for these observations (27, 28). Further activities to be unveiled might include some of the numerous properties found for other CT peptides with which ProCT shares a common structure. CGRP and ADM are potent vasodilators and modulators of nitric oxide production (29–32). In addition, pharmacological CGRP and amylin doses induce insulin resistance in rat model *in vivo* and *in vitro* (33, 34).

We recently identified human adipose tissue depots as major sepsis-related nonneuroendocrine CT mRNA expression sites leading to increased serum ProCT (20, 21). By using adipose tissue biopsies from septic patients and three different human adipocyte models, we extend our previous findings on CGRP and ADM production in response to inflammatory stimuli. To assure that the observed cytokine-mediated CT peptide mRNA expressions do indeed occur in adipocytes, experiments were successfully performed in adipocyte ceiling cultures obtained by negative buoyancy selection of lipid-laden cells. The limited availability of primary adipose cell cultures was overcome by using bone marrow-derived MSCs as adipocyte precursors (22). In accordance with our first report on *in vitro* ProCT expression in human primary adipocytes (20), IL-1β and LPS potently induced

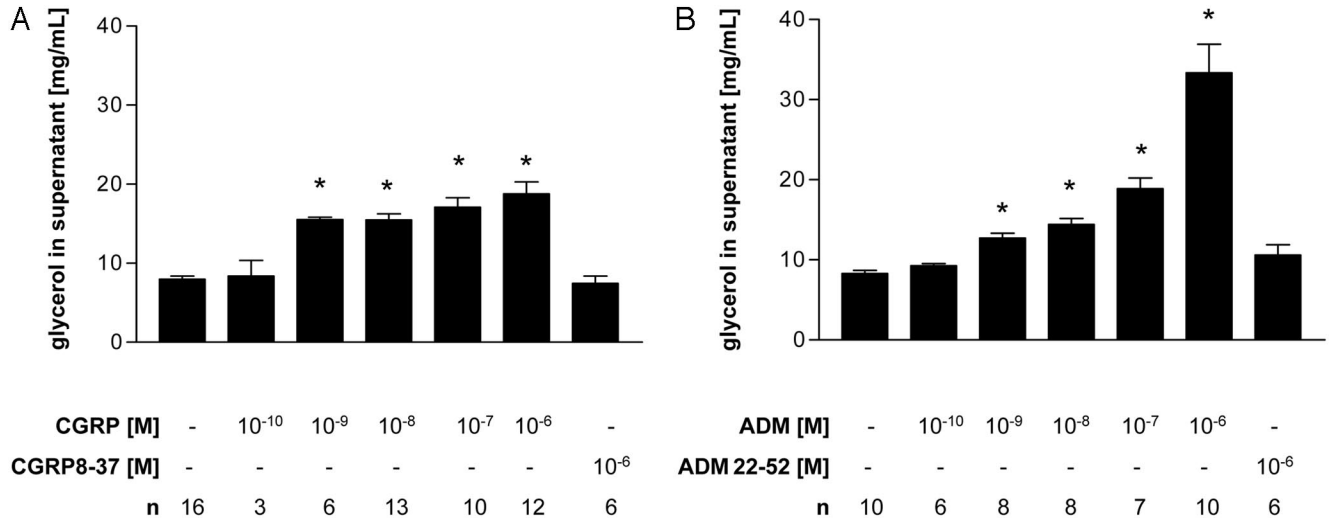

FIG. 6. CGRP- and ADM-mediated lipolysis induction in MSC-derived adipocytes. MSC-derived adipocytes were subjected to exogenous CGRP and ADM exposure between 1 nM and 1 μM for 24 h. Release of glycerol into supernatant was measured as an indicator of lipolytic activity. Results are presented as means ± SEM. n, Number of separate wells.

CALC I gene expression in MSC-derived adipocytes. Similar kinetics of CT peptide mRNA induction was observed in both adipocyte models: IL-1β-induced CT mRNA was detectable after at least 6-h incubations and long lasting ($t >$ 48 h). Furthermore, the previously shown potent inhibition by IFNγ on ProCT production was herein confirmed in MSC-derived adipocytes (20). In addition, the present data extend IFNγ-mediated gene repression to CGRP I and CGRP II.

Undifferentiated MSCs as well as preadipocytes do not respond with ProCT and CGRP production on any inflammatory treatment. This observation indicates that sepsis-related ProCT and CGRP production in adipose tissue depends on final differentiated adipocytes. We previously reported tissue-wide CT and CGRP mRNA expression (17, 18) in septic hamsters. The precise cellular origin and mechanisms of ProCT and CGRP production in nonadipose tissues will need to be addressed in future studies, but based on our findings, it is likely that the widespread up-regulation of CT and CGRP mRNA is limited to differentiated cells.

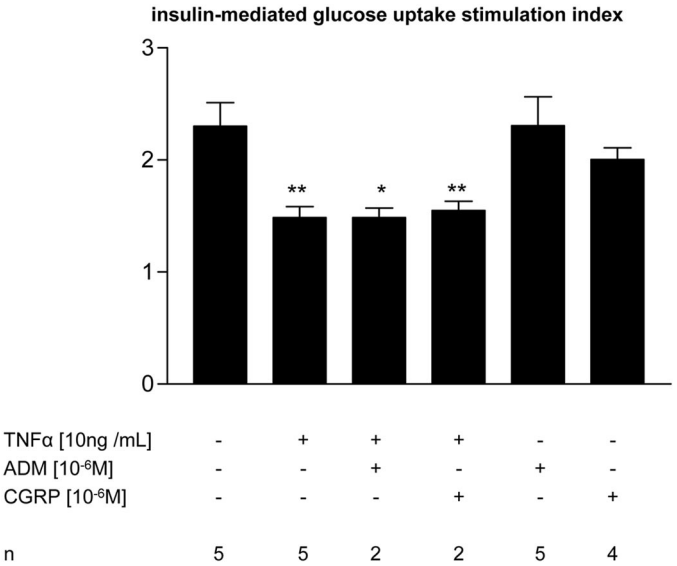

FIG. 7. Insulin-mediated glucose uptake. MSC-derived adipocytes were subjected to indicated treatments for 24 h. One hundred nanomoles insulin were applied to cells for 20 min. Then ^{3}H-deoxy-glucose was added and uptake was monitored after 15 min. Insulin stimulation indices were expressed as the ratio of basal and insulin-mediated ^{3}H-deoxy-glucose uptake. Results are expressed as means ± SEM. n, Number of independent triplicate experiments. *, $P < 0.05$, compared with controls; **, $P < 0.01$, compared with controls.

The positive effect of IFNγ on CALC IV gene-derived ADM mRNA expression and ADM protein secretion indicates major differences in gene expression in comparison with CALC I and CALC II. In contrast to ProCT and CGRP, inflammation-induced ADM expression was reported in numerous tissues and *in vitro* models, including vascular endothelial cells, vascular smooth muscle cells, fibroblasts, neurons, macrophages, TNFα-treated 3T3-L1 murine adipocytes, and very recently basal rat adipocytes (6, 35–38). Accordingly, besides differentiated adipocytes, we found ADM mRNA induction in undifferentiated MSCs and PBMC-derived macrophages. Thus, in contrast to CALC I and CALC II products ProCT and CGRP, sepsis- and inflammation-related ADM production appears to occur ubiquitously in all cell types independently of the differentiation state. Therefore, nonadipose cells possibly contribute in cytokine-mediated ADM mRNA expression and ADM peptide secretion shown herein. An approximately 10-fold induction in ADM mRNA abundance was recently shown in rat adipose tissues from obese high-fat-fed rats (38). Obesity is related to increased presence of macrophages in mice adipose tissue (39, 40). In coculture experiments we herein demonstrate ADM and CGRP mRNA induction by macrophage-secreted factors. Thus, a potential role of inflammation-mediated CT peptide expression in human obesity will need to be addressed.

The presence of CTR, CRLR, RAMP1, RAMP2, and RAMP3 mRNAs in MSC-derived adipocytes is in accordance with recent data from rat adipocytes (38). This indicates possible expression of functional CT peptide receptors, which may be responsible for the herein reported CGRP and

ADM effects (8). NEP mRNA expression in MSC-derived adipocytes is in line with a previous report on active NEP found in human adipocytes (41). NEP actively degrades CGRP and ADM (42, 43). Furthermore, ADM and NEP are inversely correlated in septic rats (44). Therefore, adipocyte NEP functions might include clearance of CT peptides in the vicinity of adipocytes.

Induction of CALC I and CALC II gene transcription was observed on exposure of adipocytes to exogenous CGRP and ADM in concentrations ranging from 1 nM to 1 μM. This induction was selective because the mRNAs of ADM, TNFα, and IL-1β were not affected by high CGRP and ADM concentrations. In contrast, the mentioned transcripts were induced by 1 ng/ml LPS administration in the present adipocyte model. This excludes that CGRP and ADM effects on CT, CGRP I, and CGRP II mRNAs are endotoxin-mediated artifacts from the recombinant peptide preparation. The CGRP(8–37)- and ADM(8–22)-mediated antagonism on mRNA inductions is a further indicator of specific CGRP and ADM effects.

When maximally stimulated with combined IL-1β/TNFα/LPS treatment, CT, CGRP I, and CGRP II mRNA appeared already after 2 h. Cycloheximide-mediated protein synthesis inhibition had positive additive effects on ADM-, CGRP-, and IL-1β-mediated CT peptide mRNA inductions, respectively. Thus, inflammatory CALC gene mRNA inductions appear not to depend on intermediate gene expression. Moreover, based on the presented data, the existence of a CALC gene transcription repressing factor with a high turnover rate may be hypothesized.

In addition to transcriptional activation, a positive dose-dependent effect of CGRP and ADM was observed on glycerol release. Thus, in analogy to proinflammatory cytokines (*e.g.* TNFα), CGRP and ADM possibly enhance lipolytic activity (45). Control experiments with ADM(22–52) or CGRP(8–37) remained negative on glycerol release. However, in contrast to mRNA inductions, preadministration of truncated peptides did not antagonize ADM and CGRP effects on lipolysis. This might be explained by a very sensitive and/or an alternative signaling pathway leading to enhanced lipolysis (46). Future studies will need to address this issue.

Intensive insulin therapy reduces mortality in intensive care unit patients, but the mechanisms responsible for sepsis-related insulin resistance induction are still poorly understood (47). Herein we assessed adipocyte insulin sensitivity by monitoring insulin-dependent glucose transport. The demonstrated TNFα-induced insulin resistance is in accordance with currently recognized concepts and expands the knowledge on MSC-derived adipocyte *in vitro* metabolic functionality (22, 48). Previous data suggested glucose transport-related antiinsulin effect by CGRP (33, 34). Herein attenuation of insulin-mediated glucose uptake by CGRP and ADM, however, was quite variable, indicating that other factors that are not yet well understood may play an important role. Therefore, a final conclusion cannot be drawn based on our data.

The process of adipocyte differentiation is characterized by increasing IL-6 production and nuclear factor-κB-related inflammation (49, 50). Cultured adipocytes are known to express inflammatory genes (51, 52). Accordingly, in MSC-derived adipocytes, we found low-level basal IL-6, TNFα, and IL-1β mRNA expression. CGRP and ADM are known to potentate inflammatory events in several cells and tissues (29, 53). Therefore, the herein shown CGRP- and ADM-mediated effects in basal adipocytes might be interpreted as modulatory events, rather than culprit inductions. Sepsis-related adipocyte CT peptide expression *in vivo* occurs in the presence of numerous proinflammatory factors, which might be significantly influenced by local CT peptide production. The precise mechanisms of inflammatory CT peptide expression and action have to be addressed in future experiments.

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IV

Somatostatin is expressed and secreted by human adipose tissue upon infection and inflammation

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Somatostatin Is Expressed and Secreted by Human Adipose Tissue upon Infection and Inflammation

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Somatostatin (SRIF) is a well-known neuroendocrine secretion product. SRIF expression and secretion are induced after inflammation in murine macrophages and in endotoxin-injected sheep and pigs. Because adipocytes have been demonstrated to produce numerous cytokines and peptide hormones, we investigated the expression of SRIF and its receptors (SSTR1–5) in human adipose tissue after inflammatory stimulation *in vitro* and in tissues from patients with septic disease.

Preadipocyte-derived adipocytes, mesenchymal stem cell-derived adipocytes, and mature explanted adipocytes expressed SRIF-mRNA after endotoxin [lipopolysaccharide (LPS)] or IL-1β treatments. LPS- and IL-1β-mediated SRIF-mRNA induction was blocked by pretreatment with dexamethasone. Using cocultures and quantitative real-time PCR, we demonstrate adipocyte SRIF induction by secretion factors from activated peripheral blood mononuclear cell-derived macrophages. In contrast to basal adipocytes, SRIF protein was detected in culture supernatants of LPS-treated and of combined TNFα/IL-1β/LPS-treated adipocytes. SRIF protein was visualized by immunohistochemistry in explanted minced adipose tissue after overnight incubation in culture medium supplemented with combined IL-1β and LPS. In septic patients, expression of SRIF-mRNA and SRIF protein was found in visceral, but not in sc, adipose tissue. Adipocyte mRNA abundance of SSTR 1–5 was differentially regulated by inflammatory treatments.

Thus, human visceral adipose tissue secretes SRIF during inflammation and sepsis and expresses several SSTRs. It is tempting to speculate that visceral adipose tissue-derived SRIF plays a modulatory role in the immunological and metabolic response to inflammation. (*J Clin Endocrinol Metab* 89: 4833–4839, 2004)

SOMATOSTATIN (SRIF) WAS described initially as a secretory product of the hypothalamus acting as a potent inhibitor of GH secretion (1). Subsequently, high densities of SRIF-producing neuroendocrine cells have been localized throughout the central and peripheral nervous systems, in the endocrine pancreas and in the gut, and to a lower extent in the thyroid, adrenals, submandibular glands, kidneys, prostate, and placenta (2–4). SRIF expression and secretion was also described in murine inflammatory and immune cells after activation (2). In the gastrointestinal tract, SRIF inhibits the secretion of numerous peptide hormones (insulin, glucagon, gastrin, and cholecystokinin), gastric emptying, gallbladder contraction, and exocrine gut secretion (3, 5). The induction of SRIF by inflammatory cytokines IL-1β, TNFα, and IL-6 was demonstrated *in vitro* in models of rat diencephalic cells (6, 7). SRIF-mRNA expression has been described in murine macrophage cell lines after cytokine stimulation (8). Accordingly, increased plasma SRIF levels were measured in jugular and portal veins in endotoxin-injected sheep and in septic pigs (9, 10).

Five distinct receptors mediating SRIF activity are widely expressed in many tissues. As a neurotransmitter, SRIF inhibits the release of GH, dopamine, norepinephrine, TRH, and CRH. Among others, additional modulatory roles have been ascribed to SRIF in inflammatory conditions and lymphocyte function (11–13). Adipose tissue emerged as a major endocrine organ in humans. Numerous peptide hormones released by adipocytes affect energy homeostasis, glucose and lipid metabolism, immune response, and reproduction (14). In addition, adipose tissue participates in the release of mediators of low-grade inflammation, which cause insulin resistance, relative impairment of insulin secretion, and, ultimately, hyperglycemia (15, 16). Knowledge of mechanisms underlying these processes is scarce. In view of the variety of endocrine factors involved in these processes, we investigated the expression of SRIF as a potential modulator in adipose tissue exposed to inflammatory cytokines. Here, we present *in vivo* and *in vitro* evidence for SRIF expression in adipose tissue in response to inflammation.

Abbreviations: DEX, Dexamethasone; FCS, fetal calf serum; HPRT, hypoxanthine-guanine phosphoribosyltransferase; LPS, lipopolysaccharide; MSC, mesenchymal stem cell; ProCT, procalcitonin; SRIF, somatostatin.

JCEM is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.

Patients and Methods

Patients

Permission was obtained from the local ethics committee for all experiments with patients. Adipose tissue samples were obtained from seven septic patients requiring laparotomy (mean age, 56 yr; range, 19–75 yr), after giving informed consent. Septicemias were due to peritonitis because of perforated sigmoid diverticulitis, perforated sigmoid carcinoma, perforated appendicitis, ischemic colitis of the sigmoid colon, and necrotisizing proctocolitis with perforation of the rectum and descending colon, respectively. In addition, adipose tissue was collected from noninfected patients undergoing elective surgery (mean age, 53 yr; range, 29–71 yr). Informed consent was obtained. Harvested tissues were incubated immediately in RNA-later (Ambion, Inc., Austin, TX) to

prevent RNA degradation. The samples were snap-frozen and stored at −70 C. Tissues were powdered under liquid nitrogen before RNA extraction using TRIzol reagent (Life Technologies, Inc., Gaithersburg, MD). Alternatively, tissues were stored in 4% formaldehyde for immunohistochemical analyses. Circulating levels of SRIF were assessed in six septic patients (mean age, 54.7 yr; range, 36.1–73.3 yr; body mass index, 28.1 ± 6.1 kg/m^2). The underlying diagnosis of these patients was sepsis due to pneumonia (n = 5) and staphylococcal infection (n = 1), respectively. In addition, we investigated SRIF plasma concentrations in four healthy subjects (mean age, 49.5 yr; range, 38–59 yr; body mass index, 24.4 ± 2.4 kg/m^2), all staff members.

Adipocyte cultures and isolation of preadipocytes

After informed consent, 50- to 500-g sc adipose tissue samples were obtained from noninfected obese patients undergoing plastic surgery. Primary cultures of human adipocytes were performed as described previously (17, 18), with modifications. Briefly, adipose tissue was minced, digested in 1 mg/ml collagenase 2 (Worthington Biochemical Corp., Freehold, NJ), filtered (150-μm nylon mesh), and centrifuged at 200 × *g*. The cell pellet was resuspended twice in erythrocyte lysis buffer, washed, and seeded in six-well plates. After a 24-h incubation in DMEM/F12 with 10% fetal calf serum (FCS; Life Technologies, Inc., Basel, Switzerland) allowing attachment, cells were washed in PBS and cultured in serum-free medium supplemented with agents [isobutylmethyl-xanthine, dexamethasone (DEX), insulin, transferrin, rosiglitazone, and triiodothyronine] that induce differentiation of preadipocytes to adipocytes. Triglyceride-storing adipocytes, representing 40–80% of cultured cells, are visible within 5–10 d. Differentiation was confirmed by RT-PCR analysis for adipocyte-specific peroxisome proliferator-activated receptor γ2 expression (19). Adipocytes were maintained for an additional 4 d in DMEM/F12 with 10% FCS before experiments, without supplementation with the above listed agents, namely rosiglitazone. In addition, floating mature adipocytes obtained after the centrifugation step were washed twice in medium (DMEM/F12 with 10% FCS). Packed adipocytes (0.5 ml) were inoculated into 50-ml flasks (Becton Dickinson Labware, Rutherford, NJ) completely filled with medium and allowed to attach to the top surface for 72 h at 37 C (20, 21). Flasks were subsequently turned around, and non-adherent cells, representing the bulk of initially inoculated cells, were removed. The adherent, triglyceride-storing mature adipocytes were cultured in 5 ml medium for 2 additional days before experiments.

Adipocytes were stimulated for 6 or 24 h with 1 μg/ml lipopolysaccharide (LPS) and 20 U/ml IL-1β, respectively. In selected experiments, cells were pretreated for 2 h with 1 μM DEX before IL-1β or LPS addition. Human-specific IL-1β was purchased from PeproTech (London, UK). LPS (*Escherichia coli* 026:B6) and DEX were from Sigma (Buchs, Switzerland).

The viability of adipocytes after stimulations was assessed via trypan blue staining, in which dead cells stain blue.

Human mesenchymal stem cells (MSCs)

Human MSCs were purchased from BioWhittaker Europe, S.p.r.l. (Verviers, Belgium). Differentiation into adipogenic lineage was performed as recommended by the manufacturer's protocol. Differentiated cells were exposed to LPS, IL-1β, and DEX as described above.

Adipocytes and macrophages in cocultures

Peripheral blood mononuclear cells were isolated by Ficoll-Plaque PLUS (Amersham, Uppsala, Sweden) and washed four times with Hank's balanced salt solution (Invitrogen, Basel, Switzerland) supplemented with 0.5% human albumin (Blutspendedienst SRK, Bern, Switzerland). Cells were resuspended in Iscove's modified Dulbecco's medium with 20% human serum and seeded in cell culture inserts with 0.4-μm pore size (Becton Dickinson Labware, Dietikon, Switzerland). After 1 h, non-adherent cells were removed by thorough washing with Hank's balanced salt solution and 0.5% human albumin. Adherent monocytes were cultured for 5 d. The obtained monocyte-derived macrophages were activated with live *E. coli* or combined 1 μg/ml LPS, 20 U/ml IL-1β, 10 ng/ml TNFα administration. After 2 h, stimulants were removed by repeated washing, and the inserts containing activated macrophages were added to *ex vivo* differentiated adipocytes kept in six-well plates for an additional 22 h. Then, adipocytes and macrophages were separately subjected to SRIF-mRNA analysis as described below.

RT-PCR

Total RNA from homogenized tissues, adipocyte cultures, or MSCs, respectively, was extracted by the single-step guanidinium-isothiocyanate method with a commercial reagent (TRIzol reagent; Life Technologies, Inc.) according to the manufacturer's protocol. Extracted RNA was quantified spectrophotometrically, and the quality was assessed by gel electrophoresis. Equal amounts of RNA per tissue or *in vitro* treatment were subjected to RT (Omniscript RT kit; Qiagen, Basel, Switzerland). PCR was performed on a conventional thermal cycler (TGradient; Biometra, Göttingen, Germany) using the PCR Taq core kit (Qiagen) and the following intron border-spanning oligonucleotides (22): SSTR1 (318-bp PCR product; GenBank accession no. BC035618), 5′-ATGGTGGCCCTCAAGGCCGG-3′ (sense) and 5′-CGCGGTGGCGTAATAGTCAA-3′ (antisense); SSTR2 (318-bp PCR product; GenBank accession no. AY236542), 5′-TCCTCTGGAATCCGAGTGGG-3′ (sense) and 5′-TTGTCCTGCTTACTGTCACT-3′ (antisense); SSTR3 (332-bp PCR product; GenBank accession no. AY277678), 5′-TGCCACCCTGGGCAACGTGT-3′ (sense) and 5′-CAGGCAGAATATGCTGGTGA-3′ (antisense); SSTR4 (323-bp PCR product; GenBank accession no. NM_ 001052), 5′-GCGCGCGGCGACCTACCGGC-3′ (sense) and 5′-GCCTGGTGATTTTCTTCTCC-3′ (antisense); SSTR5 (222-bp PCR product; GenBank NM_ 001053), 5′-CGTCTTCATCATCTACACGG-3′ (sense) and 5′-GGCCAGGTTGACGATGTTGA-3′ (antisense); SRIF (Ref. 23; 356-bp PCR product; GenBank accession no. BC032625), 5′-GATGCTGTCCTGCCGCCTCCAG-3′ (sense) and 5′-ACAGGATGTGAAAGTCTTCCA-3′ (antisense); β-actin (198-bp product; GenBank accession no. AF076191), 5′- TTCTGACCCATGCCCACCAT-3′ (sense) and 5′-ATGGATGATGATATCGCCGCGCTC-3′ (antisense).

The annealing temperature was 62 C for SSTR1–4, 58 C for SSTR5, 60 C for SRIF, and 65 C for β-actin. Thirty-five cycles of PCR were used for detection. Cycles were reduced to 28 for β-actin, to stop the reaction in the linear phase of amplification. β-Actin was used to verify equal quantities of RNA loading in each reaction. PCR products were separated and visualized on 1.5% agarose gels containing 0.5 μg/ml ethidium bromide. PCR product identity was confirmed by direct nucleotide sequencing of the PCR products by dye deoxy terminator cycle sequencing. The absence of genomic DNA contamination was confirmed by RT-negative control reactions for all RNA preparations.

Quantitative analyses of SRIF-mRNA expression

cDNA, obtained as described above, was subjected to quantitative real-time PCR analysis using the ABI 7000 Sequence detection system (PerkinElmer Life Sciences, Emeryville, CA). Specific primers yielding short PCR products suitable for Sybr-Green detection were designed using Primer Express software (version 1.0; PE Applied Biosystems, Foster City, CA). Sequences of primers were as follows: SRIF (82-bp product; GenBank accession no. BC032625), 3′-GATGCCCTGGAACCTGAAGA-5′ (sense) and 3′-CCGGGTTTGAGTTAGCAGATC-5′ (antisense); hypoxanthine-guanine phosphoribosyltransferase (HPRT; 85-bp product; GenBank accession no. M26434), 3′-TCAGGCAGTATAATCCAAAGATGGT-5′ (sense) and 3′-AGTCTGGCTTATATCCAACACTTCG-5′ (antisense). The reaction volume was 22 μl, and the conditions were set as suggested by the manufacturer. Each cDNA sample tested for quantitative SRIF-mRNA expression was also subjected to HPRT-mRNA analysis. Results were expressed as the ratio of the respective SRIF-mRNA and HPRT-mRNA threshold values. The product identity was confirmed by sequence analysis and electrophoresis on a 2.5% agarose gel containing ethidium bromide.

Peptide measurement

SRIF concentrations were determined in EDTA plasma and supernatants using a commercially available SRIF RIA kit (functional assay sensitivity, 5 pg/ml; Phoenix Pharmaceuticals Inc., Belmont, CA).

Procalcitonin (ProCT) concentrations were determined in sera by an ultra-sensitive chemiluminometric assay with a functional sensitivity of

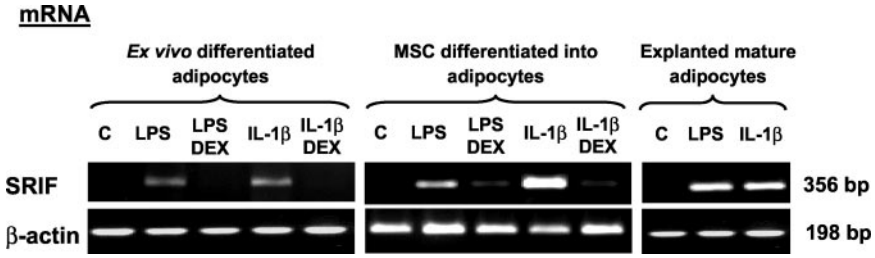

FIG. 1. Induction of SRIF-mRNA expression in cultured adipocytes and MSCs. Explanted mature adipocytes kept in so-called "ceiling cultures," *ex vivo* differentiated adipocytes, and MSCs differentiated into the adipogenic lineage were subjected to 6-h IL-1β (20 U/ml) and LPS (1 μg/ml) treatments, respectively. *Ex vivo* differentiated adipocytes and MSCs differentiated into the adipogenic lineage were preincubated with DEX for 2 h. SRIF-mRNA expression was analyzed by RT-PCR. Data are one of at least four independent experiments.

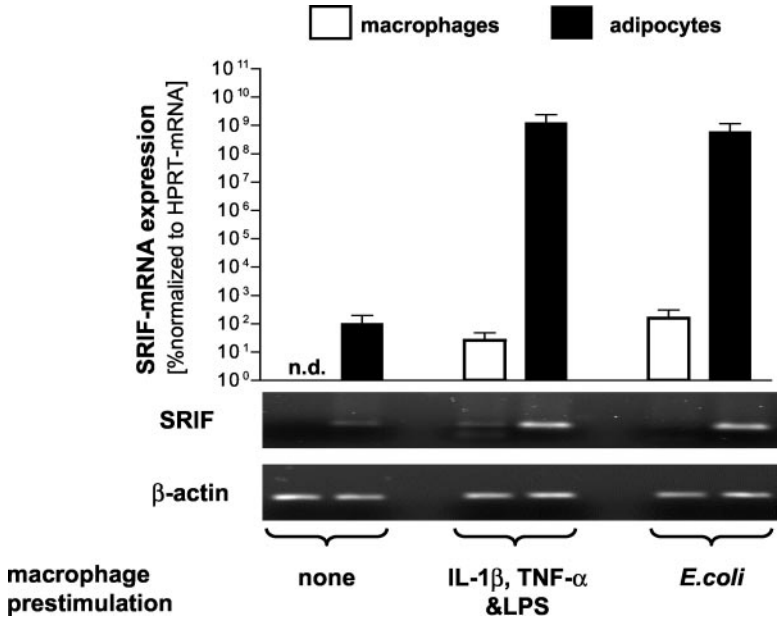

FIG. 2. Quantitative analysis of SRIF-mRNA expression in *ex vivo* differentiated adipocytes kept in coculture with macrophages. Peripheral blood mononuclear cell-derived macrophages kept in cell culture inserts with 0.4-μm pores were activated for 2 h with live *E. coli*. After repeated washing with PBS, inserts were added to *ex vivo* differentiated adipocytes in six-well plates for an additional 22 h. Total RNA from macrophages and adipocytes were separately isolated and subjected to quantitative real-time PCR analysis using specifically designed primers yielding an 82-bp product suitable for Sybr-Green detection. SRIF-mRNA threshold values were normalized with HPRT-mRNA values. In addition, conventional PCR was performed and visualized on a 2.5% agarose gel. Results are means ± SEM (n = 4) or one of four independent experiments.

5 pg/ml (ProCa-S assay; B.R.A.H.M.S. GmbH, Hennigsdorf-Berlin, Germany).

Immunohistochemistry

Adipose tissue biopsies were formalin fixed and paraffin wax embedded. The slides were dewaxed in xylene and rehydrated through graded ethanols. Heat-induced epitope retrieval was performed by immersing the slides in citrate buffer (10 mM, pH 6) and microwaving at 98 C for 30 min before cooling and rinsing with PBS. Immunohistochemical staining was performed using the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) as recommended by the manufacturer's protocol. SRIF rabbit polyclonal antibody was purchased from Novocastra Laboratories Ltd. (Newcastle, UK).

Density measurements

Density measurements of PCR amplification products were performed using Scion Image 4.02 Beta for Windows (Scion Corp., Frederick, MD).

Statistical analysis

All data are presented as means ± SEM. Unpaired *t* tests (two-sided) or Mann-Whitney *U* tests in case of nonparametric distributions were used to identify differences among the groups. For multigroup comparisons, one-way ANOVA with *post hoc* analysis for least-square differences was performed. Data were analyzed using Statistica for Windows (version 6; StatSoft Inc., Tulsa, OK).

Results

SRIF-mRNA induction in vitro and in vivo

In *ex vivo* differentiated adipocytes and in mature explanted adipocytes obtained from noninfected patients undergoing plastic surgery, SRIF-mRNA expression was almost not detectable by conventional RT-PCR analysis using 35 amplification cycles (Fig. 1). SRIF-mRNA expression was found after a 6-h exposure to LPS or the proinflammatory cytokine IL-1β in *ex vivo* differentiated adipocytes, adipogenic differentiated MSCs, and mature adipocytes. Induction of SRIF-mRNA expression was repressed after preincubation of *ex vivo* differentiated adipocytes and MSCs with DEX for 2 h (Fig. 1). Stimulation with 0.004% dimethylsulfoxide, the carrier for DEX, alone had no effect on the SRIF induction (data not shown). SRIF-mRNA induction was also observed in adipocytes kept in coculture with *E. coli*-activated or combined cytokine/LPS-activated macrophages (Fig. 2). In contrast, no SRIF-mRNA induction was observed in macrophages *per se* stimulated with *E. coli* or with combined cytokines/LPS, respectively.

In several patients with severe bacterial infection, mirrored in markedly elevated serum ProCT levels (24), we found SRIF-mRNA expression in visceral fat depots but not in sc biopsies (Fig. 3A). The SRIF gene was not expressed in

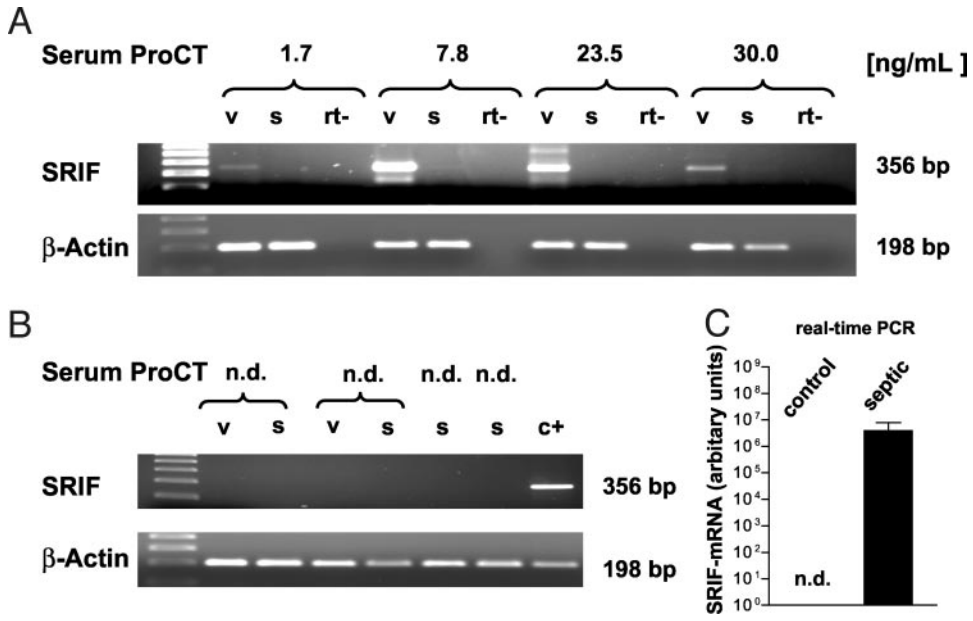

FIG. 3. SRIF-mRNA expression in adipose tissue obtained from septic and nonseptic humans. A, Subcutaneous (s) and visceral (v) adipose tissue biopsies were obtained intraoperatively from patients with markedly increased circulating ProCT levels indicating bacterial infection. B, Noninfected tissues were obtained from patients with nondetectable levels of serum ProCT as controls. SRIF-mRNA analysis was assessed by RT-PCR. Amplification products were visualized on agarose gels. Verification of mRNA as the source of amplification template was obtained by omitting the RT in reactions for pooled infected samples (rt-), resulting in no bands after PCR. RNA extracted from human islet cells was taken as a positive control (c+). C, Quantitative real-time PCR analysis using specifically designed primers yielding an 82-bp product suitable for Sybr-Green detection was performed. SRIF-mRNA threshold values were normalized with HPRT-mRNA values and are presented as means ± SEM (n = 4). Shown are data from four infected and four control patients, respectively. n.d., Not detected.

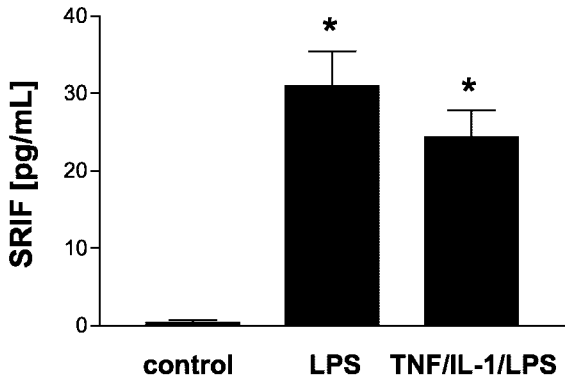

FIG. 4. SRIF release by *ex vivo* differentiated adipocytes. *Ex vivo* differentiated adipocytes were subjected to inflammatory treatment (20 U/ml IL-1β, 10 ng/ml TNFα, 1 μg/ml LPS) for 2 h in the presence of 10% fetal bovine serum. Thereafter, medium was replaced by serum-free medium containing the same inflammatory agents and incubated for an additional 22 h. SRIF protein content was analyzed by RIA. Random values between 0 and 5 were generated for measurements below the detection limit of 5 pg/ml. Data are means ± SEM (n = 6). *, $P < 0.01$, comparison of LPS alone and combined IL-1β/TNFα/LPS *vs.* control, respectively.

adipose tissue samples from noninfected control patients, neither of visceral nor of sc origin (Fig. 3, B and C).

SRIF secretion in vitro and in vivo

In supernatants of *ex vivo* differentiated unstimulated adipocytes, the SRIF protein concentration was below or at the detection limit of 5 pg/ml after a 24-h incubation (Fig. 4). In contrast, SRIF protein secretion was increased to 31 ± 5.0 pg/ml in supernatants of LPS-treated cells. The administration of LPS, IL-1β, and TNFα combined led to an average SRIF protein concentration of 24.3 ± 3.9 pg/ml (n = 6 for each treatment; ANOVA for least-square differences, $P < 0.001$). The viability of adipocytes after a 24-h exposure to LPS, IL-1β, and TNFα was unchanged as assessed by trypan blue staining (data not shown). No significant differences in peripheral blood levels of SRIF were found between infected and noninfected patients. Infected patients had a plasma SRIF level of 89.55 ± 24.1 pg/ml, and noninfected individuals had a level of 158 ± 73.73 pg/ml ($P = 0.21$).

Immunohistochemistry for the SRIF protein was negative in adipose tissue biopsies obtained from noninfected control patients (Fig. 5A) with nondetectable circulating ProCT levels. In contrast, the SRIF protein was detected in visceral adipose tissue biopsies from healthy individuals after an overnight incubation in LPS- and IL-1β-supplemented culture medium (Fig. 5B). Similarly, in visceral adipose tissue biopsies obtained from septic patients documented by increased circulating ProCT levels, the SRIF protein was detected by immunohistochemistry (Fig. 5D). No protein expression was seen in biopsies of sc origin from the same subjects (Fig. 5C).

SSTR1–5 gene expression in vitro

SSTR-mRNA expression was assessed using RT-PCR. In unstimulated human adipocytes, SSTR subtypes 1 and 2 were expressed, in contrast to subtypes 3, 4, and 5. SSTR1 down-regulation was observed in adipocytes kept in coculture with *E. coli*-activated macrophages (Fig. 6). SSTR3 and

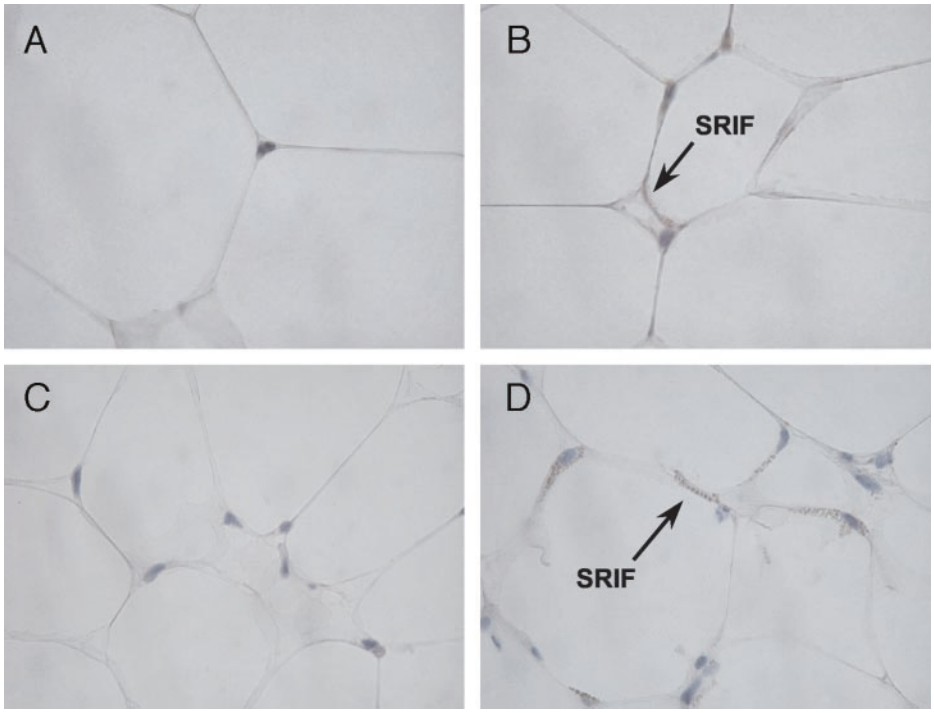

FIG. 5. SRIF immunohistochemical staining in human adipose tissue. Adipose tissue sections were immunohistochemically stained to determine the presence of SRIF. A, Subcutaneous adipose tissue biopsies obtained from noninfected individuals were taken as control tissue. B, Subcutaneous adipose tissue biopsies obtained from noninfected individuals were stimulated with LPS and IL-1β overnight in cell culture medium. Subcutaneous (C) and visceral (D) adipose tissue biopsies were obtained intraoperatively from a septic patient. All magnifications, $\times$400.

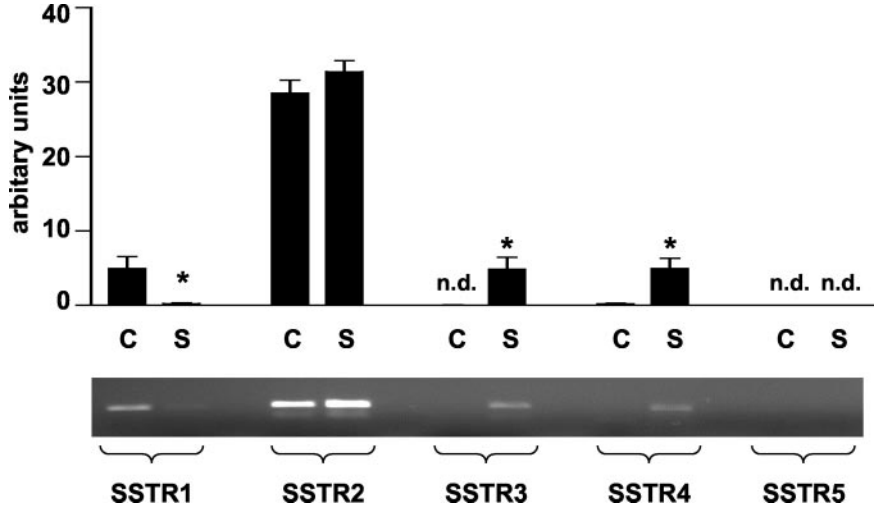

FIG. 6. Semiquantitative analysis of SSTR1–5-mRNA expression in *ex vivo* differentiated adipocytes kept in coculture with macrophages. Peripheral blood mononuclear cell-derived macrophages kept in cell culture inserts with 0.4-μm pores were activated for 2 h with live *E. coli*. After repeated washing with PBS, inserts were added to *ex vivo* differentiated adipocytes in six-well plates for an additional 22 h. Total RNA from adipocytes was separately isolated and subjected to RT-PCR analysis using specifically designed primers for each SSTR subtype. Amplification products were visualized on agarose gels. Semiquantitative analysis was performed by density measurements of the bands on the agarose gels. Results are presented as means $\pm$ SEM (n = 4) or one of four independent experiments. *, $P < 0.05$, comparison of stimulated (S) *vs.* control (C), respectively.

SSTR4 were induced in the same experiments. SSTR2 remained unchanged, and SSTR5 was never detected.

Discussion

The present study shows that SRIF is expressed and secreted in human adipose tissue in response to inflammation. Thus, adipose tissue represents a novel site of SRIF expression besides the well-described neuroendocrine cells in the brain and the gastrointestinal tract.

Ex vivo differentiated preadipocytes could be contaminated by other adipose tissue-derived cell types (*e.g.* macrophages, endothelial cells), which could provide a nonadipocyte source of SRIF expression. Therefore, we repeated the experiments with MSC-derived adipocytes, with virtually identical results. However, due to the very nature of developing MSCs, contribution of other not yet finally differentiated cells cannot be excluded. In a third model, SRIF expression in adipocytes clearly validated the initial findings using mature and purified adipocytes cultured in adipocyte-selecting "ceiling cultures" (20, 21). Accordingly, SRIF-mRNA was detectable in visceral adipose tissue of septic patients but not in noninfected subjects.

In confirmation of the transcriptional data, immunoreactive SRIF was also detected in supernatants of cytokine-stimulated adipocytes, indicating that SRIF is transcribed, processed, and secreted in fat cells. In addition, using immunohistochemistry, SRIF-positive cells were found in the visceral fat of septic patients as well as in cytokine-stimulated explants of adipose tissue.

In a coculture system, activated macrophages induced adipocyte SRIF gene expression in a similar way as did IL-1β and LPS, suggesting macrophage-derived stimulants are key

components for this induction. Interestingly, we were not able to confirm the previously reported SRIF-mRNA expression by macrophages after inflammatory activation. This may be due to species differences between rodent and human macrophages and macrophage-like cell lines (25).

SRIF was found in visceral, but not sc, adipose tissue of patients with septic disease. It could be argued that SRIF expression in visceral fat was related to the proximity of visceral fat to the site of bacterial infection in our patients (bowel infections). In this respect, secretion of cytokines in response to septic disease was sufficient to induce ProCT expression in both visceral and sc tissue in the same patients (26). Therefore, we hypothesized another modulatory factor that suppressed SRIF expression in sc adipose tissue *in vivo* despite cytokine stimulation (*e.g.* innervations).

Venous blood from visceral fat is drained via the hepatic portal vein, where SSTRs have been characterized at the nerve endings of afferent fibers of the vagal nerve (27). These receptors translate humoral SRIF signals into neural signals in the central nervous system that may contribute to the responses of the brain to sepsis and inflammation. Hence, SRIF released from visceral fat after inflammatory stimulation during infections could modulate hepato-pancreatic perfusion and/or metabolic function, thereby augmenting the relative insulin deficit and/or resistance observed in septic patients (16). Furthermore, in obese patients, enhanced inflammation-related adipose tissue SRIF production might participate in the obesity-related complications reported in patients in the intensive care unit (28). SRIF has a short plasma half-life of approximately 2–3 min and shows a diurnal rhythm (2). This may explain the present observation that there was no difference in peripheral-venous plasma SRIF levels between infected and noninfected subjects. These findings make other systemic endocrine effects of adipose tissue-derived SRIF less likely but do not rule them out because only single samples were obtained. In rats, SRIF administration lowers inflammatory markers and mediators (29). Furthermore, endogenous SRIF may participate in antiinflammatory actions of glucocorticoids (11). In this respect, the negative effect of DEX on SRIF expression in adipocytes is noteworthy. It is tempting to speculate that sepsis-induced SRIF expression described here exerts feedback effects on inflammation.

Interestingly, mRNAs for SSTR1, 3, and 4 are differentially expressed in the presence or absence of inflammatory mediators in human adipocytes. In isolated rat adipocytes, SRIF surface binding and lipolytic action has been reported (30). Hence, adipocyte-derived, inflammation-induced SRIF may exert multiple effects in an autocrine or paracrine manner, *e.g.* down-regulation of leptin secretion because plasma leptin levels decreased in human subjects after SRIF administration (31). A lipolytic effect of SRIF was also demonstrated in chicken adipocytes after prolonged exposure to the peptide (32).

Assuming that lower grades of inflammation also result in preferential SRIF expression in visceral adipose tissue, these observations may explain several previous human data on reduced GH secretion in association with visceral adiposity. Indeed, there is a highly significant negative correlation between the visceral fat mass and 24-h GH secretion in human subjects; this association was stronger than the influence of age, gender, and total body fat on GH secretion (33). In addition, GH concentrations were reduced in parallel with visceral adiposity among patients with HIV-lipodystrophy, and an increased SRIF tone in these patients was postulated (34, 35).

Visceral adipose tissue is more closely associated with the insulin resistance syndrome than sc fat. In comparison, visceral adipocytes have higher lipolytic rates, are under distinct sympathetic nervous system regulation, secrete larger amounts of IL-6 and plasminogen activator inhibitor-1, and secrete smaller amounts of adiponectin and leptin (36).

In conclusion, SRIF is secreted by human adipocytes, both *ex vivo* after stimulation with activated macrophages, LPS, and IL-1β and *in vivo* in visceral adipose tissue harvested from septic patients. Human adipose tissue expresses several SSTRs that are differentially regulated by inflammatory stimuli. It remains to be elucidated whether in sepsis visceral adipose tissue-derived SRIF plays a role as a classical hormone, thereby suppressing insulin or GH secretion, or alternatively, interferes by paracrine or autocrine action with the release of other adipocytokines.

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D.S. and P.L. contributed equally to this work.

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Cytokine-induced metabolic effects in human adipocytes are independent of endogenous nitric oxide

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[1]*Department of Research,* [2]*Division of Endocrinology, Diabetology, and Clinical Nutrition,* [3]*Institute for Surgical Research and Hospital Management, University Hospital, CH-4031 Basel; and* [4]*Division of Clinical Chemistry and Biochemistry, University Children's Hospital, Zurich, Switzerland*

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Linscheid, Philippe, Dalma Seboek, Henryk Zulewski, Arnaud Scherberich, Nenad Blau, Ulrich Keller, and Beat Müller. Cytokine-induced metabolic effects in human adipocytes are independent of endogenous nitric oxide. *Am J Physiol Endocrinol Metab* 290: E1068–E1077, 2006. First published December 27, 2005; doi:10.1152/ajpendo.00374.2005.—Nitric oxide (NO) has been recognized as a potential mediator of inflammation-induced metabolic alterations, including insulin resistance. However, expression mechanisms and potential roles of endothelial and inducible NO synthases (eNOS and iNOS, respectively) in human adipocytes are poorly understood. In the present study, we aimed to analyze several aspects of NO-related gene expression and metabolite synthesis in basal and inflammation-activated human adipocyte models. eNOS mRNA was highly expressed in omental and to a lesser extent in human subcutaneous adipose tissue biopsies, but not in purified adipocytes, in mesenchymal stem cell (MSC)- and in preadipocyte-derived adipocytes, respectively. Trace amounts of iNOS mRNA were detected in adipose tissue samples of donors with abdominal infection, as opposed to noninfected subjects. Interferon-γ, in combination with interleukin-1β or lipopolysaccharide, evoked a transient (4 h $<$ time $<$ 24 h) iNOS mRNA expression in human MSC and preadipocyte-derived adipocytes, respectively. This induction was preceded by cytokine-specific mRNAs. In addition, it was accompanied by an activation of the tetrahydrobiopterin synthesis pathway and by inhibition of peroxisome proliferator-activated receptor-γ2. In contrast to murine 3T3-L1-derived adipocytes, iNOS protein and NO oxidation products remained undetectable in iNOS mRNA-positive human adipocytes. Accordingly, coadministration of NOS inhibitors (i.e., N^{ω}-nitro-L-arginine methyl ester, N^{ω}-monomethyl-L-arginine, and 1400W) had no effects on insulin-mediated glucose uptake and lipolysis. We conclude that, in human adipocytes, endogenous NO is not involved in metabolic regulation during either basal or cytokine-activated conditions.

adipose tissue; nitric oxide synthase; insulin resistance; cytokines; lipolysis

INFLAMMATORY PROCESSES PLAY A PIVOTAL ROLE in translating obesity to metabolic disturbances (10, 36, 47). However, despite the overwhelming clinical evidence for such a relationship, very little is known about the molecular mechanisms linking obesity to insulin resistance and finally diabetes. Excessive nitric oxide (NO) production has been proposed as a mediator for insulin resistance in muscle (35, 44), liver, and adipose tissue (37, 38). Three NO synthase (NOS) isoforms produce NO in a tightly regulated, cell-specific manner (3, 8, 33). Activity of the constitutively expressed endothelial and neuronal isoforms is regulated by Ca^{2+}-mediated calmodulin binding. A complex regulation mechanism requires both nuclear factor (NF)-κB and interferon (IFN)-γ mediated STAT1 activations for triggering inducible NOS (iNOS) transcription in humans (19). Reflecting the potential toxicity resulting from the enzyme's high activity, the expression is additionally regulated on mRNA stability and translational and posttranslational levels (8, 40).

Basal endothelial NOS (eNOS) and iNOS expressions were reported in human adipose tissue and in adipocytes obtained from collagenase-digested adipose tissue (14, 15, 43). iNOS expression and activity was induced in white adipose tissue of lipopolysaccharide (LPS)-treated rats and in murine 3T3-L1 adipocytes subjected to combined LPS, tumor necrosis factor (TNF)-α, and IFN-γ (25, 39). Tetrahydrobiopterin (BH_4) is essential and rate-limiting on NOS activity in several cells, including activated 3T3-L1 adipocytes (29).

NO-releasing chemicals (e.g., sodium nitroprusside) and NO gas increase basal lipolysis in rat adipocytes (20). Catecholamine-stimulated lipolysis, however, is inhibited by NO donors but not by authentic NO gas, indicating additional, not yet well-defined, regulatory mechanisms on NO-mediated metabolic effects. Administration of N^{ω}-nitro-L-arginine methyl ester (L-NAME), an NOS inhibitor, increases lipolysis in vivo in humans (4). In contrast, diphenyliodonium (DPI)-mediated NOS blockade decreases both basal and dibutyryl cAMP-stimulated lipolysis (21). In LPS- and cytokine-challenged 3T3-L1 adipocytes, iNOS-derived NO was proposed as a negative lipolysis modulator (34). NOS inhibition resulted in impaired insulin-mediated glucose uptake in rat adipocytes and myocytes (41). However, $iNOS^{-/-}$ knockout mice are protected against obesity-induced insulin resistance (35).

In view of these complex and partially conflicting findings, we herein aimed to clarify the presence and relevance of endogenous NO synthesis in human adipocytes. Therefore, we analyzed NOS expressions in adipose tissue biopsies, in freshly isolated and in cultured mature adipocytes, in preadipocyte-derived adipocytes, and in mesenchymal stem cell (MSC)-derived adipocytes. In addition, BH_4 synthesis was assessed in basal and activated adipocytes. Finally, we tested the potential influence of NOS inhibitors on lipolytic activity and on insulin-mediated glucose transport in human adipocytes.

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METHODS

Patients. Omental and subcutaneous adipose tissue biopsies were obtained from four donors requiring elective surgery (mean age 46 yr, range 19–71). Informed consent was obtained. Two of the patients had abdominal infections resulting from perforated sigmoid diverticulitis and perforated appendicitis. Harvested tissues were immediately incubated in RNA-later (Ambion, Austin, TX) to prevent RNA degradation. The samples were snap-frozen in liquid nitrogen and stored at −70°C. Tissues were powdered under liquid nitrogen before RNA extraction using Tri Reagent.

Adipocyte cultures. Human preadipocyte-derived adipocytes were obtained from patients undergoing plastic surgery, as previously described (30). Bone marrow aspirates (20–40 ml) were obtained from healthy donors (18–63 yr) during routine orthopedic surgical procedures, in accordance with the local ethical committee (University Hospital Basel) and after informed consent. Nucleated cells were isolated from the aspirate by Ficoll density gradient centrifugation (Histopaque1; Sigma, Buchs, Switzerland). Human MSC were thereafter selected within the nucleated cells in culture on the basis of adhesion and proliferation on the plastic substrate. Cells were expanded in DMEM supplemented with 10% FBS and 5 ng/ml basic fibroblast growth factor (all from Invitrogen, Basel, Switzerland). For experiments, cells between passages 4 and 10 were seeded in six-well plates or 100-mm dishes. Adipogenic differentiation was induced by incubating confluent cells in DMEM-F-12 (Invitrogen) containing 3% FBS and supplements as follows: 250 μM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, 0.2 nM 3,3,5-triiodo-L-thyronine, 5 μM transferrin (all from Sigma), 100 nM insulin (Novo Nordisk, Küsnacht, Switzerland), and 1 μM rosiglitazone (GlaxoSmithKline, Worthing, UK). Typically, 80–90% of MSCs underwent adipogenic differentiation as assessed by lipid droplets formation. Expression of adipocyte-specific mRNAs [e.g., peroxisome proliferator-activated receptor-(PPAR)γ2, leptin, adiponectin, GLUT4] was confirmed by RT-PCR. After 15–18 days, supplements were removed by washing three times with warm PBS. Adipocytes were kept in DMEM-F-12 containing 3% FBS for three additional days before initiation of experiments in fresh medium.

Human IFN-γ and interleukin (IL)-1β were purchased from PeproTech (London, UK). LPS (*Escherichia coli* 026:B6), N^{ω}-monomethyl-L-arginine (L-NMMA), L-NAME, *N*-[3-(aminomethyl)benzyl]acetamidine, and 1400W were from Sigma. Sepiapterin was from Schircks Laboratories (Jona, Switzerland). Viability of adipocytes after treatments was assessed with trypan blue staining, with viable cells excluding trypan blue and dead cells staining blue.

RT-PCR. Total RNA was extracted from liquid nitrogen-powderized tissues or adipocyte cultures with TRI Reagent (Molecular Research Center, Cincinnati, OH). Extracted RNA was quantified spectrophotometrically at 260 nm with a Biophotometer (Vaudaux-Eppendorf, Schönenbuch, Switzerland). Ratio of extinction at 260 and 280 nm was between 1.5 and 2.0, and the quality was assessed by gel electrophoresis. Total RNA (1 μg) was subjected to reverse transcription (Omniscript RT kit; Qiagen, Basel, Switzerland). PCR was performed on a conventional thermal cycler (TGradient; Biometra, Göttingen, Germany) using PCR Taq core kit (Qiagen). Human gene-specific, intron-spanning primers and conditions were as indicated in Table 1. Amplification products were visualized on 1.5% (amplicons >250 bp) or 2.5% (amplicons <250 bp) agarose gels containing 0.5 μg/ml ethidium bromide. A 100-bp Molecular Ruler (Bio-Rad, Reinach, Switzerland) was run as size reference. PCR product identity was confirmed by nucleotide sequencing (Microsynth, Balgach, Switzerland).

Determination of total NO. NO is rapidly oxidized to nitrite (NO_2^-) and nitrate (NO_3^-). After incubation periods up to 72 h, cell culture supernatants were supplemented with 60 mU nitrate reductase and 50 μM NADPH and kept for 2 h at room temperature in the dark. NO_2^- were detected by Griess assay (22). $NaNO_3$ dilutions were used as concentration reference.

Pterin analysis. MSC- and preadipocyte-derived adipocytes were cultured in 100-mm dishes, treated with IFN-γ-IL-1β-LPS for 48 h, washed with PBS, and scraped in 200 μl of 0.1 M HCl. Cells were lysed by three cycles of freeze/thaw. Lysis supernatants obtained by centrifugation and culture supernatants were oxidized for 5 min with MnO_2 and deproteinized by filtration through Ultrafree-MC 5,000 NMWL filters (Millipore, Volketswil, Switzerland). Samples of 200 μl were supplemented with 15 μl of 1 M HCl. Pterins (i.e., BH_4 and neopterin) were analyzed by HPLC as described previously (9). BH_4 was measured as total biopterin (sum of biopterin, dihydrobiopterin, and BH_4).

Western blot analysis. MSC-derived adipocytes from two 100-mm dishes were scraped in 500 μl buffer containing 150 mM NaCl, 10 mM Tris·HCl (pH 7.4), 1 mM EGTA, 0.5% Nonidet P-40 (Igepal; all from Sigma), and 1% Triton and protease inhibitor cocktail (Roche Diagnostics, Rotkreutz, Switzerland). Lysis was carried out on ice for 30 min. Alternatively, cytosolic lysates were obtained by repeated freeze/thawing in 25 mM Tris·HCl (pH 7.4) supplemented with protease inhibitor cocktail. After 30 min cold centrifugation at 14,000 rpm, a 21-gauge needle was used to aspirate lysates. When needed, centrifugation was repeated for 15 min to clear lysates of floating lipids. Protein concentrations were determined using Bradford reagent as indicated by the supplier (Sigma). Lysate aliquots containing 40 μg total protein were mixed 1:1 with Laemmli buffer and run on 7.5% SDS-PAGE (Ready Gel; Bio-Rad). Proteins were transferred to nitrocellulose (Optripan BA-S 85; Schleicher & Schuell, Dassel, Germany) in a Mini Trans-Blot system (Bio-Rad). Blots were blocked for

Table 1. *List of human gene-specific primers for RT-PCR analysis*

mRNA	Sense Primer	Antisense Primer	Size, bp	GeneBank No.	Annealing Temperature, °C	No. of Cycles
eNOS	CACCGCTACAACATCCTGGAG	CTGTGTTACTGGACTCCTTCC	799	M95296	55	35
iNOS	ACGTGCGTTACTCCACCAACAA	CATAGCGGATGAGCTGAGCATT	114	L09210	55	35
GTPCH	TTGGTTATCTTCCTAACAA	GTGCTGGTCACAGTTTTGCT	226	U19523	50	35
TNF-α	GGCCCAGGCAGTCAGATCAT	GGGGCTCTTGATGGCAGAGA	310	M10988	65	35
IL-1β	TTCCCTGCCCACAGACCTTC	AGGCCCAAGGCCACAGGTAT	298	BC008678	65	35
IL-6	GCAAAGAGGCACTGGCAGAAA	CAGGCTGGCATTTGTGGTTG	284	M54894	60	25
PPARγ2	GCGATTCCTTCACTGATAC	GCATTATGAGACATCCCCAC	580	NM_015869	55	28
PPARγ1	TCTCTCCGTAATGGAAGACC	GCATTATGAGACATCCCCAC	474	NM_138712	55	30
Leptin	TGCCCATCCAAAAAGTCCA	GAAGTCCAAACCGGTGACTTTCT	121	NM_000230	58	35
Adiponectin	TGGGCCATCTCCTCCTCA	AATAGCAGTAGAACAGCTCCCAGC	102	NM_004797	58	35
β-Actin	TTCTGACCCATGCCCACCAT	CCTCGCCTTTGCCGATCC	198	NM_001101	65	28

eNOS, endothelial nitric oxide synthase; iNOS inducible nitric oxide synthase; GTPCH, GTP cyclohydrolase; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; IL-6, interleukin-6; PPARγ2, peroxisome proliferative-activated receptor-γ transcript variant 2; PPARγ1, peroxisome proliferative-activated receptor-γ transcript variant 1.

1 h in PBS with 0.1% Tween 20 and 10% nonfat dry milk and exposed overnight at 4°C to primary antibody in PBS with 0.1% Tween 20 and 1% nonfat dry milk. A monoclonal mouse anti-iNOS antibody reactive for both human and mouse iNOS (BD Transduction Laboratories, Basel, Switzerland) was diluted 1:2,500. Monoclonal mouse anti-PPARγ was diluted 1:200 (Santa Cruz, Heidelberg, Germany). Bound antibodies were visualized by the Immun-star horseradish peroxidase chemiluminescent kit (Bio-Rad).

Insulin-mediated glucose uptake. On *day 1*, differentiation medium was removed from MSC-derived adipocytes. Cells were washed three times in warm PBS and kept in DMEM-F-12 containing 5 mM glucose and 3% FBS. Supplements were added on *day 2*. On *day 3*, at $t = 0$ min, 100 nM insulin was added to one-half of the wells. At $t = 20$ min, 1 μC 2-deoxy-D-[^{3}H(G)]glucose (PerkinElmer, Boston, MA) was added to all wells. After 15 min, cells were washed three times in ice-cold PBS and lysed in 0.1% SDS. Radioactivity was measured in a scintillation counter. Results are expressed as the ratio of insulin and noninsulin-mediated glucose uptake (RIMGU).

Glycerol release in culture medium. On *day 1*, differentiation medium was removed from MSC-derived adipocytes. Cells were washed three times in warm PBS and kept in phenol red-free DMEM-F-12 (Invitrogen) containing 3% FBS. Medium was changed on *day 2*, and supplements were added. On *day 3*, 800 μl supernatant aliquots were collected and kept frozen at −20°C until used for glycerol measurement (Glycerol UV-method; Boehringer Mannheim/R-Biopharm, Darmstadt, Germany).

Statistical analysis. Data are presented as means ± SD. Two-group comparisons were performed using the Mann-Whitney *U*-test. For multigroup comparisons, one-way ANOVA was used with Dunnett's Multiple Comparison posttest.

RESULTS

eNOS and iNOS mRNA expression in human adipose tissue. eNOS mRNA was detected in subcutaneous and omental human adipose tissue. The transcript abundance was higher in omental fat biospies compared with subcutaneous samples (Fig. 1*A*). Lipid-laden mature adipocytes were separated from stromal cells (e.g., preadipocytes, endothelial cells) by collagenase digestion and several cycles washing and centrifugation at 400 *g*. Low eNOS mRNA levels were found in adipocyte preparations after the first wash but not after repeating the procedure five times (Fig. 1*B*). In contrast, eNOS mRNA was abundant in stromal cells in which adipocyte-specific PPARγ2 mRNA was absent.

Trace amounts of iNOS mRNA were detected in adipose tissue biopsies from patients with abdominal infection when 40 PCR cycles were applied instead of the usual 35 cycles (Fig. 1*A*). In contrast, iNOS mRNA was absent in noninfected subjects.

Transient iNOS mRNA induction in human adipocytes. In addition to visible lipid accumulation, the adipocyte character of MSC-derived adipocytes was documented by RT-PCR analysis. Several adipocyte marker genes, including adiponectin, leptin, and PPARγ2, were induced during adipogenic differentiation (Fig. 2). In contrast, adipocyte-unspecific PPARγ1 transcripts were also detected in undifferentiated MSC.

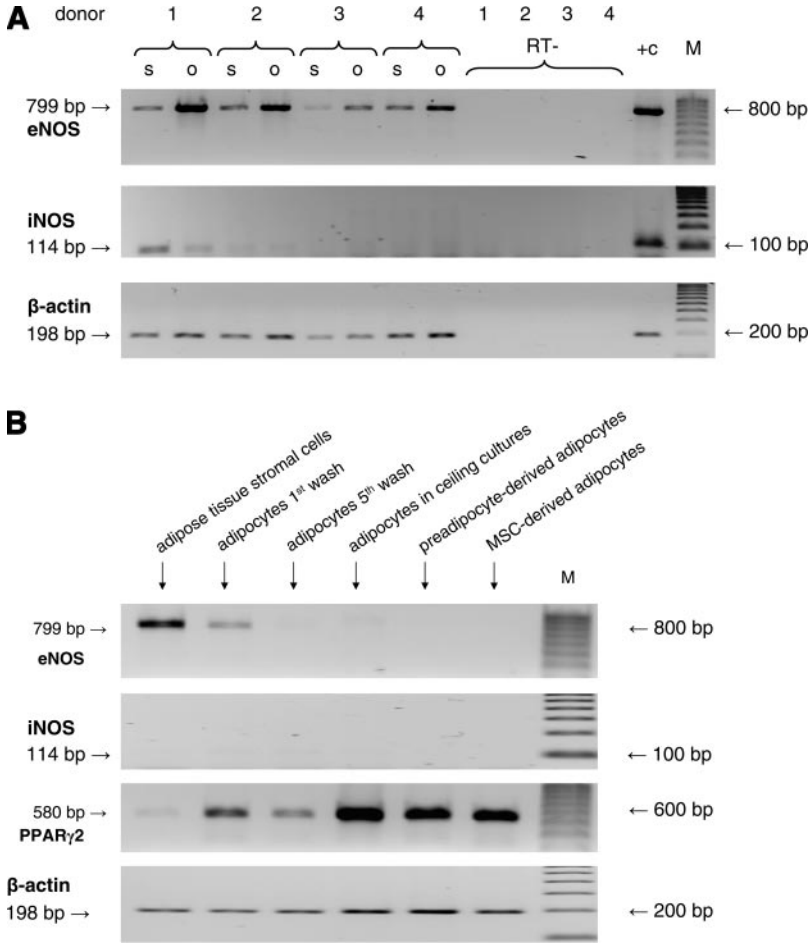

Fig. 1. RT-PCR analysis of endothelial (e) and inducible (i) nitric oxide synthase (NOS) mRNA. *A*: subcutaneous (s) and omental (o) adipose tissue biopsies were obtained from donors with infection (1 and 2) or without infection (3 and 4). Isolated RNA was subjected to RT-PCR analysis for eNOS and iNOS mRNAs. Absence of genomic DNA contamination was verified by performing reactions without RT (RT−). *B*: eNOS, iNOS, and peroxisome proliferator-activated receptor (PPAR)-γ2 mRNA were analyzed in mature explanted adipocytes at initial and final purification steps. In addition, eNOS mRNA content was assessed in adipocytes kept in ceiling cultures (preadipocyte) and in mesenchymal stem cell (MSC)-derived adipocytes. +c, positive control; M, 100-bp Molecular Ruler.

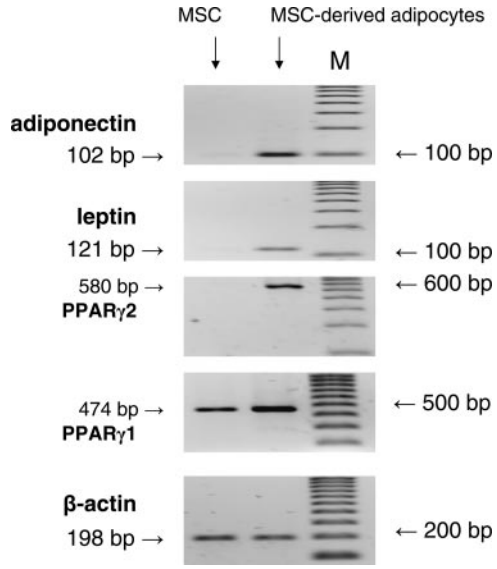

Fig. 2. Induction of adipocyte markers in MSC-derived adipocytes. MSC were subjected to RT-PCR analysis before and after adipogenic differentiation.

iNOS mRNA was induced in MSC-derived adipocytes after 6 h exposure to combined IFN-γ-IL-1β and IFN-γ-LPS but not by single treatments (Fig. 3*A*). In contrast, guanosine triphosphate cyclohydrolase (GTPCH) mRNA, the rate-limiting enzyme for BH_4 synthesis, was also induced by single treatments. TNF-α, IL-1β, and IL-6 mRNAs were induced by IL-1β and LPS alone but not by IFN-γ alone. Administration of IFN-γ-IL-1β and IFN-γ-LPS did not evoke iNOS mRNA induction when adipocytes were kept in adipogenic differentiation medium (data not shown).

iNOS mRNA induction was detectable ~4 h after IFN-γ-IL-1β and IFN-γ-LPS administrations (Fig. 3*B*). Under identical conditions, GTPCH, TNF-α, IL-1β, and IL-6 mRNA induction was observable after 2 h. iNOS and IL-1β mRNA were no longer detected after 24 h. GTPCH and TNF-α mRNA expression declined after incubation periods exceeding 10 h. IFN-γ-IL-1β- and IFN-γ-LPS-induced IL-6 mRNA remained elevated compared with controls for at least 48 h. In contrast, administration of IFN-γ-IL-1β and IFN-γ-LPS diminished PPARγ2 mRNA abundance.

In preadipocyte-derived adipocytes similar mRNA expression patterns were observed (Fig. 3*C*).

NO_2^- and NO_3^- analysis in culture supernatants. Putative cytokine-induced iNOS enzyme activity was assessed by analyzing NO-derived stable oxidation products NO_2^- and NO_3^- in adipocyte culture supernatants. Detection limit for NO_3^- after nitrate reductase treatment was 1 μM as determined in $NaNO_3$ dilutions (data not shown). Murine 3T3-L1 adipocytes served as controls: NO_2^- were increased at least 10-fold in 24-h conditioned supernatants from IFN-γ-TNF-α-IL-1β-treated 3T3-L1-derived adipocytes compared with basal conditions. In contrast, no NO_2^-/NO_3^- induction was found in supernatants from MSC-derived adipocytes incubated for up to 72 h with several combinations of inflammatory mediators, including IFN-γ-IL-1β, IFN-γ-LPS, IFN-γ-IL-1β-TNF-α, and IFN-γ-IL-1β-LPS. NO_2^-/NO_3^- induction was also negative in the additional presence of exogenous 10 μM sepiapterin, which is converted intracellularly to iNOS cofactor BH_4.

NOS activity was not detected in IFN-γ-IL-1β-TNF-α-exposed preadipocyte-derived adipocytes, even in the presence of sepiapterin (data not shown).

BH_4 synthesis in MSC- and in preadipocyte-derived adipocytes. Cytokine-induced BH_4 synthesis was assessed in MSC- and in preadipocyte-derived adipocytes. In basal cells, BH_4 concentrations were below the detection limit in lysates and 5.4 ± 0.5 nM in supernatants (Fig. 4). Upon 48 h exposure to combined IFN-γ-IL-1β-LPS, BH_4 concentration was 67.6 ± 13.2 nM in cell lysates. Under the same conditions, BH_4 secretion was increased 7.1-fold (38.4 ± 2.8 nM) in supernatants compared with untreated controls.

In basal cells, neopterin concentrations were 4.4 ± 2.8 nM in lysates and below the detection limit in supernatants. Neopterin concentration was augmented 36.2-fold (159.5 ± 38.13 nM) in cell lysates upon 48 h treatments with IFN-γ-IL-1β-LPS. In the respective supernatants, neopterin concentration was 14.9 ± 1.4 nM.

iNOS and PPARγ protein analysis in human adipocytes. We aimed to determine cytokine-induced iNOS protein expression by Western blot analysis. Basal and IFN-γ-TNF-α-IL-1β-treated 3T3-L1 adipocytes served as negative and positive controls, respectively (Fig. 5*A*). iNOS protein was not detected in protein lysates from MSC-derived adipocytes treated with IFN-γ-IL-1β for 8, 10, or 24 h. In addition, iNOS was neither found in IFN-γ-LPS (data not shown) nor in IFN-γ-, IL-1β-, and TNF-α-treated adipocytes (Fig. 5*B*). Lysates were probed in parallel for PPARγ protein to confirm integrity of lysates and the activity of administrated treatments. PPARγ1 and PPARγ2 were detected in lysates from basal adipocytes (Fig. 5, *A* and *B*). In accordance with mRNA data, PPARγ2 protein was downregulated by inflammatory treatments, including IFN-γ-IL-1β and IFN-γ-IL-1β-TNF-α.

Insulin-mediated glucose uptake. The RIMGU was measured in the presence or absence of IFN-γ-IL-1β and IFN-γ-LPS, respectively. After 2 h in fresh culture medium, basal RIMGU was 3.75 ± 0.32 (Fig. 6*A*). The addition of IFN-γ-IL-1β and IFN-γ-LPS diminished RIMGUs to 2.0 ± 0.1 (P 0.01) and 1.7 ± 0.1 ($P < 0.01$), respectively.

Upon 10 h exposure to either basal medium, IFN-γ-IL-1β, or IFN-γ-LPS, RIMGUs were 2.23 ± 0.06, 1.49 ± 0.07 ($P <$ 0.01), and 1.30 ± 0.06 ($P < 0.01$), respectively (Fig. 6*B*). IFN-γ-IL-1β- and IFN-γ- and LPS-mediated RIMGUs remained virtually unchanged when agents known to block NOS activity were coadministered (i.e., L-NAME, L-NNMA, and 1400W).

In further experiments we measured insulin-mediated glucose uptake after 24 h.

In untreated control cells, RIMGU was 2.21 ± 0.24 (Fig. 6*C*). Exposure to IFN-γ and IL-1β alone slightly reduced RIMGU to 1.56 ± 0.12 and 1.70 ± 0.21, respectively. Combined IFN-γ-IL-1β administration clearly reduced insulin-mediated glucose uptake throughout the experiments, with a RIMGU of 1.184 ± 0.05 ($P < 0.01$). Coadministration of L-arginine-derived NOS inhibitors L-NAME and L-NMMA had no influence on basal or IFN-γ- and IL-1β-modulated RIMGUs.

Glycerol release. Lipolytic activity in MSC-derived adipocytes was assessed by measuring glycerol accumulation in culture medium. IL-1β-induced glycerol release was time dependent for at least 24 h (Fig. 7*A*). Coadministration of IFN-γ

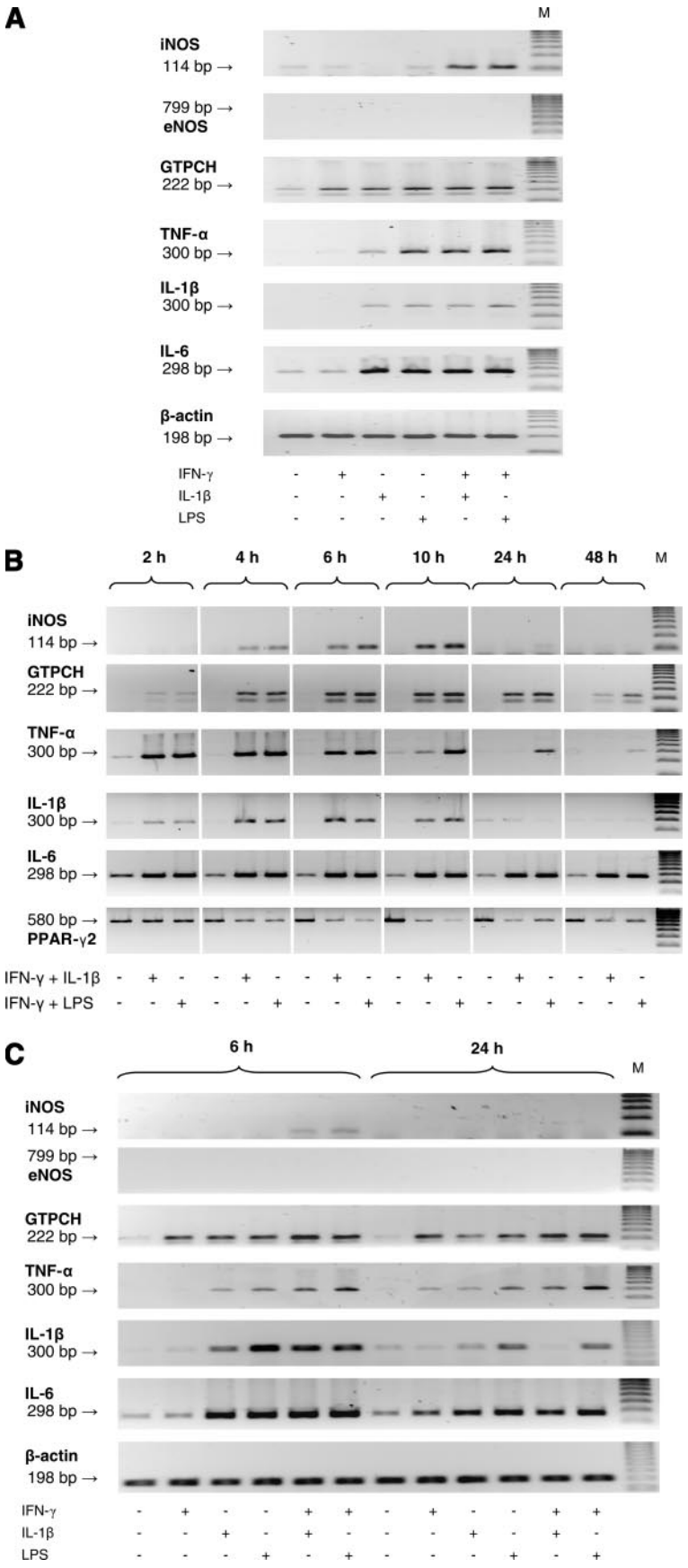

Fig. 3. Induction of iNOS and cytokine mRNAs in human adipocytes. MSC-derived adipocytes were subjected to inflammatory agents. RT-PCR analysis was performed after 6 h (*A*) or at the indicated time points (*B*). Similar experiments were performed in preadipocyte-derived adipocytes (*C*). Results are representatives from at least 3 separate experiments. TNF, tumor necrosis factor; IL, interleukin; IFN, interferon; LPS, lipopolysaccharide; +, with; −, without.

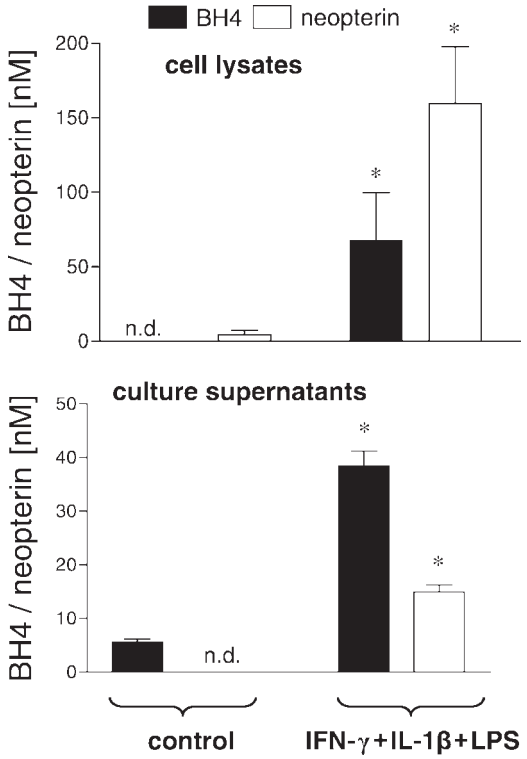

Fig. 4. Tetrahydrobiopterin (BH_4) and neopterin synthesis induction in human adipocytes. Human MSC- and preadipocyte-derived adipocytes were exposed to IFN-γ-IL-1β-LPS for 48 h. BH_4 and neopterin were measured both in cell lysates and in culture supernatants. Data were combined from experiments performed in MSC ($n = 4$)- and preadipocyte ($n = 2$)-derived adipocytes. Results are expressed as means ± SD. ND, not detectable. *$P < 0.01$ compared with untreated controls.

reduced IL-1β-mediated glycerol secretion by 38% ($P < 0.05$), 40% ($P < 0.05$), and 47% ($P < 0.05$) after 12, 16, and 24 h, respectively. Glycerol release remained at basal levels in the presence of IFN-γ, L-NAME, or L-NMMA alone (data not shown). Administration of L-NAME, L-NMMA, and 1400W had no effect on IL-1β- and IFN-γ-modulated lipolysis after 12 and 16 h (Fig. 7*B*).

DISCUSSION

In the present study, we analyzed expression kinetics and the potential role of NOS enzymes in several human adipocyte models before and after inflammatory stimulation. In contrast to rodent adipocytes, the presented data raise serious doubts on a relevant endogenous NO production in human adipocytes under both physiological and inflammatory conditions.

Adipose tissue is a complex conglomerate of several cell types, including adipocytes, preadipocytes, vascular endothelial cells, smooth muscle cells, macrophages, nerve endings, and others. Mature adipocytes account for only 16% of adipose tissue cells (23). In our hands, eNOS mRNA was not present in the final, purest adipocyte fraction. The contrast to published data (14, 15) is most likely because of contaminating stromal cells, which were still present in the adipocyte fraction after initial washes. To decontaminate adipocytes from adhering stromal cells, the negative buoyancy, lipid-laden fraction from collagenase-digested adipose tissue was washed five times. Moreover, the efficiency of centrifugation was increased by applying 400 *g* instead of 200 *g* as we generally used for adipose tissue stromal cell isolation (30). Accordingly, the abundant eNOS mRNA signal found in stromal cells disappeared after expansion of preadipocytes and differentiation to adipocytes. During the process of differentiation, preadipocytes and MSC adopted several characteristics of adipocytes, including insulin-mediated glucose uptake, inflammation-induced lipolysis, PPARγ2, leptin, and adiponectin mRNA expression. In contrast, eNOS mRNA was not induced. Insulin- and ANG II-modulated NO formation was recently demonstrated in human primary preadipocyte preparations (15). However, this report contained no evidence on functional NOS expression and NO synthesis in differentiated adipocytes. Taken together, the above-mentioned observations advocate that eNOS expression is restricted to the endothelium in adipose tissue. Our finding of increased eNOS mRNA abundance in omental compared with subcutaneous adipose tissue is in agreement with previous data (43). We hypothesize that this finding is attributable to the high density of vascular vessels in omental fat.

Tight regulation of iNOS activity is cell- and species-specific (19, 27). In rodent adipose tissue and adipocytes, the cytokine- and LPS-induced sustained NO production is well

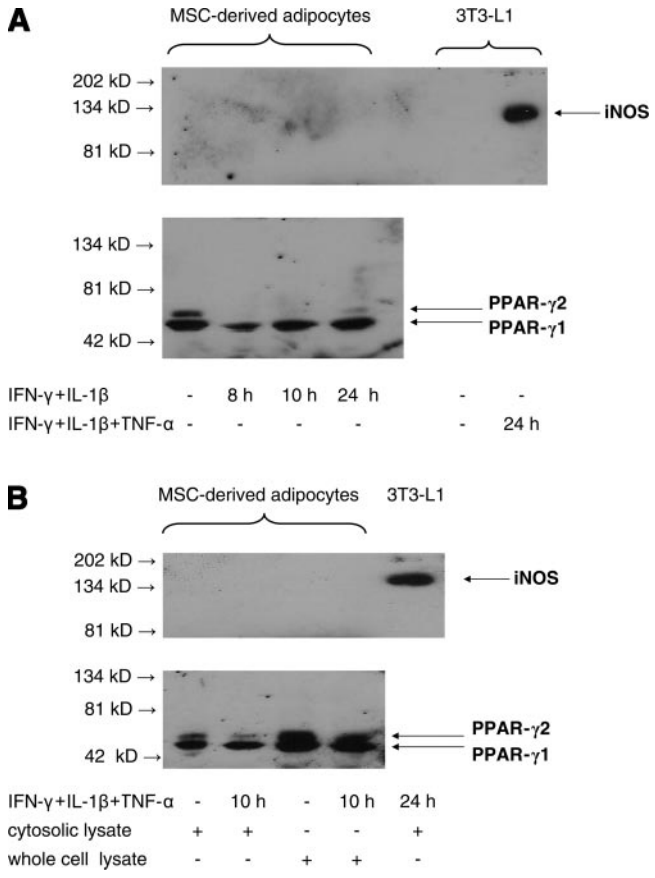

Fig. 5. iNOS and PPARγ protein expression in MSC-derived adipocytes. Adipocytes were subjected to iNOS mRNA-inducing treatments as indicated. Whole cell lysates (or cytosolic lysates where indicated) containing 40 μg (MSC-derived adipocytes) or 10 μg (3T3-L1) total protein were subjected to iNOS and PPARγ2 detection by Western blot. *A*: adipocytes were exposed for 8, 10, or 24 h to IFN-γ-IL-1β. *B*: adipocytes were treated for 10 h with IFN-γ-IL-1β-TNF-α. Results are representatives from 4 (*A*) and 2 (*B*) separate experiments.

established (25, 29, 34, 39). Obesity is increasingly recognized as an inflammatory condition (10, 47), and human obesity-related iNOS induction in adipose tissue has been proposed (14, 15, 43). In agreement with previous data on iNOS protein expression in adipose tissue biopsies, we found trace amounts of iNOS mRNA in some donors (4). Interestingly, rodent obesity is associated with macrophage accumulation in adipose tissue (46, 48). Therefore, it may be hypothesized that obesity-related inflammatory processes, including iNOS expression, are attributable to macrophages and other nonadipocytes within adipose tissue. In human preadipocytes, a modest increase in iNOS mRNA abundance was recently reported during the initial 8 days of in vitro adipogenesis (15). However, data of functional iNOS enzyme induction were not provided. The transcriptional iNOS activation may be explained by differentiation-related NF-κB activation, as observed in 3T3-L1 (7).

Primary preadipocyte-derived adipocytes are a well-established, albeit laborious, cell model allowing in vitro studies of

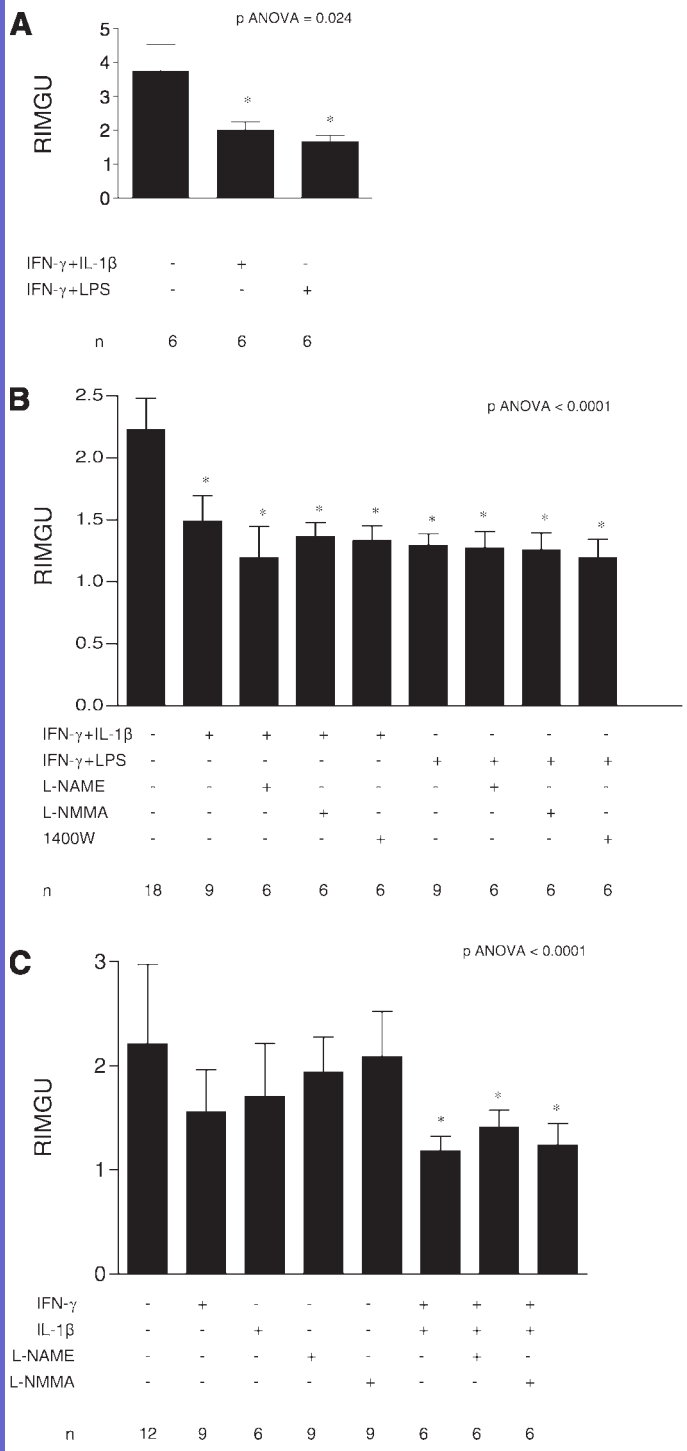

Fig. 6. Insulin-mediated glucose uptake is not affected by NOS inhibitors. MSC-derived adipocytes were exposed to IFN-γ, IL-1β, LPS, N^{ω}-nitro-L-arginine methyl ester (L-NAME), N^{ω}-monomethyl-L-arginine (L-NMMA), and 1400W as indicated for 2 h (*A*), 10 h (*B*), and 24 h (*C*). Glucose uptake was measured in the presence or absence of 100 nM insulin. Results are expressed as means ± SD of the ratio of insulin- and non-insulin-mediated glucose uptake (RIMGU). Cells from at least 2 separate donors were used; *n* indicates the no. of separate wells. *$P < 0.01$ compared with untreated controls.

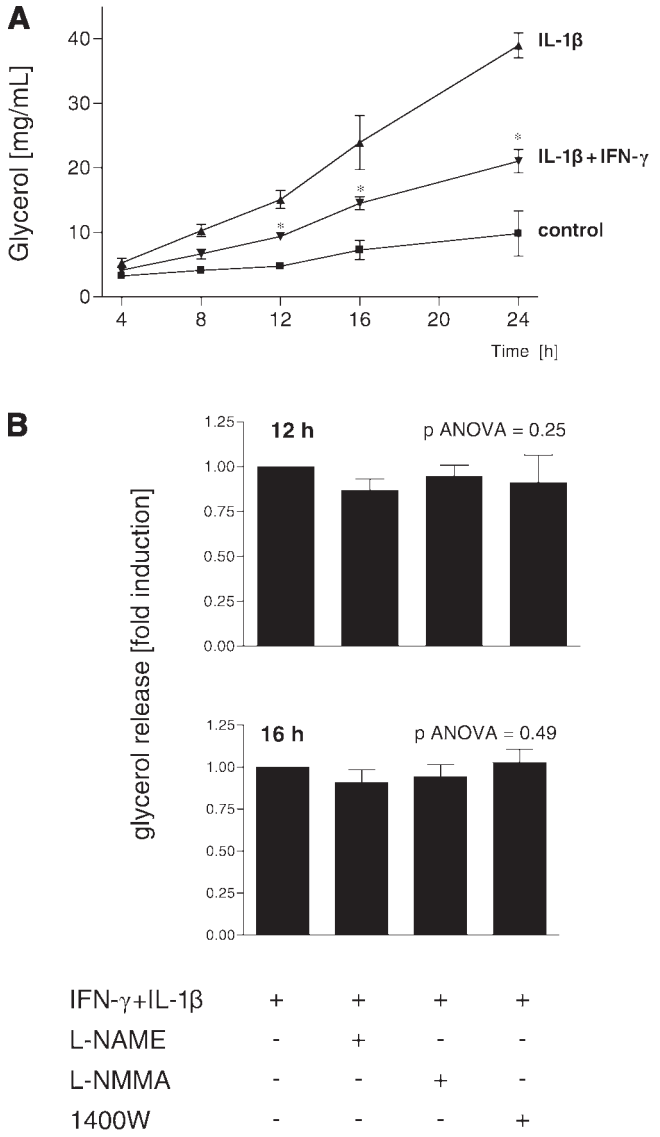

Fig. 7. Lipolysis is not affected by NOS inhibitors. IFN-γ, IL-1β, LPS, L-NAME, L-NMMA, and 1400W were added to MSC-derived adipocytes as indicated at time = 0. Glycerol concentration was measured in culture supernatants. *A*: time dependence of IL-1β- and IFN-γ- and IL-1β-induced glycerol release. *B*: IFN-γ- and IL-1β-induced glycerol release in the presence or absence of NOS inhibitors. Results are expressed as means ± SD from $n = 4$ (*A*) and $n = 6$ (*B*) separate wells. Cells from 2 separate donors were used. *$P < 0.05$ compared with IL-1β alone.

Primary preadipocyte-derived adipocytes are a well-established, albeit laborious, cell model allowing in vitro studies of

human fat cells (24). Expanding MSC cultures appear as a useful alternative for adipocyte precursor cells. MSC-derived adipocytes express and secrete several adipocyte markers, including immunodetectable leptin and adiponectin, and exhibit inducible lipolytic activity (42). In addition to adipocyte-specific mRNA expression (e.g., PPARγ2, leptin, adiponectin), lipid storage, and lipolytic activity, the herein presented insulin-mediated glucose uptake experiments further document the adipocyte character of the MSC-derived model. The observed inflammation-mediated PPARγ2 mRNA and protein down-regulation validates previous observations made in 3T3-L1-derived adipocytes (17, 45).

To our knowledge, induction of NO generation in human adipocyte models exposed to inflammatory mediators has not yet been addressed. In MSC- and in preadipocyte-derived adipocytes, iNOS mRNA was induced by combined IFN-γ-IL-1β and IFN-γ-LPS, respectively, but not by IFN-γ, IL-1β, and LPS alone. IL-1β- and LPS-mediated gene induction occurs mainly via NF-κB, whereas STAT1 mediates IFN-γ signaling (1, 13). Accordingly, the human *NOS-2* gene is known to depend on both STAT1- and NF-κB-mediated promoter activation (19). In agreement with previous human data, inflammation-induced iNOS mRNA expression was transient in the present human adipocyte models (12). However, at the time points with the highest iNOS mRNA abundance, iNOS protein was not detected by Western blot analysis using a monoclonal antibody suitable for human iNOS detection (31, 32). Accordingly, we failed to measure cytokine-induced NO oxidation products. Thus, similar to other human cells, translational mechanisms appear to block human adipocyte iNOS expression (31). In human monocytes and macrophages, the activity of existing iNOS protein is blocked by low BH_4 synthesis capacity (28). This appeared not to be the case in our adipocytes: the inflammation-induced expression of the BH_4-synthesizing enzyme GTPCH evoked the production of both BH_4 and its side product neopterin. The presence of a functional BH_4 synthesis pathway was previously described in rodent (18, 29), but not in human, adipocytes. In the absence of functional NOS, the role of inflammation-induced BH_4 in human adipocytes remains to be elucidated.

Low NO synthesis may occur at undetectable levels and modulate glucose metabolism and lipolytic activity. Therefore, we performed additional experiments with metabolic read outs (i.e., glucose uptake and glycerol release). MSC-derived adipocytes exposed for 2 h to IFN-γ-IL-1β or IFN-γ-LPS displayed markedly reduced insulin-mediated glucose uptake. A role of iNOS-derived NO can be ruled out since iNOS mRNA induction initiated approximately after 4-h incuabtions. Further glucose uptake experiments were performed after 10-h exposures when iNOS mRNA reached highest abundance. However, the additional presence of NOS inhibitors (i.e., L-NAME, L-NMMA, and 1400W) had no effect on insulin-mediated glucose uptake. Similar results were previously shown in murine 3T3-L1 and T37i adipocytes (38). Interestingly, obesity-induced insulin resistance is not ameliorated by *NOS-2* gene inactivation in mouse adipose tissue (35). In contrast, muscle tissue is protected against obesity-linked insulin resistance in the same animal model. Accordingly, L-NAME-mediated NOS inhibition prevents cytokine- and LPS-induced insulin resistance in L6 myocytes (6). Thus NO may play a role in glucose metabolism in muscle. A similar role appears unlikely in adipocytes.

Complex and partially conflicting data have been presented on NO as a potential modulator of adipose tissue lipolysis (4, 14, 20, 21, 25, 34, 37–39, 41, 43). The interpretation of experiments using exogenous NO sources appears to be problematic. Different NO-generating chemicals (e.g., *S*-nitroso-*N*-acetyl-penicillamine, NO gas, PAPA-NONOate) give raise to distinct, highly reactive NO-related molecules (e.g., nitrosonium, $NO^{\cdot}$, nitroxyl anion). Consequently, experiments using different NO donors have suggested both pro- and anti-lipolytic NO-mediated effects (20). Moreover, the proposed NO-mediated attenuation of isoprotereonol-mediated lipolysis (2) might be because of extracellular, oxidation-linked inactivation of the β-adrenergic agonist (11, 26). The impact of endogenous NOS inhibition in adipocytes and adipose tissue has been addressed in other reports. L-NMMA administration increases lipolysis in human adipose tissue, as shown in a microdialysis study (4). The absence of eNOS expression and the ineffective NOS inhibitor administration in the present adipocyte models indicates an indirect effect possibly mediated by vascular eNOS. In contrast, basal and dibutyryl cAMP-induced lipolysis were decreased by DPI, another NOS inhibitor, in isolated rat adipocytes (21). These conflicting results may be explained by species specificity, different adipose tissue models, and the different NOS inhibitors.

Inflammatory cytokines have pro-lipolytic effects (16, 42). In accordance with this view, IL-1β induced a time-dependent glycerol release in MSC-derived adipocytes. As opposed to previous 3T3-F442A adipocyte-related observations, IFN-γ had no effect on basal lipolysis in MSC-derived adipocytes. Surprisingly, glycerol release was markedly reduced in IFN-γ- and IL-1β-administered cells compared with IL-1β alone. Based on studies in murine adipocyte models, including 3T3-L1, T37i, and adipose tissue explants from NOS $2^{-/-}$ knockout mice, iNOS-derived NO has been proposed as a negative feedback inhibitor containing excessive inflammation-induced lipoysis (34). In contrast, the lipolytic activity under inflammatory conditions appears to be NO independent in the present human adipocyte model.

In summary, we conclude that, in human adipocytes, endogenous NO does not play a mandatory role during IL-1β- and IFN-γ-induced gene expression and metabolic modulation. In adipose tissue, however, NO from other sources (e.g., endothelial cells, macrophages) may exert effects on neighboring adipocytes. The present study provides further support for the species-specific regulation of adipose tissue metabolism, such as the previously reported production and release of cytokines and adipokines (5), emphasizing the importance of investigating human adipose tissue to improve our understanding of human physiology.

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VI

Human bone marrow-derived mesenchymal stem cells differentiate into insulin, somatostatin and glucagon expressing cells

Seboek D, Timper K, Eberhardt M, Linscheid P, Keller U, Müller B and Zulewski H (submitted)

Human bone marrow-derived mesenchymal stem cells differentiate into insulin, somatostatin and glucagon expressing cells

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ABSTRACT

Replacement of insulin producing cells represents an almost ideal treatment for patients with diabetes mellitus type 1. Here we show that human bone marrow-derived mesenchymal stem cells (MSC) harbour the potential to differentiate into insulin, glucagon and somatostatin expressing cells *in vitro*. MSC from 5 healthy donors and 3 patients with diabetes type 1 were expanded and differentiated using defined culture conditions. Upon differentiation we observed the expression of various pancreatic genes including the transcription factors Isl-1, Ipf-1, Ngn-3, Pax-4, Pax-6, Nkx-2.2 and Nkx-6.1 as well as the islet proteins insulin, glucagon, somatostatin and the glucose transporter glut-2.

Keywords: diabetes, mesenchymal stem cell, differentiation, human, pancreas

Introduction

Stem/progenitor cells with the potential to differentiate into insulin expressing cells were described in pancreatic ducts[1], islets of Langerhans [2,3], the liver [4,5], the central nervous system [6], the spleen [7] and bone marrow [8,9]. Mesenchymal stem cells (MSC) from mouse bone marrow were shown to harbour the potential to differentiate into insulin secreting cells *in vitro* and to reverse hyperglycaemia in an animal model of diabetes [9]. Similarly, mesenchymal precursor cells from mouse spleen were able to regenerate insulin producing cells in a mouse model of autoimmune diabetes [7]. Recently human MSC were described to express at low level the islet transcription factor Nkx-6.1 and to differentiate into insulin producing cells upon adenoviral transduction with vectors over-expressing the transcription factors Ipf-1, Hlxb-6 or Foxa-2 [10]. The mechanisms underlying this apparent developmental plasticity of MSC are unknown. Interestingly, MSC were also shown to bear the potential to adopt a neural phenotype *in vitro* and *in vivo* [11-14] in rodents and humans [13] suggesting a neuro-endocrine developmental capacity of these cells. Expanding MSC express several stem cell marker like stem cell factor (SCF) and Thy-1 [15,16] but also nestin [13,14,17], a gene initially characterized as a marker of neural stem or progenitor cells [18] and later also suggested to be a marker for multipotent pancreatic stem cells [3]. Neural precursor cells express beside nestin also the side population stem cell marker ABCG2 [19,20]. Nestin and possibly ABCG2 expression could therefore represent a possible link between MSC and their ability to differentiate into neuro-endocrine cells. ABCG2 expression was not yet described in human bone marrow derived MSC.

Stem/progenitor cells with the capacity to adopt a pancreatic endocrine phenotype should follow at least in part the complex program of normal pancreas development, for review see [21]. Initiation of pancreas development requires the induction of the transcription factor Ipf-1 that is prerequisite for pancreas formation in mouse and man [22,23]. Shortly thereafter the transcription factor islet-1 (Isl-1) that is required for generation of endocrine cells is induced at day E9 [24] followed by induction of the transcription factor Ngn-3, another crucial step toward pancreatic endocrine

cells [25,26]. In the present study we show that MSC from human bone marrow of non-diabetic subjects as well as patients with longstanding type 1 diabetes are positive for the stem cell markers nestin and ABCG2 and display the potential to activate pancreatic developmental genes in response to defined culture conditions. This includes the transcription factors Ipf-1, Isl-1, Ngn-3, Pax-4, Pax-6, Nkx-2.2, Nkx-6.1 as well as the islet genes insulin, glucagon, somatostatin and glut-2.

RESEARCH DESIGN AND METHODS

Isolation and expansion of MSC

Five non-diabetic subjects (age 26 to 64 years) referred to the University Hospital for routine orthopaedic surgery were asked to donate 20 ml bone marrow during the surgical procedure. Three consecutive c-peptide negative patients (age 31, 50 and 53 years) with diabetes mellitus type 1 for 14, 35, and 37 years, respectively donated 20 ml bone marrow that was obtained by aspiration biopsy. All patients gave informed written consent and the study protocol was approved by the local ethics committee for human studies.

After diluting the marrow aspirates with phosphate buffer saline (PBS) at a ratio of 1:4, nucleated cells were isolated using a density gradient solution (Histopaque, Sigma Chemical, Buchs, CH). The cells were cultured in Minimum Essential Medium (MEM) α medium with 5.5 mM glucose containing 10% fetal bovine serum (FBS), 1% HEPES (1M), 1% sodium pyruvate MEM 100 mM, 1% penicillin-streptomycin glutamate (10,000 U/ml penicillin; 10,000 µg/mL streptomycin) (all from Invitrogen AG, Basel, CH). Nucleated cells were plated at a density of 100,000 cells/cm^2 in the medium supplemented with 5 ng/mL fibroblast growth factor-2 (FGF, R&D Systems, Wiesbaden, D) and cultured in a humidified incubator at 37°C, 5% CO_2. FGF was supplemented in order to enrich the fraction of true progenitor cells [27]. MSC were cultured in 175cm^2 Flasks (Becton Dickinson AG, Basel, CH), allowing attachment. Medium was changed twice a week. MSC were selected on the basis of adhesion and proliferation on the plastic substrate.

MSC culture

After the 3rd passage medium was changed to DMEM with glucose 25 mM supplemented with 10% heat inactivated FBS, 5 ng/mL FGF, 1% sodium pyruvate MEM 100 mM and 1% penicillin/streptomycin 5000 U/mL (all from Invitrogen AG, Basel, CH). Media and supplements were changed every 72 h. At a confluency of 95%, cells were collected using trypsin (Invitrogen AG,

Basel, Switzerland) and washed twice with serum-free DMEM/F12 medium.
For induction of differentiation, MSC were replated at a cell density of 2-3 x 10^5/well and cultured for 3 days in ultra low attachment 6 well plates (Vitaris AG, Baar, Switzerland) allowing the formation of islet-like clusters. MSC from non-diabetic subject 1 were analyzed on two occasions, at passage 4 and passage 7. The gene expression profile of each differentiation study was analyzed every 24 hours for 3 days in every subject or patient. For each differentiation day cells from 4 separate wells were harvested and analyzed independently and a mean value was calculated. Differentiation medium consists of serum-free DMEM/F12 medium with 17.5 mM glucose in the presence of nicotinamide 10mM, activin-A 2 nM, exendin-4 10nM, hepatocyte growth factor 100 pM and pentagastrin 10 nM (all from Sigma, Basel, Switzerland) as well as B-27 serum-free supplement, N-2 Supplement and 1% penicillin/streptomycin 5,000 U/l (all from Invitrogen AG, Basel, Switzerland). Betacellulin (Sigma Chemical, Buchs, Switzerland) was added in a concentration of 2 nmol/L.

RNA isolation and Reverse transcription (RT)

Total RNA was extracted using TRIzol reagent (Lucerna Chemie AG, Luzern, Switzerland) according to the manufacturer's protocol. RNA samples were treated with DNase (Ambion, Cambridgeshire, UK) in order to remove possible genomic DNA. RNA was quantified spectrophotometrically at 260 nm (Biophotometer, Eppendorf-Vaudaux, Schönenbuch, Switzerland). The quality was assessed by gel electrophoresis on an agarose gel containing ethidium bromide (EtBr, BioRad Laboratories AG, Reinach, Switzerland). 1 µg of total RNA was subjected to reverse transcription (RT) (Omniscript RT kit; Quiagen, Basel, Switzerland). Human islet RNA was a gift from Wolfgang Moritz, University Hospital Zürich, Switzerland.

Polymerase chain reaction (PCR)

PCR was performed on a conventional thermal cycler (TGradient, Biometra, Göttingen, Germany) using PCR Taq core kit (Qiagen). Primers were as mentioned in table 1. Amplification products were visualized on agarose gels containing 0.5 μg/mL ethidium bromide. 100 bp Molecular Ruler (BioRad, Reinach, Switzerland) was run as size reference. PCR product identity was confirmed by nucleotide sequencing (Microsynth AG, Balgach, Switzerland).

Quantitative Real-Time polymerase chain reaction (PCR)

cDNA, obtained as described above, was subjected to quantitative real-time PCR analysis using the ABI 7000 Sequence (Perkin Elmer, USA) detection system. Specific primers yielding short PCR products suitable for Sybr-Green (Abgene, Epsom, UK) detection were designed using Primer Express software (version 2.0; PE Applied Biosystems, Foster City, CA). For sequences of primers see Table 1. The reaction consisted of 50 µL, containing 25 µL Sybr-Green, 2 µL sense primer (10 µmol/L), 2 µL antisense primer (10 µmol/L), 16 µL H_2O; and 5 µL cDNA. Conditions were set as suggested by the manufacturer. Each cDNA sample tested for quantitative gene mRNA expression was also subjected to Hypoxanthine PhosphoRibosyl Transferase (HPRT) mRNA analysis. For quantitative analysis of gene expression the standard curve method was used with dilution of HPRT up to 10^{-8}. Results were expressed as the ratio of the respective gene mRNA and HPRT mRNA threshold values. Data are expressed as percentage expression as compared to day 0. For combined analysis of gene expression on days 0, 1, 2 and 3 in non-diabetic subjects the mean value of each single differentiation experiment (calculated from the results of 4 wells that were analyzed independently) was used. The product identity was confirmed by sequence analysis and electrophoresis on a 3% agarose gel containing EtBr.

Analysis of mRNA expression of pancreatic developmental genes as compared to human islets.

cDNA of human islets was a kind gift from Wolfgang Moritz, University Hospital Zürich. Real-time PCR was carried out as mentioned above with the same primer pairs. Gene copy numbers were calculated as the ratio of the respective gene mRNA and HPRT mRNA threshold values. mRNA expression of the respective gene in human islets was then expressed as the ratio of the respective gene on day 3 of the differentiated MSC.

Peptide measurements

Somatostatin concentration was determined in culture supernatants using a commercially available somatostatin radioimmunoassay (RIA) kit (functional assay sensitivity: somatostatin 5 pg/tube, Phoenix Pharmaceuticals Inc., Belmont, CA)

Immunocytochemistry

Cultured MSC were transferred into Lab-Tek chamber slides (Nunc, Naperville, IL) and incubated overnight in expansion medium. Islet-like clusters were collected after 3 days in differentiation medium. Some of them were dissociated by trypsin-ethylenediaminetetraacetic acid (EDTA) in order to obtain single cells. Single cells or islet-like clusters were transferred on glass slides coated with poly-L-lysine (Sigma, Buchs, Switzerland) and incubated overnight in DMEM/F12 medium containing 10% FBS allowing them to attach. All cells were then fixed with 4% paraformaldehyde in PBS (pH 7.4) for 30 min at room temperature. After several rinses in PBS, cells were permeabilized with chilled methanol for 10 min. Unspecific binding was prevented by incubation with 10 % heat inactivated FBS in PBS at RT for 30 min. Fixed cultured MSC were incubated with primary antisera for 3 h at RT or overnight at 4°C, rinsed off with PBS and incubated with secondary antisera for 1 h at RT. Fixed islet-like clusters or single cells were incubated with primary antisera for 60-90 min at 37°C, rinsed off with PBS and incubated with secondary goat antisera for 45 min at 37°C. After several washes with PBS, cells were coverslipped with non-fluorescing mounting medium. The primary

antibodies used were rabbit anti-human C-peptide for dispersed cells (dilution 1:500) (Linco, St. Charles, MO), sheep anti-human C-peptide for islet like cluster (dilution 1:100) (Abcam, Cambridge, MA) and rabbit anti- glucagon (dilution 1:100) (Linco, St. Charles, MO). Mouse monoclonal antibody 39.4D5 raised against Isl1 (dilution 1:100) was obtained from Developmental Studies Hybridoma Bank, University of Iowa, IA. The rabbit anti-human Ipf1 (dilution 1:1,000) was a generous gift from J.F. Habener, Boston, MA. The secondary antibodies from Molecular Probes (Invitrogen AG, Basel. Switzerland) were goat anti-mouse (dilution 1:1,000), donkey anti-sheep (1:100) and, goat anti-rabbit (dilution 1:1,000). Goat anti mouse IgG and donkey anti-sheep IgG were labeled with alexa fluor 488 dye. Goat anti rabbit IgG was labeled with alexa fluor 546 dye. 4, 6-diamidino-2-phenylindole (DAPI, 5 µg/ml) from Sigma (Basel, Switzerland) was used to label the nuclei (10 min at 37°C). Cells were then examined either by fluorescence microsope (Axiophot, Zeiss, Germany) or by confocal microscope (LSM 510, Zeiss, Germany).The insulinoma cell line INS-1E was kindly provided by Claes Wollheim, Geneva, Switzerland.

RESULTS

Isolation of MSC of non-diabetic subjects. Human bone marrow derived MSC from five independent donors were isolated on the basis of adhesion to tissue culture dishes and proliferation in FGF-2 containing culture medium. The initial population of mononuclear cells purified by Ficoll density gradient technique was positive for classical markers of haematopoietic cells like CD45 and c-Kit but also ABCG2 that is known to be expressed in erythroid precursors and natural killer lymphocytes (Fig. 1a). These cells were mostly of haematopoietic origin. They were negative for Thy-1, nestin and Isl-1. During the process of MSC isolation haematopoietic cells don't adhere to plastic surface and are discarded with medium change leading to loss of CD45 positive cells. Consequently, proliferating MSC were negative for CD45, according to their non-haematopoietic origin and showed a strong expression of SCF and Thy-1, markers typically expressed in MSC (Fig. 1a). C-kit mRNA expression was clearly reduced as compared to initial population of mononuclear cells (Fig.1 a). In addition, MSC expressed nestin, ABCG2 and Isl-1 mRNA (Fig. 1a) while Ipf-1 was not detected with standard RT-PCR. (data not shown). Immunocytochemistry revealed Isl-1 protein in the nuclei of approximately 10% to of cultured MSC (Fig. 1b).

Induction of differentiation was performed in serum-free medium (17.5 mM glucose) supplemented with exendin-4, pentagastrin, activin-A, betacellulin, nicotinamide and hepatocyte growth factor. Over the three day period we could observe a sharp step wise decrease in the expression level of the stem cell marker ABCG2 (Fig. 2a). This was mirrored by up-regulation of Isl-1, Ipf-1, Ngn-3 and Pax-6 (Fig. 2a). In addition, we observed an up-regulation of the glucose transporter glut-2, insulin, glucagon and somatostatin (Figure 2b).

After having finished the first series of quantitative real time PCR studies we also analyzed the expression of additional transcription factors of the β-cell lineage i.e. Pax-4, Nkx2.2 and Nkx6.1 using standard PCR technique. As compared to undifferentiated cells these developmental markers were also up-regulated at day 3 (Fig. 3).

Analysis of islet proteins in differentiated MSC. Formation of islet like clusters was observed already 24 hours after initiation of the differentiation process (Figure 4a). For immunocytochemistry analysis of Ipf-1 cells in the cluster were dissociated, and stained with the Ipf-1 antiserum. Cells that stained positive for Ipf-1 after 3 days displayed a nuclear staining pattern (Figure 4b). Ipf-1 was not detected in undifferentiated cells (data not shown). C-peptide and glucagon positive cells were found in differentiated islet-like cluster (Fig. 4c-f). Although insulin or glucagon release was not found in differentiated islet like clusters we were able to measure somatostatin release into the culture medium. Somatostatin was detectable already 24 hours after initiation of differentiation and reached plateau levels at days 2 and 3 (Fig. 4g).
When compared to adult human islets the expression levels for Ipf-1, Isl-1, Ngn-3 and Pax-6 found in our islet like cluster reached values of 0.07%, 21%, 290% and 20% respectively, of the expression levels found in human islets. Conversely, the corresponding values for mRNA expression for glut2, somatostatin, glucagon and insulin reached 6.1%, 0.2%, 0.3% and less than 0.01% of the values found in human islets.

Differentiation of bone marrow derived MSC from type 1 diabetic patients. We next sought to determine if MSC from type 1 diabetic patients would also be able to adopt a pancreatic endocrine phenotype. Bone marrow derived MSC from three patients with long-standing type 1 diabetes expressed similar stem cell markers during the expansion period together with Isl-1 as did MSC from non-diabetic subjects (Fig. 5). In proof of principle experiments induction of differentiation resulted in an up-regulation of the transcripts for Ipf-1, Ngn-3 as well as glut-2, insulin, somatostatin and glucagon as illustrated in figure 6. Although all three patients with diabetes expressed Isl-1 and ABCG2 in proliferating MSC and up-regulated insulin, glucagon and somatostatin mRNA during the differentiation period we observed a substantial variation in the expression level of these genes between patients. C-peptide was also detected with immunocytochemistry on single dispersed cells (Fig. 6c)

DISCUSSION

The data presented herein show that human bone marrow-derived MSC are able to adopt a pancreatic endocrine phenotype *ex vivo* in non-diabetic subjects as well as patients with longstanding type 1 diabetes. This was achieved without genetic modification of the cells and in response to defined culture conditions. Initially these cells were cultured in high glucose (25mM) and FGF (5ng/ml) and expressed not only the stem cell markers nestin and ABCG2 but also the transcription factor Isl-1. ABCG2 is a known marker for the side population phenotype stem cells in bone marrow [20] and was recently found in pancreatic islet derived precursor cells and neural stem cells [19,28]. Together with Isl-1 it may thus point to a subpopulation of MSC with a neuro-endocrine developmental potential. During the differentiation experiments a strong down-regulation of ABCG2 gene expression was observed indicating that the cells indeed changed their developmental state from a stem cell to a more differentiated cell type. ABCG2 however is known to be expressed not only in stem cells but also in erythroid precursors and natural killer lymphocytes [20]. Both cell populations are initially co-purified by Ficoll density gradient leading to the positive ABCG2 signal (Fig.1) in the primary mononuclear cell population that was isolated from the bone marrow and contain mostly haematopoietic cells. During the expansion period however only CD45 negative cells continued to proliferate indicating a non-haematopoietic origin of these cells. In addition classical markers for MSC were up-regulated like Thy-1 and SCF. Similar cells are routinely differentiated into adipocytes or osteoblasts in our institution [29,30] demonstrating their mesenchymal phenotype.

The transcription factor Isl-1 is crucial for the development of pancreatic endocrine cells. Disruption of Isl-1 expression is associated with absence of dorsal mesenchyme and a marked reduction of Ipf-1 gene expression in dorsal epithelium in mice [24]. Embryonic explants of the pancreatic anlage from Isl-1 (-/-) mice did not generate insulin, glucagon or somatostatin positive cells *in vitro* as did explants from Isl-1 (+/-) animals [24]. Isl-1 expression is found at embryonic day 9 in the mouse and is together with Ipf-1 one of the earliest pancreatic markers detected during development [24]. We

hypothesize that induction of Isl-1 expression in human MSC may represent the primary critical event that allows adoption of a pancreatic endocrine phenotype. Signals in the culture medium that triggered Isl-1 expression in a subpopulation of MSC could be the high glucose concentration, FBS, FGF-2 or all factors together. The exact mechanisms however, remain to be elucidated.

Beside its crucial role for development of pancreatic endocrine cells Isl-1 is also involved in the development of the central nervous system and the heart [31-34]. Therefore, some of these Isl-1 positive MSC could also represent potential progenitors for motor neurons [31] or cardiomyocytes [34].

Using serum-free medium supplemented with factors known for their beneficial effects on differentiation of pancreatic or hepatic precursors into insulin producing cells [35-38] we have induced the activation of various crucial pancreatic transcription factors including Ipf-1, Ngn-3, Pax-4, Pax-6, Nkx-2.2 and Nkx-6.1 as well as the islet genes insulin, glucagon, somatostatin and glut-2. The differentiation process *in vitro* described herein may represent -at least in part- replication of ontogeny in response to the defined culture conditions. Noteworthy, these results were obtained in normal subjects as well as in patients with diabetes mellitus type 1 and, without genetic manipulation of MSC. As a limitation, these cells were not yet able to secret insulin or glucagon (data not shown) indicating a certain level of developmental immaturity although they did secrete somatostatin. Viewing the very low expression of insulin as compared to human islets we are still far away from clinically meaningful insulin production in bone marrow derived MSC. But, for the first time we show that cultured human MSC may have such potential without genetic modification.

The real time PCR studies over 3 days revealed that many pancreatic genes were activated already 24h after induction of differentiation. These profound changes in the transcriptional program occurred surprisingly fast. But, observations of early activation of insulin gene expression were also made in mice *in vivo* in response to hyperglycaemia [39]. Here the authors found insulin positive cells in the bone marrow and adipose tissue of hyperglycaemic mice already after 3 days. These cells could represent MSC as described in

the present study and our previous report showing induction of pancreatic developmental genes in human adipose tissue derived MSC [40].

Several studies addressed the possibility that bone marrow derived stem cells could contribute to β-cell turnover *in vivo*. Some of them presented positive results and suggested the presence of a circulating pool of stem cells that could participate in the process of β-cell neogenesis [8]. These results however were not confirmed by others [41] and a recent report questioned the entire concept of β-cell stem/progenitor cells with studies using genetic lineage tracing experiments [42]. With this approach it has been shown that pre-existing β-cells rather than adult stem/progenitor cells retained a proliferative capacity and may thus represent the major source of new β-cells in adult life, at least in mice [42]. While *in vivo* studies are not conclusive regarding the role of adult stem cells for generation of new β-cells this does not exclude the differentiation of adult stem cells into insulin producing cells *in vitro*. It has been shown that mouse mesenchymal stem cells from bone marrow cultured in high glucose over 4 months induced several β-cell specific genes including insulin and glut-2 [9]. These cells were also able to reverse hyperglycaemia in an animal model of diabetes although other crucial transcription factors like Pax4 and Isl-1 were not expressed [9]. In accordance with this report by Tang et al. our human MSC are also CD45 negative and express nestin during the expansion period.

In summary Isl-1 positive MSC can be isolated from human bone marrow and are able to adopt a pancreatic endocrine phenotype in non-diabetic as well as type 1 diabetic subjects. These cells could be used as a human model to study development of pancreatic endocrine cells *ex vivo* and may help to develop stem cell based therapies for diabetes mellitus type 1.

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FIGURE LEGENDS

Figure 1. Expression of stem cell markers in mononuclear cells and MSC. (a) Presence of CD45, c-kit, ABCG2, SCF, Thy1, nestin and Isl-1 mRNA was analyzed by RT-PCR in freshly isolated mononuclear cells (Ficoll separation) and after the 6^{th} passage in expansion medium. (b) Immunocytochemistry for the transcription factor Isl-1 (magnification x 200). Arrows indicate positive staining in nuclei of MSC. Nuclei were counterstained with DAPI. Positive control was carried out with INS1E cells (right panel).

Figure 2. Induction of pancreatic developmental genes in MSC in response to defined culture conditions in non-diabetic subjects. Gene expression was monitored every 24h for 3 days. Data are normalized to HPRT and expressed as percentage expression as compared to day 0. Results are means ± SEM of quadruplicates of six independent experiments from five independent donors.
(a) mRNA expression of the side population marker ABCG2 and the pancreatic transcription factors, Isl-1, Ipf-1, Ngn-3 and Pax-6 was analyzed by real-time PCR. (b) Induction of glut-2, insulin, somatostatin and glucagon (logarithmic scale).

Figure 3. Expression of Pax4, Nkx2.2 and Nkx6.1 mRNA was analyzed by conventional PCR in separate experiments (n=3).

Figure 4. Analysis of islet like clusters generated from MSC.
(a) Phase contrast image of islet like clusters after differentiation of MSC (magnification x 200). For immunocytochemistry islet-like clusters were collected after 3 days in differentiation medium. Some of them were dissociated by trypsin-EDTA to obtain single cells that were stained for Ipf-1 *(b). (c-d)* Staining of differentiated clusters for c-peptide and glucagon (magnification x 400). The panels *(e-f)* shows the non-specific affinity of the secondary antibodies. Images were obtained with a laser scanning confocal microscope. Undifferentiated MSC were used as negative control. Nuclear

staining in blue with DAPI. (g) Somatostatin release during the differentiation period was measured with RIA in supernatants collected each day (n=8).

Figure 5. Expression of stem cell markers in MSC from Type 1 diabetic patients. (a) Presence of CD45, c-kit, ABCG2, SCF, Thy1, nestin and Isl-1 mRNA was analyzed by RT-PCR after the 6th passage in expansion medium.

Figure 6. Example for induction of pancreatic developmental genes in MSC of a patient with type 1 diabetes. MSC were kept in differentiation medium for 3 days. Data are normalized to HPRT and expressed as percentage expression as compared to day 0. Each time point was analyzed in quadruplicates.

(a) mRNA expression of the side population marker ABCG2 and the pancreatic transcription factors, Isl-1, Ipf-1, Ngn-3 and Pax-6 was analyzed by real-time PCR. (b) Real-time PCR analysis for mRNA of glut-2, insulin, sand glucagon. (c) Immunocytochemistry for c-peptide in undifferentiated and differentiated MSC. Nuclei are stained with DAPI (magnification x 400).

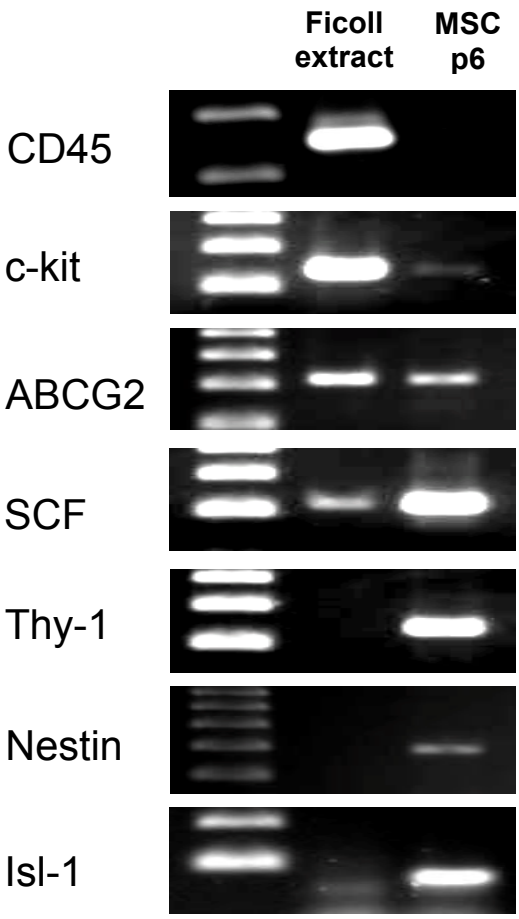
Table 1

Primer sequences for RT-PCR. All primers were run at 60ºC.

gene	sense primer	antisense primer	amplicon	accession number	cycles
HPRT	5´-TCAGGCAGTATAATCCAAAGATGGT-3´	5´-AGTCTGGCTTATATCCAACACTTCG-3´	85bp	M26434	40
Insulin	5´-GCAGCCTTTGTGAACCAACA-3´	5´-TTCCCCGCACACTAGGTAGAGA-3´	69bp	NM_000207	40
Ipf-1	5´-TGATACTGGATTGGCGTTGTTT-3´	5´-TCCCAAGGTGGAGTGCTGTAG-3´	70bp	NM_000209	40
ABCG2	5´-GGTTACGTGGTACAAGATGATGTTG-3´	5´-AGCCGAAGAGCTGCTGAGAA-3´	80bp	AY289766	40
Pax-6	5´-TGCGACATTTCCCGAATTCT-3´	5´-GATGGAGCCAGTCTCGTAATACCT-3´	81bp	NM_001604	40
Isl-1	5´-CAACTGGTCAATTTTTCAGAAGGA-3´	5´-TTGAGAGGACATTGATGCTACTTCAC-3´	75bp	NM_002202	40
Nestin	5´- CGTTGGAACAGAGGTTGGAG -3´	5´- TAAGAAAGGCTGGCACAGGT -3´	396bp	BC032580	40
Glut-2	5´-AGCACTTGGCACTTTTCATCAG-3´	5´-GCCCAAGATAAATTCAAGACCAAT-3´	82bp	J03810	40
Ngn-3	5`-CTATTCTTTTGCGCCGGTAGA-3`	5`-CTCACGGGTCACTTGGACAGT-3`	73bp	NM_020999	40
Somatost	5´-GATGCCCTGGAACCTGAAGA-3´	5´-CCGGGTTTGAGTTAGCAGATCT-3´	82bp	BC032625	40
Glucagon	5´-CCCAAGATTTTGTGCAGTGGTT-3´	5´-CAGCATGTCTCTCAAATTCATCGT-3´	80bp	NM_002054	40
Thy-1	5´-GTCCTTTCTCCCCCAATCTC-3´	5´-GGGAGACCTGCAAGACTGTT-3´	239bp	NM_033209	40
SCF	5´-GGTGGCAAATCTTCCAAAAG-3´	5´-TCTTTCACGCACTCCACAAG-3´	222bp	BC074725	40
c-kit	5´-GGCATCACGGTGACTTCAAT-3´	5´-GGTTTGGGGAATGCTTCATA-3´	244bp	L04143	40
CD45	5´-CAGGCAGCAATGCTATCTCA-3´	5´-CTGTGATGGTGGTGTTGGAG-3´	153bp	Y00638	40

Figure 1

a


Ficoll extract
MSC p6
CD45
c-kit
ABCG2
SCF
Thy-1
Nestin
Isl-1

b

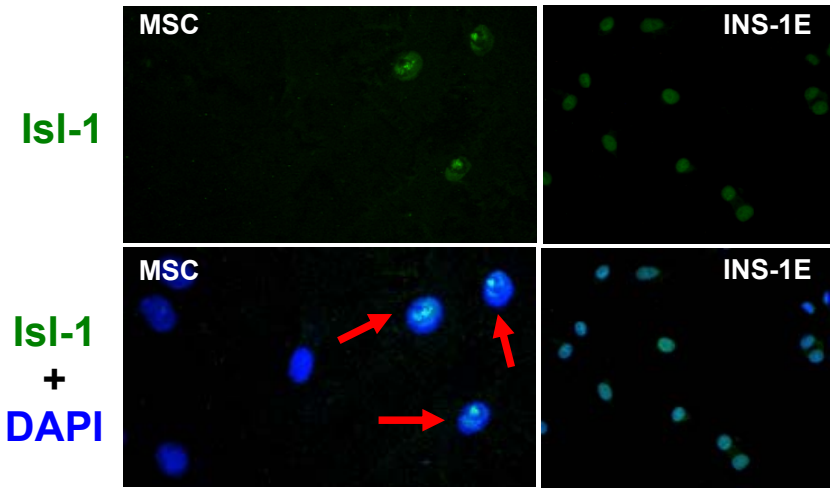

Isl-1
Isl-1 + DAPI
MSC
INS-1E
MSC
INS-1E

Figure 2a

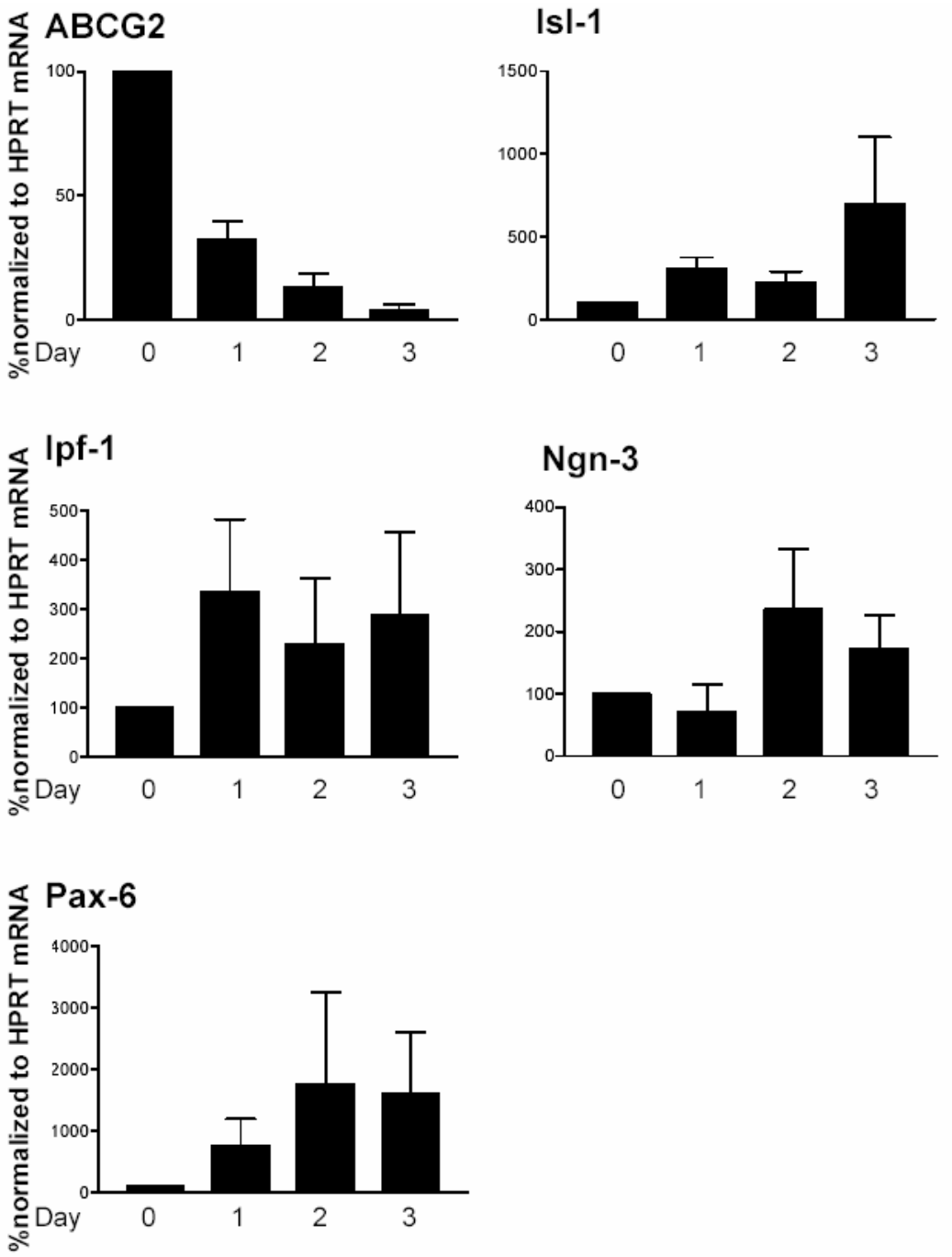

ABCG2
%normalized to HPRT mRNA
100
50
0
Day 0 1 2 3
Isl-1
1500
1000
500
0
0 1 2 3
Ipf-1
%normalized to HPRT mRNA
500
400
300
200
100
0
Day 0 1 2 3
Ngn-3
400
300
200
100
0
0 1 2 3
Pax-6
%normalized to HPRT mRNA
4000
3000
2000
1000
0
Day 0 1 2 3

Figure 2b

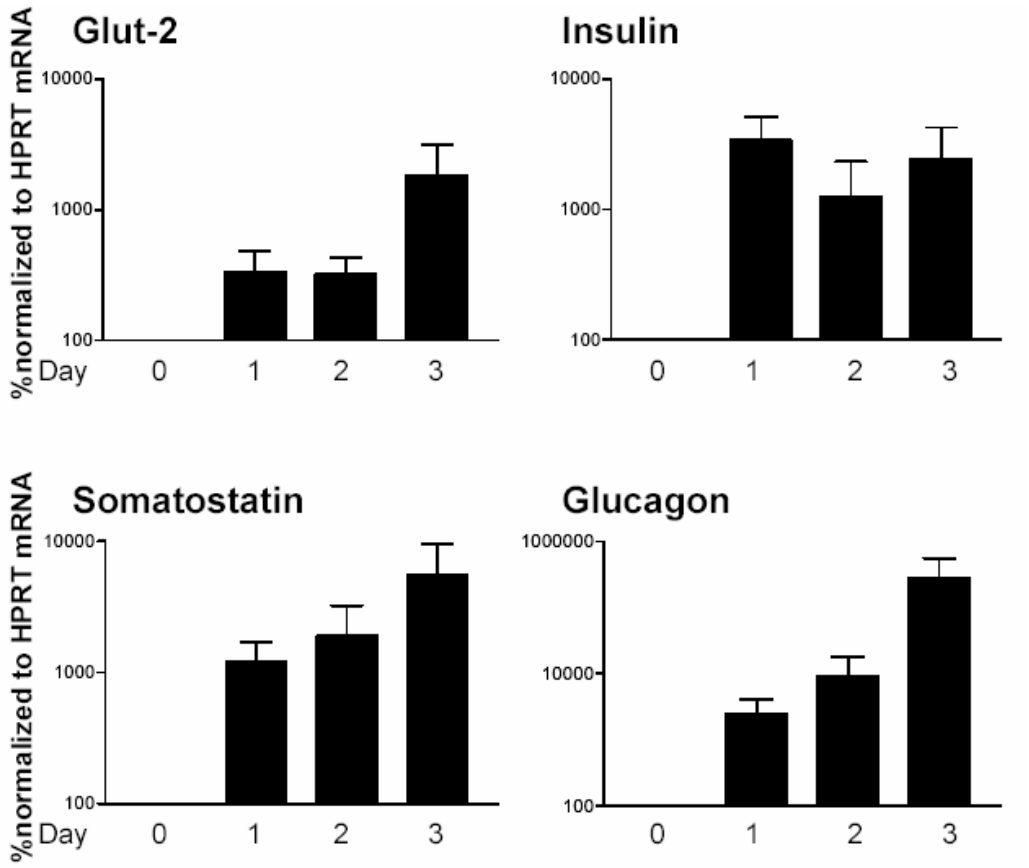


Figure 3

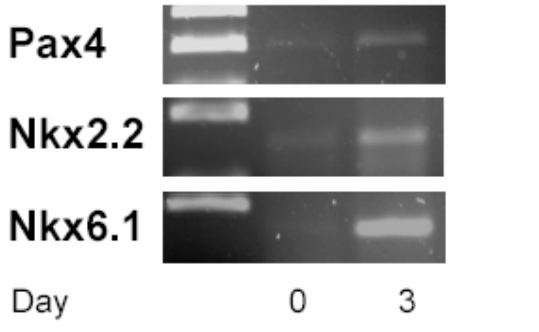


Figure 4

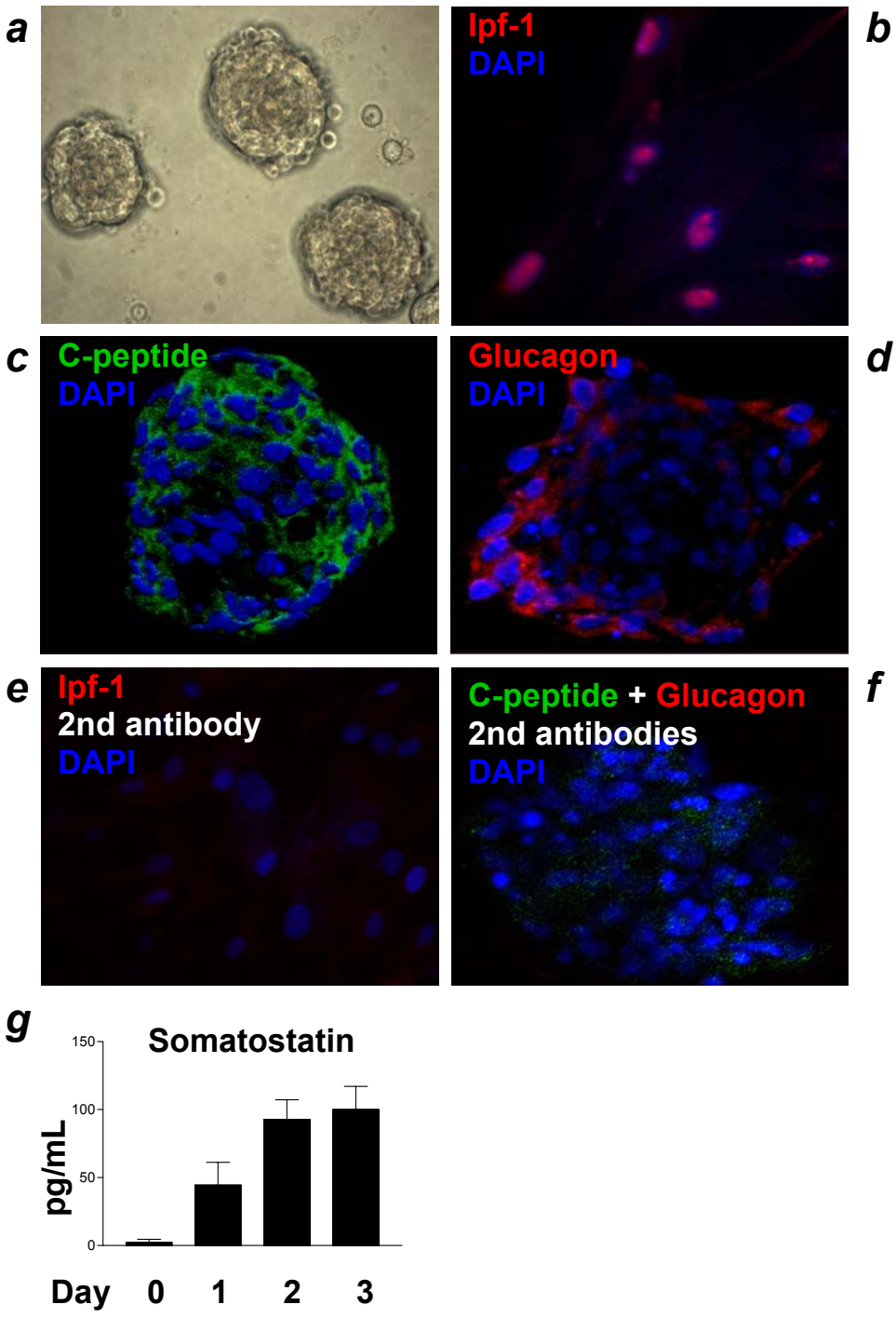

a
b
Ipf-1
DAPI
c
C-peptide
DAPI
d
Glucagon
DAPI
e
Ipf-1
2nd antibody
DAPI
f
C-peptide + Glucagon
2nd antibodies
DAPI
g
Somatostatin
150
100
50
0
pg/mL
Day 0 1 2 3

Figure 5

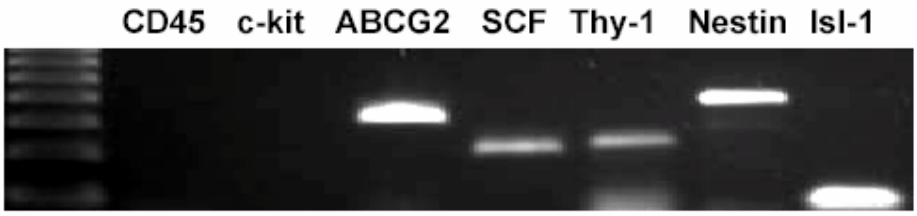


Figure 6a

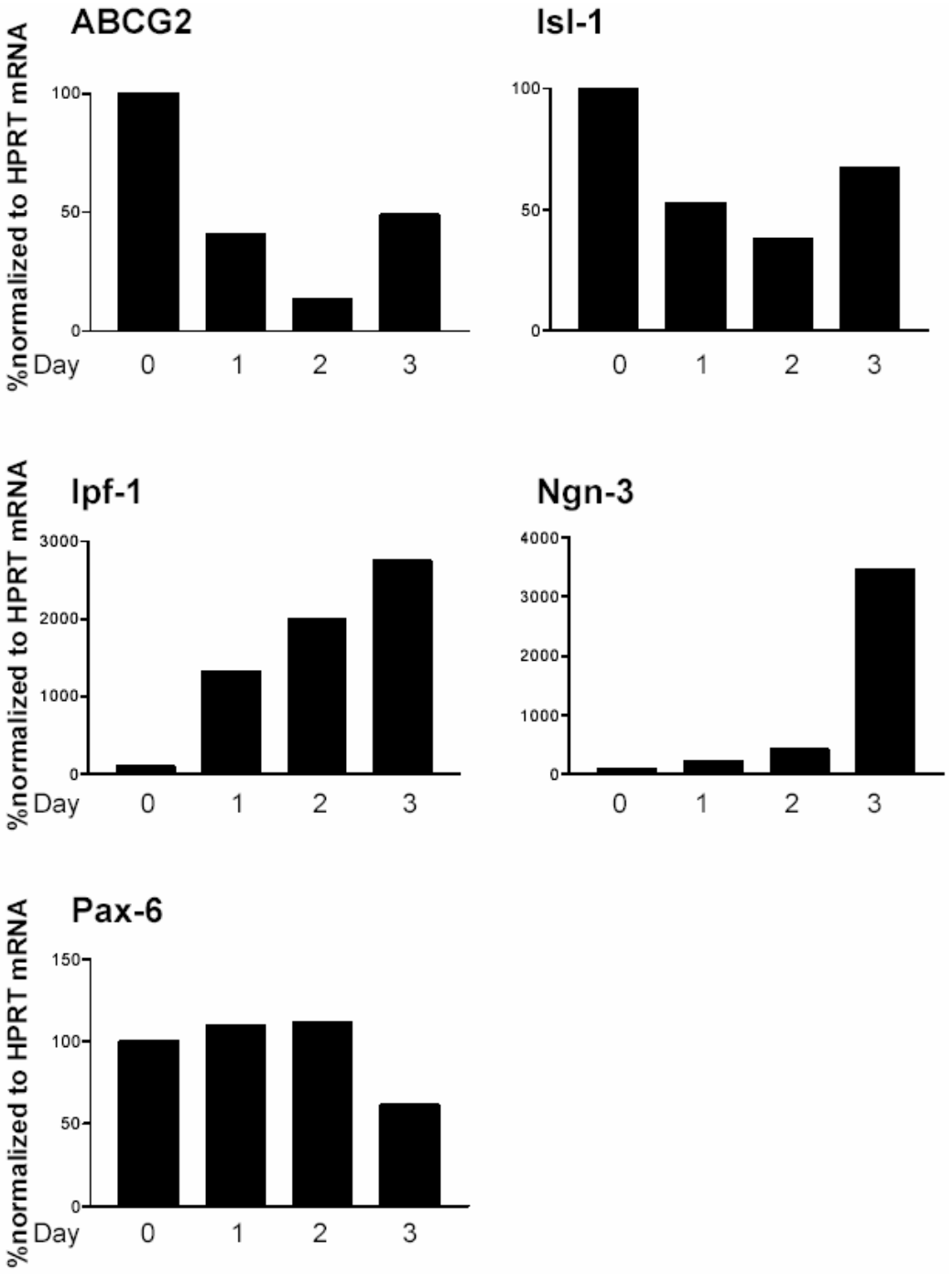

ABCG2
%normalized to HPRT mRNA
100
50
0
Day 0 1 2 3
Isl-1
100
50
0
0 1 2 3
Ipf-1
%normalized to HPRT mRNA
3000
2000
1000
0
Day 0 1 2 3
Ngn-3
4000
3000
2000
1000
0
0 1 2 3
Pax-6
%normalized to HPRT mRNA
150
100
50
0
Day 0 1 2 3

Figure 6b

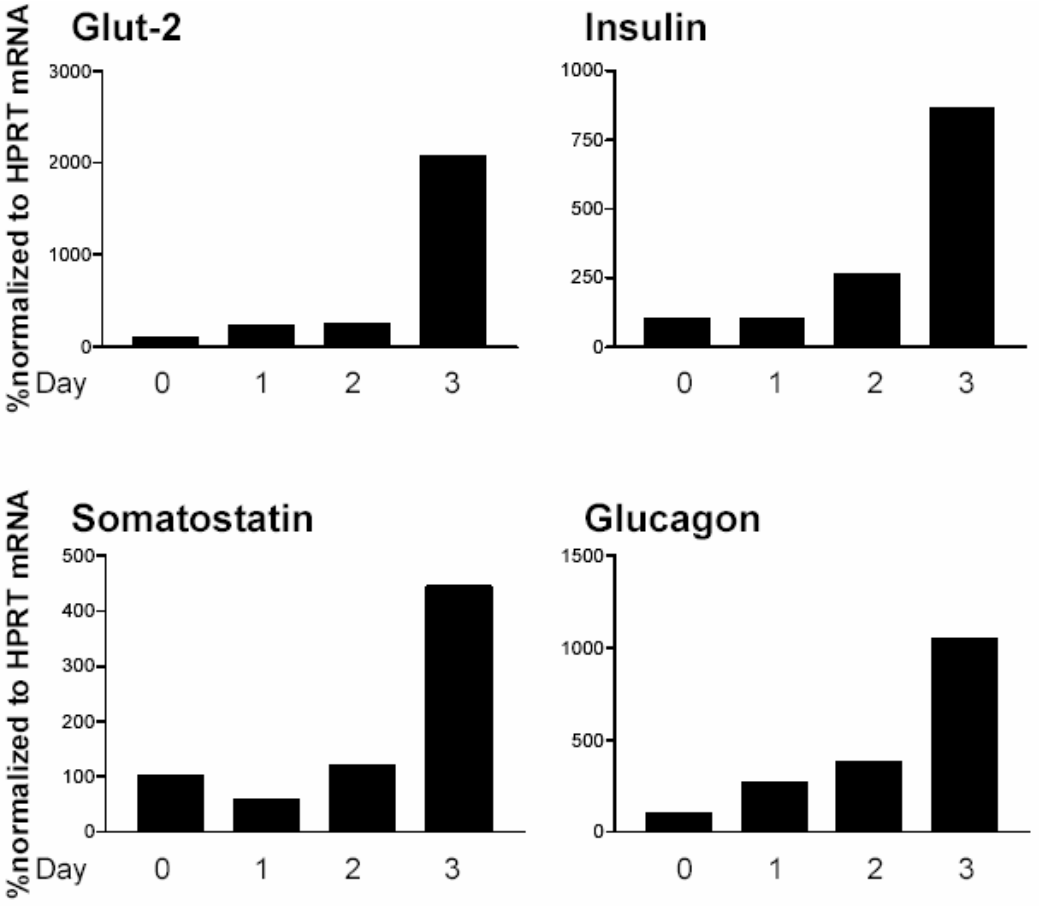
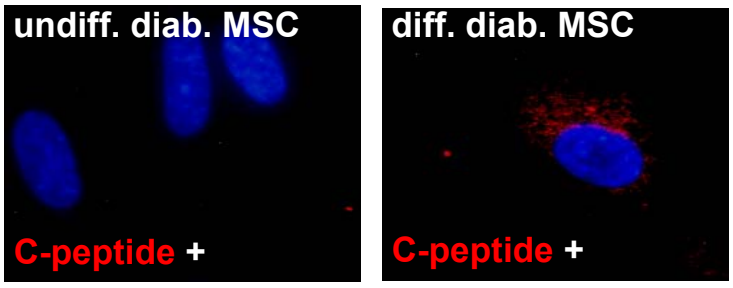


Figure 6c



VII

Human adipose tissue-derived mesenchymal stem cells differentiate into insulin, somatostatin, and glucagon expressing cells

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BBRC
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Human adipose tissue-derived mesenchymal stem cells differentiate into insulin, somatostatin, and glucagon expressing cells

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Abstract

Mesenchymal stem cells (MSC) from mouse bone marrow were shown to adopt a pancreatic endocrine phenotype in vitro and to reverse diabetes in an animal model. MSC from human bone marrow and adipose tissue represent very similar cell populations with comparable phenotypes. Adipose tissue is abundant and easily accessible and could thus also harbor cells with the potential to differentiate in insulin producing cells. We isolated human adipose tissue-derived MSC from four healthy donors. During the proliferation period, the cells expressed the stem cell markers nestin, ABCG2, SCF, Thy-1 as well as the pancreatic endocrine transcription factor Isl-1. The cells were induced to differentiate into a pancreatic endocrine phenotype by defined culture conditions within 3 days. Using quantitative PCR a down-regulation of ABCG2 and up-regulation of pancreatic developmental transcription factors Isl-1, Ipf-1, and Ngn3 were observed together with induction of the islet hormones insulin, glucagon, and somatostatin.

Keywords: Mesenchymal stem cells; Isl-1; Human; Adipose tissue; Nestin; ABCG2; Differentiation; Insulin; Glucagon

Mesenchymal stem cells have been initially described as clonal, plastic adherent cells from bone marrow [1] capable of differentiating into adipocytes, chondrocytes, and osteoblasts [2,3]. They have been later identified in various other tissues including muscle, brain, and adipose tissue [4–6]. In addition to their ability to differentiate into adipocytes, osteoblast, and chondrocytes, these stem cells were also found to adopt a neural and hepatic phenotype in vitro and in vivo [7–12]. Proliferating MSC express the stem cell marker nestin [9,11,13], a gene initially characterized as a marker of neural stem or progenitor cells [14] and later also suggested to be a marker for multipotent pancreatic stem cells [15]. MSC from mouse bone marrow were recently shown to harbor the potential to differentiate into insulin secreting cells in vitro and to reverse hyperglycemia in an animal model of diabetes [16]. Similarly, mesenchymal CD45-negative precursor cells from mouse spleen were able to regenerate insulin producing cells in a mouse model of autoimmune diabetes [17]. The mechanisms underlying this apparent developmental plasticity of MSC are unknown. MSC from human bone marrow and adipose tissue represent a very similar cell population with comparable phenotypes [6,18–20]. Thus, MSC with the potential to adopt a pancreatic endocrine phenotype could also exist in human adipose tissue. In the light of the actual worldwide diabetes epidemic, the generation of insulin producing cells from adipose tissue-derived stem cells represents an attractive treatment option for patients who have lost their residual insulin production. In the present study, we show that MSC from human adipose tissue express the stem cell markers nestin and ABCG2, and display the potential to activate pancreatic developmental genes in response to

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defined culture conditions. This includes the transcription factors Ipf-1, Isl-1, Ngn-3, and Pax-6 as well as the islet proteins insulin, glucagon, and somatostatin.

Materials and methods

Isolation and expansion of human adipose tissue-derived MSC. Human adipose tissue was obtained from patients undergoing plastic surgery in accordance with the Local Ethics Committee (University Hospital Basel, Switzerland). Adipose tissue-derived MSC were isolated and expanded as previously described [21]. Culture medium contained DMEM supplemented with 10% fetal bovine serum (FBS) and 5 ng/mL fibroblast growth factor (bFGF), 1% sodium pyruvate MEM 100 mM, and 1% penicillin/streptomycin 5000 U/mL (all from Invitrogen, Basel, Switzerland). MSC were cultured in 175 cm^2 Flasks (Becton–Dickinson AG, Basel, Switzerland). Medium was changed twice a week. At a confluence of 95%, cells were collected using trypsin (Invitrogen AG, Basel, Switzerland) and washed twice with DMEM/F12 medium.

For induction of differentiation, the cells were seeded at a cell density of $2–3 \times 10^5$/well of a 6-well plate and cultured for 3 days in ultra-low attachment plates (Vitaris AG, Baar, Switzerland). MSC from four donors were analyzed at passages 4–7. The gene expression profile of each differentiation study was analyzed every 24 h for 3 days in every subject. For each differentiation day cells from four separate wells were harvested and analyzed independently and a mean value was calculated. Differentiation medium consisted of serum-free DMEM/F12 medium with 17.5 mM glucose in the presence of nicotinamide 10 mM, activin-A 2 nM, exendin-4 10 nM, hepatocyte growth factor 100 pM, and pentagastrin 10 nM (all from Sigma, Basel, Switzerland) as well as B-27 serum-free supplement, N-2 Supplement, and 1% penicillin/streptomycin 5000 U/L (all from Invitrogen AG, Basel, Switzerland).

RNA isolation and reverse transcription. Total RNA was extracted using TRIzol reagent (Lucerna Chemie AG, Luzern, Switzerland) according to the manufacturer's protocol. RNA samples were treated with DNase (Ambion, Cambridgeshire, UK) in order to remove possible contaminating genomic DNA. RNA was quantified spectrophotometrically at 260 nm (Biophotometer, Eppendorf-Vaudaux, Schönenbuch, Switzerland). The quality was assessed by gel electrophoresis on agarose gel containing ethidium bromide (EtBr, Bio-Rad Laboratories AG, Reinach, Switzerland). One microgram of total RNA was subjected to reverse transcription (RT) (Omniscript RT kit; Qiagen, Basel, Switzerland).

Polymerase chain reaction. Polymerase chain reaction (PCR) was performed on a conventional thermal cycler (TGradient, Biometra, Göttingen, Germany) using PCR Taq core kit (Qiagen). Human gene-specific, intron spanning primers were used as mentioned in Table 1 with exception of primer for Ipf-1 and insulin that were used for real-time PCR. Amplification products were visualized on agarose gels containing 0.5 μg/mL EtBr. 100 bp Molecular Ruler (Bio-Rad, Reinach, Switzerland) was run as size reference. PCR product identity was confirmed by nucleotide sequencing (Microsynth AG, Balgach, Switzerland).

Quantitative real-time polymerase chain reaction. cDNA, obtained as described above, was subjected to quantitative real-time PCR analysis using the ABI 7000 Sequence (Perkin-Elmer, USA) detection system. Specific primers yielding short PCR products suitable for Sybr-Green (Abgene, Epsom, UK) detection were designed using Primer Express software (version 2.0; PE Applied Biosystems, Foster City, CA). For sequences of primers, see Table 1. The reaction consisted of 50 μL, containing 25 μL Sybr-Green, 2 μL sense primer (10 μmol/L), 2 μL antisense primer (10 μmol/L), 16 μL H_2O, and 5 μL cDNA. Conditions were set as suggested by the manufacturer. Each cDNA sample tested for quantitative gene mRNA expression was also subjected to hypoxanthine-phosphoribosyltransferase (HPRT) mRNA analysis. Results were expressed as the ratio of the respective gene mRNA and HPRT mRNA threshold values. Gene induction in differentiated cells is expressed as percentage of values found in undifferentiated MSC. The product identity was confirmed by

Table 1
Primer sequences for RT-PCR

Gene	Sense primer	Antisense primer	Amplicon (bp)	Accession number	Cycles
HPRT	5′-TCAGGCAGTATAATCCAAAGATGGT-3′	5′-AGTCTGGCTTATATCCAACACTTCG-3′	85	M26434	40
Insulin	5′-GCAGCCTTTGTGAACCAACA-3′	5′-TTCCCCGCACACTAGGTAGAGA-3′	69	NM_000207	40
Ipf-1	5′-TGATACTGGATTGGCGTTGTTT-3′	5′-TCCCAAGGTGGAGTGCTGTAG-3′	70	NM_000209	40
ABCG2	5′-GGTTACGTGGTACAAGATGATGTTG-3′	5′-AGCCGAAGAGCTGCTGAGAA-3′	80	AY289766	40
Pax-6	5′-TGCGACATTTCCCGAATTCT-3′	5′-GATGGAGCCAGTCTCGTAATACCT-3′	81	NM_001604	40
Isl-1	5′-CAACTGGTCAATTTTTCAGAAGGA-3′	5′-TTGAGAGGACATTGATGCTACTTCAC-3′	75	NM_002202	40
Nestin	5′-CGTTGGAACAGAGGTTGGAG-3′	5′-TAAGAAAGGCTGGCACAGGT-3′	396	BC032580	40
Ngn-3	5′-CTATTCTTTTGCGCCGGTAGA-3′	5′-CTCACGGGTCACTTGGACAGT-3′	73	NM_020999	40
Somatost	5′-GATGCCCTGGAACCTGAAGA-3′	5′-CCGGGTTTGAGTTAGCAGATCT-3′	82	BC032625	40
Glucagon	5′-CCCAAGATTTTGTGCAGTGGTT-3′	5′-CAGCATGTCTCTCAAATTCATCGT-3′	80	NM_002054	40
Thy-1	5′-GTCCTTTCTCCCCCAATCTC-3′	5′-GGGAGACCTGCAAGACTGTT-3′	239	NM_033209	40
SCF	5′-GGTGGCAAATCTTCCAAAAG-3′	5′-TCTTTCACGCACTCCACAAG-3′	222	BC074725	40
c-kit	5′-GGCATCACGGTGACTTCAAT-3′	5′-GGTTTGGGGAATGCTTCATA-3′	244	L04143	40

All primers were run at 60 °C.

sequence analysis and electrophoresis on a 3% agarose gel containing EtBr.

Peptide measurements. Somatostatin concentration was determined in culture supernatants using a commercially available somatostatin radioimmunoassay (RIA) kit (functional assay sensitivity: somatostatin 5 pg/tube, Phoenix Pharmaceuticals, Belmont, CA).

Immunocytochemistry. Cultured MSC were transferred on glass slides and incubated overnight in expansion medium. Islet-like clusters were collected after 3 days in differentiation medium and dissociated by trypsin-ethylenediaminetetraacetic acid (EDTA) in order to obtain single cells. Single cells were transferred on glass slides coated with poly-L-lysine (Sigma, Buchs, Switzerland) and incubated overnight in DMEM/F12 medium containing 10% FBS allowing them to attach. Cells were then fixed with 4% paraformaldehyde in PBS (pH 7.4) for 30 min at room temperature. After several rinses in PBS, cells were permeabilized with chilled methanol for 10 min. Unspecific binding was prevented by incubation with 10% heat-inactivated FBS in PBS at RT for 30 min. Cells were incubated with primary antisera for 60–90 min at 37 °C, rinsed off with PBS, and incubated with secondary goat antisera for 45 min at 37 °C. After several washes with PBS, cells were coverslipped with non-fluorescing mounting medium. The primary antibody rabbit anti-human c-peptide (dilution 1:500) was from Linco, St. Charles, MO. Mouse anti-Isl-1 (dilution 1:100) (number 39.4D5) was obtained from Developmental Studies Hybridoma Bank, University of Iowa, IA. The rabbit anti-human Ipf-1 (dilution 1:1000) was a generous gift from J.F. Habener, Boston, MA. The secondary antibodies from Molecular Probes (Invitrogen AG, Basel, Switzerland) were goat anti-mouse (dilution 1:1000) and goat anti-rabbit (dilution 1:1000). Goat anti-mouse IgG was labeled with Alexa fluor 488 dye. Goat anti-rabbit IgG was labeled with Alexa fluor 546 dye. 4,6-Diamidino-2-phenylindole (DAPI, 5 μg/mL) from Sigma was used to label the nuclei (10 min at 37 °C). Cells were examined by fluorescence microscope (Axiophot, Zeiss, Germany).

Results

Human adipose tissue-derived MSC from four donors were isolated and proliferated in bFGF containing culture medium. The initial cell population was positive for stem cell factor (SCF) and its receptor (c-kit). This cell population was negative for ABCG2, nestin, Thy-1, and Isl-1 as assessed by RT-PCR (Fig. 1A). Proliferating MSC, however, expressed ABCG2, nestin, Thy-1, and Isl-1 mRNA (Fig. 1A). Using immunocytochemistry we found Isl-1 protein in the nuclei of approximately 10% of cultured MSC (Fig. 1B). The mesenchymal characteristic of adipose tissue-derived MSC was described by previous studies [6,18–20] and confirmed with internal control experiments showing adoption of adipocytic and osteocytic phenotypes of these cells in response to standard protocols (data not shown).

Induction of pancreatic endocrine differentiation with our defined culture conditions was associated with stepwise decrease in the expression level of the stem cell marker ABCG2 as analyzed by quantitative real-time PCR. This was mirrored by up-regulation of Isl-1, Ipf-1, and Ngn-3 expression during the observed 3-day period (Fig. 2). Interestingly, expression of Pax-6 was also found in proliferating MSC and was not further induced by the 3-day differentiation procedure. Genes known to be positively regulated by Ipf-1 like insulin and somatostatin were also induced during the differentiation period. In addition, an activation of glucagon gene expression was observed (Fig. 3).

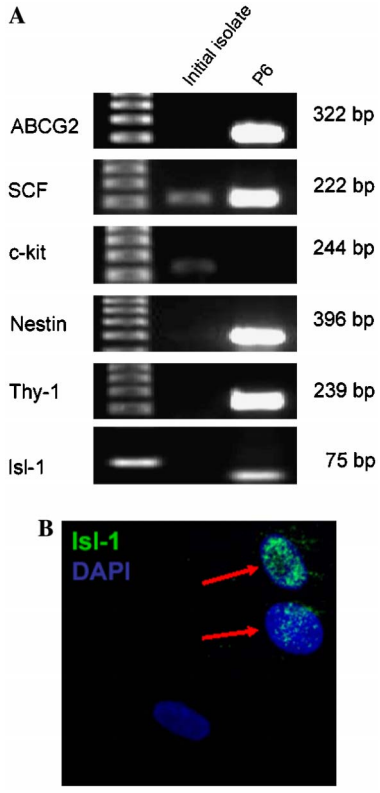

Fig. 1. Expression of stem cell markers in adipose tissue-derived MSC. (A) Presence of ABCG2, SCF, c-kit, nestin, Thy1, and Isl-1 mRNA was analyzed by RT-PCR in primary cell isolates and MSC (passage 6) in expansion medium. (B) Immunocytochemistry for the transcription factor Isl-1 (magnification 400×). Arrows indicate positive staining. Nuclei were counterstained with DAPI (in blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Nuclear staining for Ipf-1 was found in approximately 10% of the cells after 3 days (Figs. 4A and B). Ipf-1 was not detected by immunocytochemistry in undifferentiated cells. C-peptide positive cells were found in differentiated MSC (Figs. 4C and D). In addition, we found a release of somatostatin into the medium that reached plateau levels at day 3 (Fig. 4E).

Discussion

The present study demonstrates that human adipose tissue-derived MSC are able to adopt a pancreatic endocrine phenotype ex vivo. This was achieved without genetic modification and in response to defined culture conditions. Initially, these cells were cultured in high glucose (25 mM) and bFGF (5 ng/mL) and expressed after expansion not only the stem cell markers nestin, ABCG2, SCF, and Thy-1 but also the pancreatic transcription factor Isl-1 (Fig. 1). ABCG2 is a known marker for the side population phenotype stem cells in bone marrow [22] and was recently found in pancreatic islet-derived precursor cells and neural stem cells [23,24]. Together with Isl-1 it may thus point to a subpopulation of MSC with

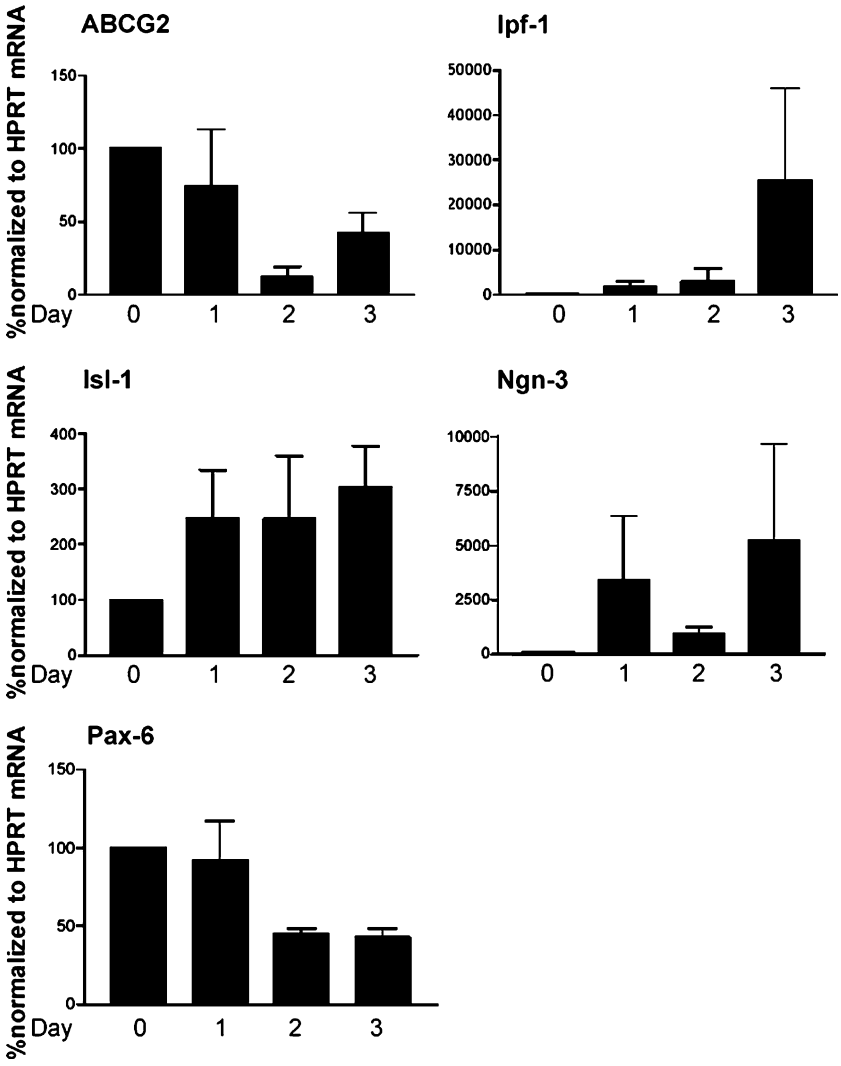

Fig. 2. Induction of pancreatic developmental genes in adipose tissue-derived MSC in response to defined culture conditions. Gene expression was monitored every 24 h for 3 days. Data are normalized to HPRT and expressed as percentage expression as compared to day 0. Results are means ± SEM of quadruplicate of four independent experiments from four independent donors. mRNA expression of pancreatic transcription factors Isl-1, Ipf-1, Ngn-3, and the side population marker ABCG2 and Pax-6 was analyzed by real-time PCR.

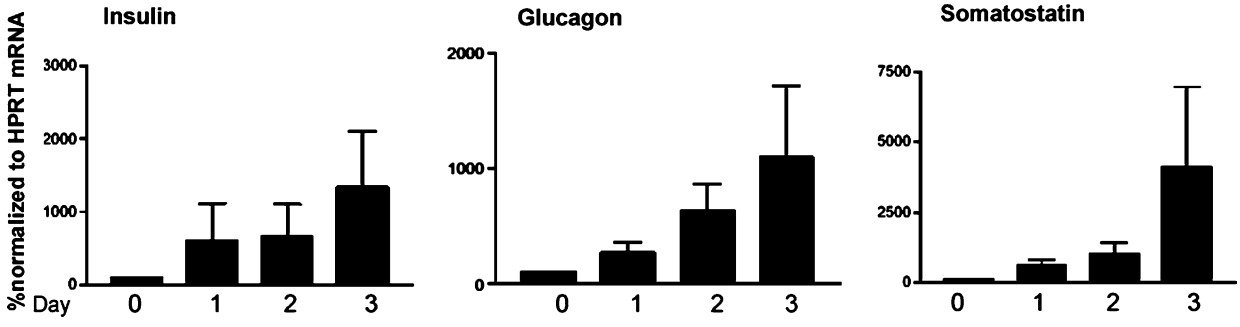

Fig. 3. Induction of the islet genes insulin, glucagon, and somatostatin in adipose tissue-derived MSC in response to defined culture conditions.

neuro-endocrine developmental potential. The transcription factor Isl-1 is crucial for the development of pancreatic endocrine cells. Disruption of Isl-1 expression is associated with absence of dorsal mesenchyme and a marked reduction of Ipf-1 gene expression in dorsal epithelium in mice [25]. Embryonic explants of the pancreatic anlage from Isl-1 (−/−) mice did not generate insulin, glucagon or somatostatin positive cells in vitro as did explants from Isl-1 (+/−) animals [25]. Isl-1 expression is together with Ipf-1 one of the earliest pancreatic transcription factors detected during development [25]. Induction of Isl-1 expression in our human MSC

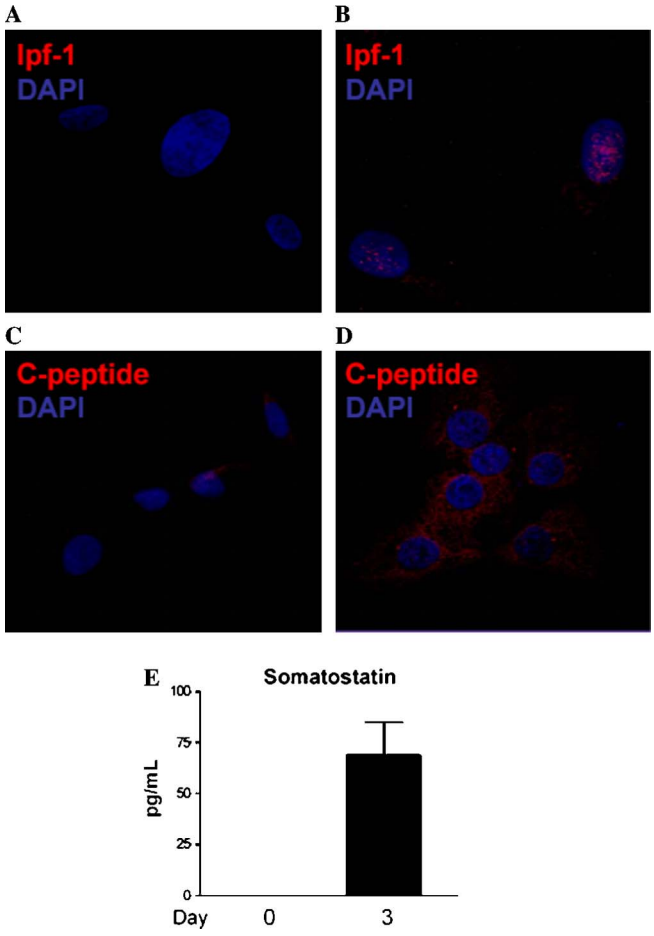

Fig. 4. Immunocytochemistry for Ipf-1 and C-peptide in undifferentiated and differentiated adipose tissue-derived MSC. Single cells were stained for Ipf-1 (A and B, magnification 400×) and c-peptide (C and D, magnification 200×). Undifferentiated MSC were used as negative control (A and C). Nuclear staining was performed in blue with DAPI. (E) Somatostatin release during the differentiation period was measured with RIA in supernatants collected on day 0 and 3 ($n = 8$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

may represent a critical event that allows adoption of a pancreatic endocrine phenotype. Other early markers of pancreas development like Ipf-1 and Ngn-3 were not detected by PCR in undifferentiated cells.

Using serum-free medium supplemented with factors known for their beneficial effects on differentiation of precursor cells into insulin producing cells (i.e., exendin-4, pentagastrin, activin-A, betacellulin, nicotinamide, and hepatocyte growth factor) [26–29] we have induced an activation of pancreatic transcription factors including Ipf-1, Isl-1, Ngn-3, as well as the islet proteins insulin, glucagon, and somatostatin. The real-time PCR studies revealed an activation of some of these pancreatic genes already 24 h after induction of differentiation (Figs. 2 and 3). Similar early induction of insulin gene expression was reported recently in mice in vivo in response to hyperglycemia [30]. Here, insulin positive cells were identified in adipose tissue, spleen, and also bone marrow of hyperglycemic mice after 3 days.

Several studies addressed the possibility that bone marrow-derived stem cells could contribute to β-cell turnover in vivo. Some of them presented positive results, suggesting a circulating pool of stem cells that could participate in the process of β-cell neogenesis [31]. These results, however, were not confirmed by others [32] and a recent report questioned the entire concept of β-cell stem/progenitor cells with studies using genetic lineage tracing experiments [33]. With this approach it has been shown that pre-existing β-cells rather than adult stem/progenitor cells retained a proliferative capacity and may thus represent the major source of new β-cells in adult life, at least in mice [33]. While in vivo studies are not conclusive, this does not exclude the differentiation of adult stem cells into insulin producing cells in vitro. It has been shown that mouse mesenchymal stem cells from bone marrow cultured in high glucose over 4 months induced several β-cell-specific genes including insulin and Ipf-1 [16]. These cells were also able to reverse hyperglycemia in an animal model of diabetes [16].

In summary, Isl-1 positive MSC can be isolated from human adipose tissue and are able to adopt a pancreatic endocrine phenotype. These cells could be used as a human model to develop stem cell-based therapies for diabetes mellitus.

Acknowledgments

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VIII

Lentiviral vectors efficiently transduce human mesenchymal stem cell- and preadipocyte derived mature adipocytes

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Lentiviral vectors efficiently transduce human mesenchymal stem cell- and preadipocyte derived mature adipocytes

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Abbreviations: central polypurin tract (cPPT), Feline immunodeficiency virus (FIV), herpes simplex virus (HSV), Human immunodeficiency virus (HIV), multiplicity of infection (moi), murine leukemia virus (MLV), mesenchymal stem cell (MSC), green fluorescent protein (GFP)

Abstract

Adipose tissue is thought to play a crucial role in translating obesity into associated metabolic diseases. In the present study we aimed to establish a procedure for introducing genetic information into both human mesenchymal stem cell (MSC)- and human preadipocyte-derived adipocytes. Expression of the green fluorescent protein (GFP) reporter gene was assessed after exposure to lentiviruses (53RPK PGK GFP and FIV CMV GFP VSVG). Preserved adipocyte function was evaluated by insulin-induced glucose uptake.

Adipocytes expressed GFP after 5 days post-transduction. Up to 60% of mature adipocytes were GFP positive. Transduction of undifferentiated bone marrow-derived MSCs and preadipocytes did not affect their capacity to adopt an adipocyte phenotype upon differentiation. Insulin-induced glucose uptake was not affected in transduced mature adipocytes. Transduction efficiency by adenoviral-mediated gene transfer into both differentiated and undifferentiated cells was similar to lentiviral infection. However, this procedure was associated with over 50% cell death within the first 5 days.

Lentiviral vectors provide an effective gene transfer techniques for the genetic modification of MSC- and preadipocyte-derived human adipocytes without apparent loss of cell-specific functions. This technique may provide a basis for further studies on the biology of adipose tissue and has the potential to become a target for gene therapy in obesity-related disorders.

Keywords: human immunodeficieny virus, felin immunodeficinecy virus, adenovirus, glucose uptake

Introduction:

Adipose tissue is increasingly recognised as an endocrine organ secreting hormones, cytokines and other signalling molecules (1). Obesity-associated pathologies, including type 2 diabetes, hypertension and atherosclerosis, have a high prevalence in modern societies (2). Improvement of the understanding of the physiological function of adipocytes is a prerequisite for understanding the molecular mechanisms that underpin these diseases (3). Rodent cell models including the 3T3-L1 cell line have been used in numerous studies. However, physiological differences between human and murine adipocytes undermine the transfer of knowledge obtained with murine cells (4). Human preadipocyte- and mesenchymal stem cell (MSC)-derived adipocytes represent an attractive alternative to rodent models (5). Introducing genetic information in non-dividing mature adipocytes is not an easy task. Gene transfer into murine 3T3-L1 cell line has been achieved by electroporation (6) and different viral vectors (7). A transduction efficiency of up to 15% transduced cells was achieved by electroporation in human mature adipocytes (8). Fiber-modified adenovirus vectors mediate efficient but transient gene transfer into undifferentiated and differentiated human MSC(9).

Human immunodeficiency virus (HIV) and HIV-derived pseudotyped lentiviral vectors efficiently infect and integrate into the genome of human cells, irrespective of the state of cell proliferation (10-19). In addition, retroviral vectors based on murine leukemia virus (MLV) are attractive gene delivery systems. The major drawback of MLV-derived vectors is there inability to transduce non-dividing cells (20). Feline immunodeficiency virus (FIV), a vector used for lentiviral vaccine development and antiviral therapy, is another appealing candidate for vector development. It has been shown to efficiently transduce dividing and non-dividing cells (21). Using the herpes simplex virus (HSV) based gene vector system promising results were achieved in human preadipocytes and rabbit adipose tissue (22). In this study, differentiation of preadipocytes was not hampered by the transduction procedure. Undifferentiated adipose tissue-derived mesenchymal progenitor cells have been successfully transduced with adenoviral, oncoretroviral and lentiviral vectors (23).

Herein, we present a method that allows stable lentiviral-mediated transduction of human mature MSC- and preadipocytes-derived adipocytes. We show that the method is efficient and does not compromise adipocyte physiology. Infected adipocytes display a stable transduction and cell viability for prolonged periods of time.

Materials and Methods

Cell cultures

Human preadipocyte-derived adipocytes were obtained from patients undergoing plastic surgery as previously described (24). Bone marrow aspirates (20-40 mL) were obtained from healthy donors (18-63 years) during routine othopaedic surgical procedures, in accordance with the local ethics committee (University Hospital Basel, Switzerland) and after informed consent. Nucleated cells were isolated from the aspirate by Ficoll density gradient centrifugation (Histopaque1, Sigma, Buchs, Switzerland). Human mesenchymal stem cells (MSC) were selected within the nucleated cells in culture by adhesion and proliferation on the plastic substrate. Cells were expanded in DMEM supplemented with 10% fetal bovine FBS and 5 ng/mL bFGF (all from Invitrogen, Basel, Switzerland). For experiments, cells between passages 4 and 10 were seeded in 6 well plates. Adipogenic differentiation was induced by incubating confluent cells in DMEM/F12 (Invitrogen) containing 3% FBS and supplements as follows: 250 µM 3-isobutyl-1-methylxanthine (IBMX), 1 µM dexamethasone, 0.2 nM 3,3,5-triiodo-L-thyronine, 5 µM transferrin (all from Sigma), 100 nM insulin (Novo Nordisk, Küsnacht, Switzerland), 1 µM rosiglitazone (GlaxoSmithKline, Worthing, UK). Typically, 80% to 90% of MSCs underwent adipogenic differentiation as assessed by lipid droplets formation. Expression of adipocyte-specific mRNAs (e.g. PPARγ2, PPARγ1, leptin, adiponectin) was confirmed by RT-PCR.

Human embryonic kidney cell line 293T (ATCC; SD3515) was cultured in DMEM (Invitrogen) with 10% FBS in 100 mm petridishes. All media contained 100 U/ml penicillin and 100 µg/mL streptomycin (Invitrogen).

Generation of recombinant lentiviruses.

Lentivirus production was carried in 293T cells cotransfected with a plasmid containing enhanced green fluorescent protein (GFP) under the control of the phosphoglyceratekinase (PGK) promoter (53RPA-PGK-GFPas). The envelope vector was pMD2.G and the packaging vector psPAX2. Transient transfection of the plasmid set into 293T cells was achieved by the

calcium phosphate method as described previously (http://tronolab.com). All schematics of the plasmids are available online. Virus containing supernatants were collected on day 2 and 3 post transfection and kept in -80°C freezer until the start of the experiments.

Ready to use feline immunodeficiency-virus derived lentivirus containing GFP under the control of the cytomegalovirus promoter (FIV CMV GFP VSVG) was prepared as described previously (21). Detailed schematics are presented in the study by Johnston JC *et al* (21).

Generation of adenovirus

The adenovirus vector used contained enhanced GFP under the control of the CMV promoter (AD5 CMV eGFP). It expresses the nontoxic gene derived from jellyfish *Aequoria Victoria*. AD5 CMV eGFP was prepared as described by Vasquez *et al* (25).

Infection of cells

Infection of differentiated MSC- and preadipocyte-derived adipocytes was carried out overnight with aliquots of the cell medium derived from the infected 293T cells containing the lentivirus (53RPK PGK GFP). Infection with FIV CMV GFP VSVG and AD5 CMV eGFP was carried out over night in serum free growth medium. On the following day, infected adipocytes were washed with pre-warmed PBS and maintained in adipogenic differentiation medium with 3% FBS for up to one month.

Microscopy

Transduction efficiency was visualized by fluorescence microscopy (Olympus IX 50). The microscope was equipped with an Olympus U-TVO 5XC camera.

Reverse transcriptase-polymerase chain reaction (RT-PCR*)*

Total RNA was extracted from adipocyte cultures with TRI Reagent® (Molecular Research Center, Inc., Cincinnati, OH). Extracted RNA was quantified spectrophotometrically at 260 nm with a Biophotometer (Vaudaux-Eppendorf AG, Schönenbuch Switzerland). Ratio of the

absorbance at 260 nm and 280 nm was between 1.5 and 2.0 and the quality was assessed by gel electrophoresis. 1 µg total RNA was subjected to reverse transcription (Omniscript^{TM} RT kit, Qiagen, Basel, Switzerland). PCR was performed on a conventional thermal cycler (TGradient, Biometra, Göttingen, Germany) using PCR Taq core kit (Qiagen). Human gene-specific, intron spanning primers and conditions were as follows: adiponectin (102-bp product; GenBank accession no. NM_004797), 5'-TGGGCCATCTCCTCCTCA-3' (sense) and 5'-AATAGCAGTAGAACAGCTCCCAGC-3' (antisense); leptin (121-bp product; GenBank accession no. NM_000230), 5'-TGCCCATCCAAAAAGTCC-3' (sense) and 5'-GAAGTCCAAACCCGTGACTTTCT-3' (antisense); PPARγ2 (580-bp product, GenBank accession no. NM_015869), 5'-GCGATTCCTTCACTGATAC-3' (sense) and 5'-GCATTATGAGACATCCCCAC-3' (antisense); PPARγ1 (474-bp product, GenBank accession no. NM_138712), 5'-TCTCTCCGTAATGGAAGACC-3' (sense) and 5'-GCATTATGAGACATCCCCAC-3' (antisense); β-actin (198-bp product, GenBank accession no. NM_001101), 5'-TTCTGACCCATGCCCACCAT-3' (sense) and 5'-ATGGATGATGATATCGCCGCGCTC-3' (antisense).65 28

The annealing temperature was 58°C for adiponectin and leptin (35 cycles), 55°C for PPARAγ2 and PPARγ1 (28 and 30 cycles, respectively), for β-actin 65°C (28 cycles). Amplification products were visualized on 1.5% (amplicons > 250 bp) or 2.5% (amplicons <250 bp) agarose gels containing 0.5 µg/mL ethidium bromide. 100 bp Molecular Ruler (BioRad, Reinach, Switzerland) was used as size reference. PCR product identity was confirmed by nucleotide sequencing (Microsynth AG, Balgach, Switzerland).

FACS

GFP expression was determined by fluorescent activated cell sorter (FACS) analysis. Adherent cells were detached with trypsin-EDTA and washed with PBS prior to analyses.

Insulin-mediated glucose uptake

Three days prior to glucose uptake experiment differentiation medium was removed from differentiated and transduced adipocytes. Cells were washed three times in warm PBS and kept in DMEM/F12 containing 5 mM glucose and 3% FBS. On day 3, at timepoint 0, 100 nM insulin was added to half of the wells. At t = 20 min 1 µC deoxy-d-glucose, 2-[^{3}H(G)] (PerkinElmer, Boston, MA) was added to all wells. After 15 min cells were washed three times in ice cold PBS and lysed in 0.1 % SDS. Radioactivity was measured by scintillation counting.

Statistics

Results are presented as means ± SEM. Analyses of variance with post-hoc analysis for least square differences was carried out.

Results:

Bone marrow-derived MSCs and preadipocytes were differentiated into mature adipocytes and expression of adipocyte-specific markers was assessed by RT-PCR before and after adipogenic differentiation. Adiponectin, leptin and PPARγ2 mRNA expression was detected in MSC- and preadipocyte-derived adipocytes, but not in undifferentiated cells. In contrast, PPARγ1 mRNA was also expressed before and after adipogenic differentiation (Figure 1).

Two lentiviral vectors encoding the GFP reporter gene were used (53PRK-PGK-GFP and FIV CMV GFP VSVG) for transduction experiments of MSC- and preadipocyte-derived adipocytes. Adipocytes were exposed overnight to the culture medium derived from lentivirus producing 293T cells, and subsequently washed and maintained in differentiation medium for adipocytes. Visible GFP expression in the cytoplasm was evident after the third day of infection. Five days post-infection GFP expression reached a steady state level (Figure 2a-d) and remained constant for at least 30 days. No significant perturbation of cell viability was observed in the 30 day period (not shown).

To determine transduction efficiency of the two lentiviruses, FACS analyses were carried out. 10%-50% of the cells were detected positive (Figure 3). The high variability of the FACS analysis may be attributable to the difficult trypsinization procedure of differentiated adipocytes. Furthermore, fat droplets let these cells float in the cuvettes used for FACS analysis. Hence, it was difficult to keep the cells in homogene suspension over time. For this reason, transduced cells were also counted under the microscope. Comparing total cell number as visualized with DAPI staining and transduced cells, visualized with GFP, up to 60% of differentiated cells were GFP positive.

In further experiments, MSC- and preadipocyte-derived adipocytes were exposed overnight to adenovirus at different multiplicity of infection (moi) ranging from 10 to 800. GFP expression was visible on day 1 post-infection. However, adenovirus exposure appeared to have toxic side effects as adipocytes began to detach 3 or 4 days, and after 7 days all cells were dead (data not shown).

In undifferentiated cell cultures (MSC and preadipocytes) 40% of the cells showed GFP expression after lentiviral infection. Transduction of undifferentiated MSCs did not affect their capacity to adopt an adipocyte phenotype upon differentiation (data not shown). However, undifferentiated cells, within the culture of the differentiated adipocytes did not show any GFP expression (data not shown).

The metabolic integrity of the transduced adipocytes was evaluated after 14 days of infection. Glucose uptake in transduced and not transduced adipocytes were measured in the presence or absence of 100 nM insulin, respectively (Figure 4). As compared to basal glucose uptake, insulin-mediated glucose uptake increased 2.34-fold in MSC- and 2.07-fold in preadipocyte-derived adipocytes transduced with 53PRK-PGK-GFP, respectively. In MSC- and in preadipocyte-derived adipocytes transduced with FIV CMV GFP VSVG the insulin-mediated increases were 1.74-fold and 1.65-fold, respectively. Similar results were obtained in non-transduced control adipocytes.

Discussion

Adipocytes are difficult to be transduce by common chemical methods due to negative buoyancy, quiescence and physical fragility. Herein, we have successfully established a lentivirus-based method for post-differentiation transduction of human adipocytes.

Lentiviruses have the ability to transduce a wide variety of cells, both proliferating and quiescent (10-19). After infecting the host, they integrate into the genome. Sustained gene expression is mediated by HIV *pol* region-derived promoter central polypurin tract (cPPT) (26). Lentiviral transduction of undifferentiated adipose tissue-derived preadipocytes was previously demonstrated (23). Infected cells were differentiated into adipogenic and osteogenic lineages. However, an infection-related influence on the differentiation process cannot be excluded and functional studies were not presented in these previous reports. This potential drawback can be excluded by the herein demonstrated post-differentiation transduction procedure. The method allows stable and sustained inclusion of genetic information as we observed GFP-expression 4 to 30 days after infection.

Differentiated adipocytes were previously transduced by HSV-mediated lentiviral gene transfer. However, after only 12 days (latest time point investigated) only about 10-20% of the cells remained GFP positive (22). A laborious lentivirus-based method has been used for transduction of human primary mature adipocytes. However, only about 15% of the cells were successfully transduced. Moreover, cells could only be kept 20 h in culture post transduction (8). Adenoviruses are alternative candidates for transduction of non-dividing cells. However, the coxsackie virus and adenovirus receptor (CAR) is only scarcely expressed in several cell cultures types (27-30). Therefore, we subjected MSC and preadipocyte-derived adipocytes to high adenovirus titers for efficient transduction. In accordance with previous data in murine 3T3-L1 adipocytes (7), adenovirus treatment had cytotoxic effects on human adipocytes. Introduction of fiber-modified adeno vectors might circumvent these problems (9). Adenoviral vectors can be extremely efficient in some cell types, such as hepatocytes, but remain episomal and non-replicating and therefore

progressively lost over time.(31) Similarly, MLV-derived retroviral vectors require cell division and nuclear membrane dissolution for genome integration.(16, 20, 32-34)

Typically, about 20% of MSCs and preadipocytes remained undifferentiated after subjecting them to adipogenic medium. Intriguingly, lentiviral- and adenoviral-mediated transduction were strictly limited to differentiated adipocytes. This observation correlates with previous results also obtained in differentiated human adipocytes (35). Hence, it is tempting to speculate that differentiated adipocytes might have more efficient plasma membrane binding and internalization components for the adenovirus as compared to preadipocytes.

The titer of lentivirus was only determined for the FIV-derived vector. Successful transduction was achieved with a low of moi 2. Increased virus concentration did not result in higher transduction efficiency. Titrating lentivectors by determining the concentration of vector genome RNA in the supernatant may overestimate the vector titer by 1'000-10'000 fold because such analysis counts defective or otherwise non-infectious particles as well as free-floating vector genomes in the supernatant (36, 37). Therefore, with the HIV-derived virus we used always the supernatants directly regardless of the virus amount. We could not observe any differences in transduction efficiency. Most importantly, insulin-mediated glucose uptake experiments performed 14 days post-infection demonstrated that the adipocytes were still fully functional. These results were independent of the origin of the adipocyte (i.e. MSC-or preadipocyte- derived adipocytes) and the type of lentivirus. FIV-derived transduction systems include characteristics inherent in lentivirus vectors, such as large coding capacity, stable gene transfer and the ability to infect non-dividing cells. Due to their non-human origin their use might be safer as compared to HIV-derived lentiviruses.

In conclusion, based on our data lentiviral vectors are the gene-transfer system of choice for genetic modification of MSC- and preadipocyte-derived human adipocytes without apparent loss of cell specific functions. The availability of an efficient vector system may stimulate the use of adipose tissue, the largest endocrine gland, as a target for gene therapy in obesity and related disorders.

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Titles and legends to figures

Figure 1: Induction of adipocyte markers in MSC- and preadipocyte derived adipocytes MSC and preadipocytes were subjected to RT-PCR analysis of leptin, adiponectin, PPARγ2 and PPARγ1 before and after adipogenic differentiation. M indicates 100 bp molecular weight marker.

Figure 2: **Transduction of human MSC- and preadipocyte-derived adipocytes.** a) Mature MSC derived adipocytes and b) preadipocyte derived human adipocytes were exposed to lentiviral vectors containing GFP under a PGK promoter (53RPA PGK GFP). c) Mature MSC derived adipocytes and d) preadipocyte derived adipocytes were transduced with a lentiviral vector containing GFP under a CMV promoter (FIV CMV GPF VSVG). Pictures were taken 4 days after transduction with the Olympus IX 50 fluorescent microscope.

Figure 3: FACS analysis of transduced MSC- derived adipocytes. The upper panels show total cell counts and the area defined as living mature adipocytes. 10% - 12 % of total cell count was used for statistical analyses. Lower panel show GFP positive cells as compared to non transduced cells. a) mature adipocytes without transduction were used as control cells to optimize FACS conditions. b) mature adipocytes were transduced with 53RPK-PGK-GFP lentivirus. 30.39 % of the cells were GFP positiv. c) mature adipocytes were transduced with with FIV CMV GFP VSVG lentivirus. 9.58% of cells were GFP positive.

Figure 4: Insulin-induced glucose uptake assay. a) MSC- and b) preadipocyte-derived human adipocytes were exposed to 53RPK PGK GFP. c) MSC- and d) preadipocyte-derived adipocytes were exposed to FIV CMV GFP VSVG at moi 2 pfu/cell for two weeks. Transduced cells were stimulated by insulin (100nM) for 20 min and assayed for intracellular ^{3}H deoxy glucose uptake. Glucose uptake is expressed in cpm. Results are expressed as means ± SEM from three separate experiments performed in triplicate.

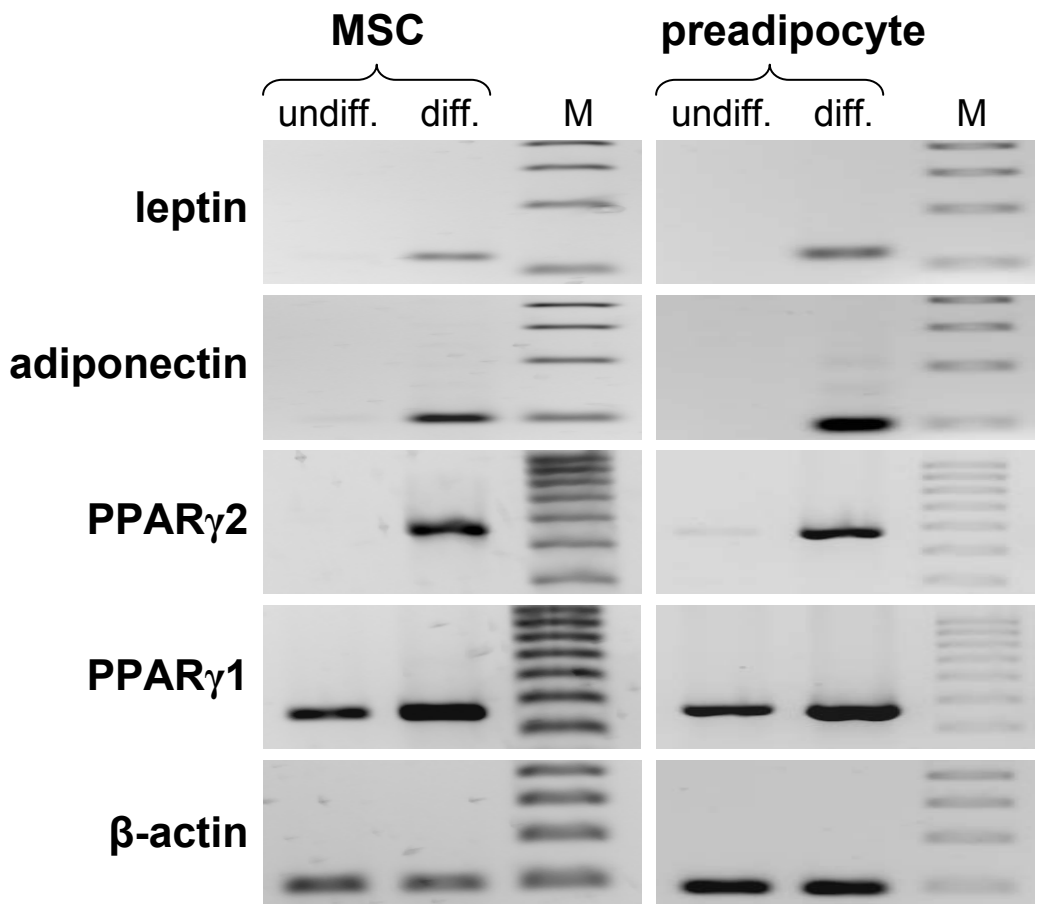
MSC
preadipocyte
undiff.
diff.
M
undiff.
diff.
M
leptin
adiponectin
PPARγ2
PPARγ1
β-actin

Figure 1

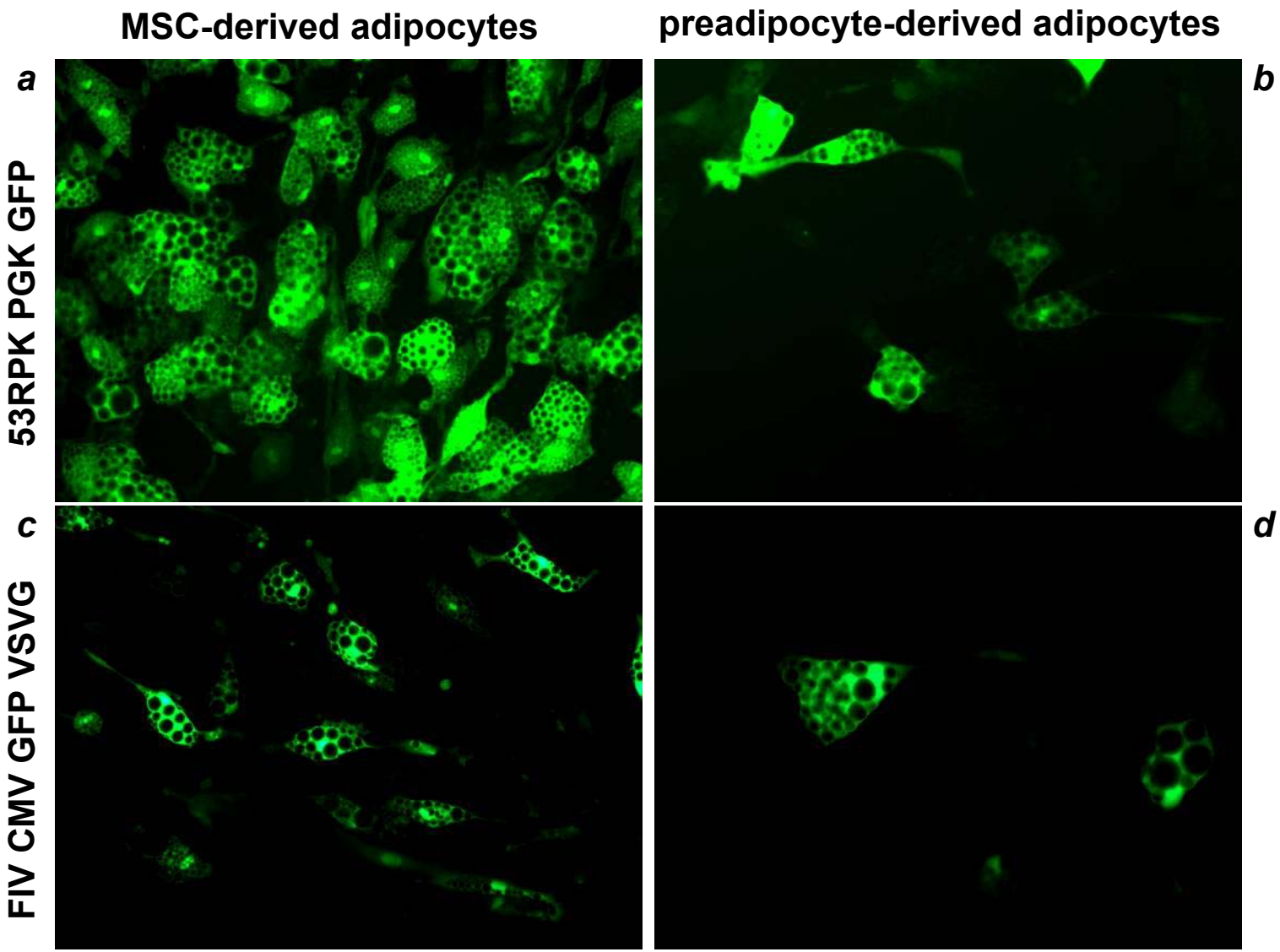
MSC-derived adipocytes
preadipocyte-derived adipocytes
a
b
c
d
53RPK PGK GFP
FIV CMV GFP VSVG

Figure 2

a) MSC-derived adipocytes no virus

b) MSC-derived adipocytes 53RPK PGK GFP

c) MSC-derived adipocytes FIV CMV GFP VSVG

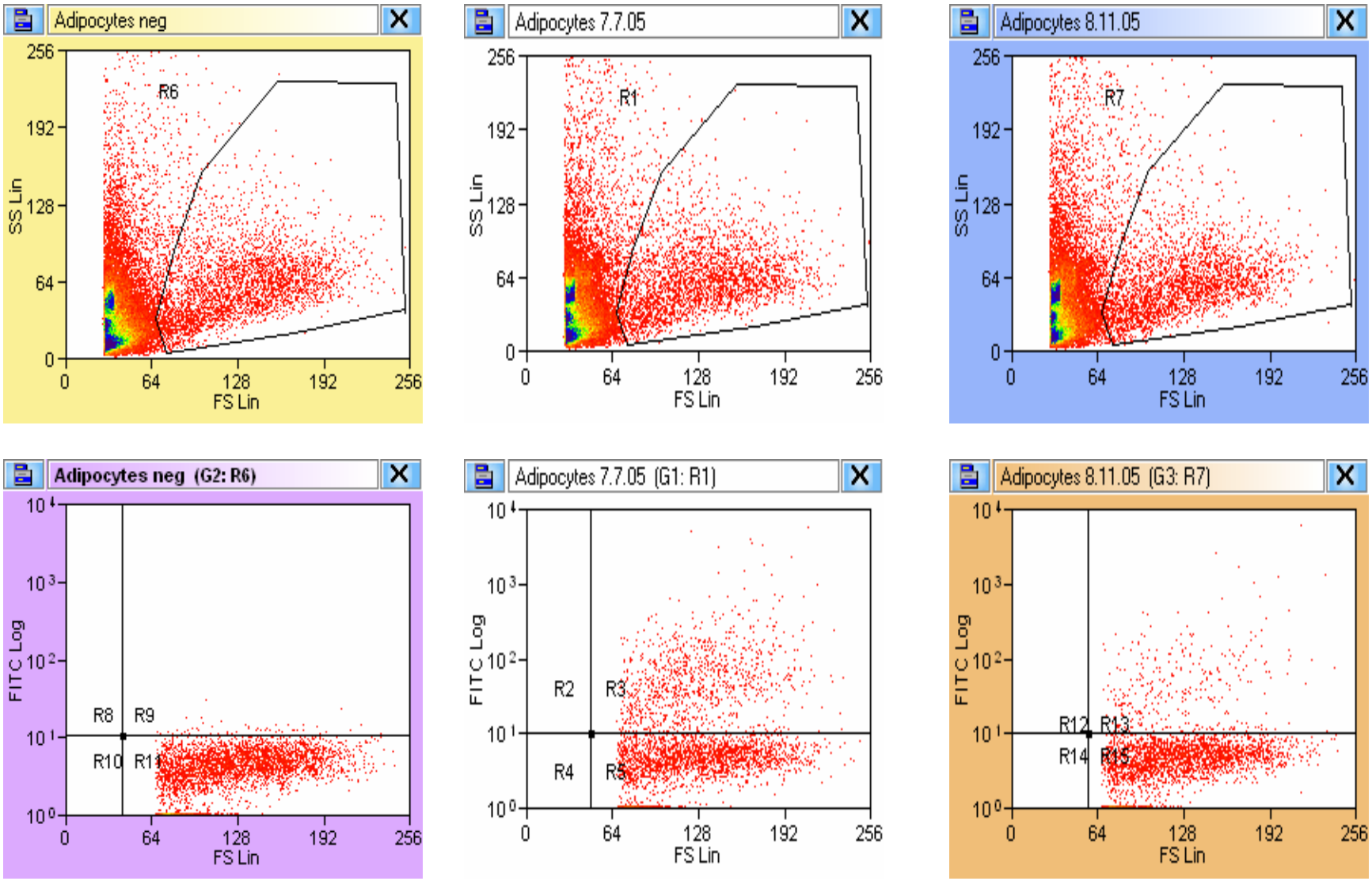

Figure 3

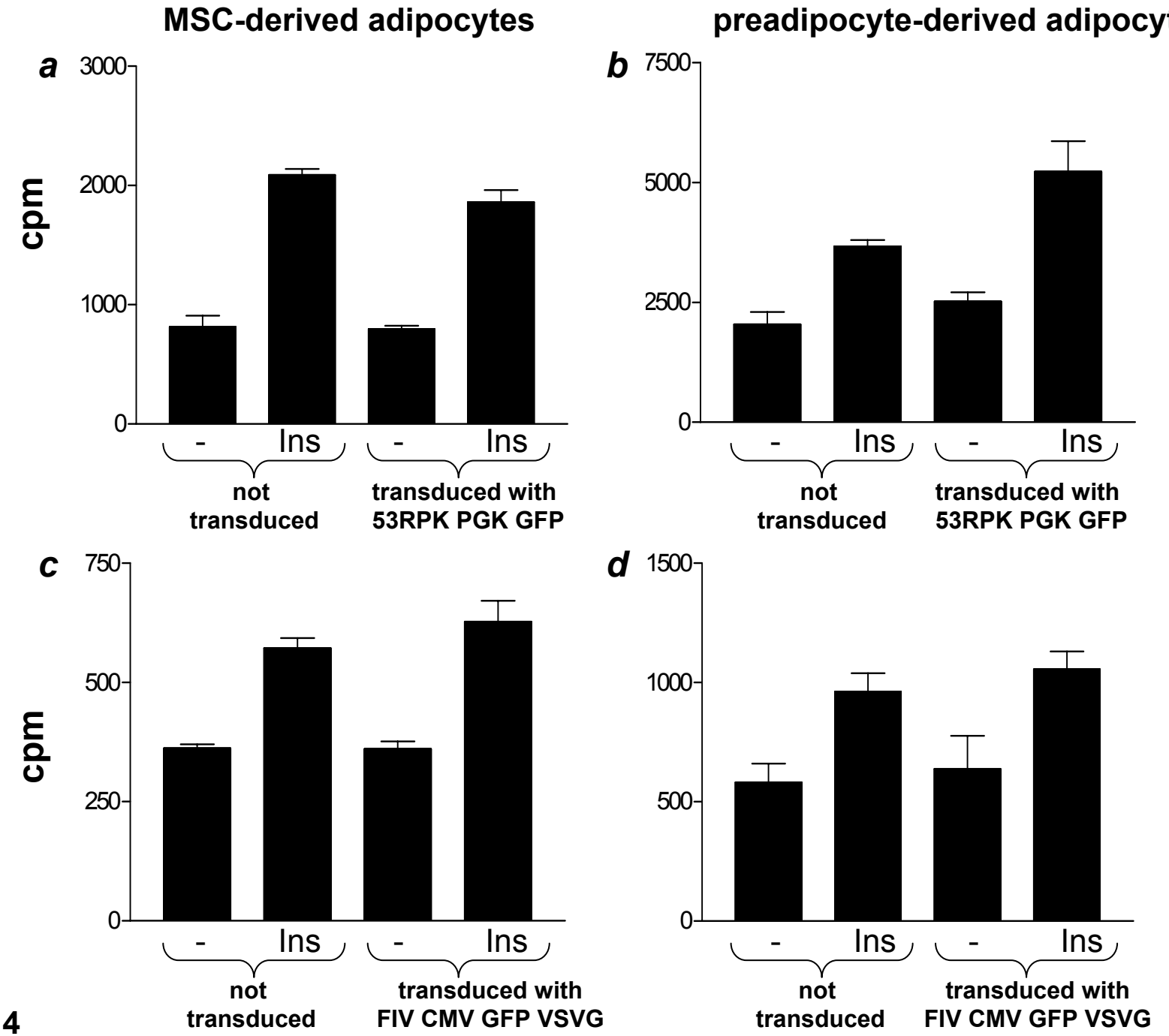
MSC-derived adipocytes
preadipocyte-derived adipocytes
a
b
c
d
cpm
3000
2000
1000
0
7500
5000
2500
750
500
250
1500
-
Ins
not transduced
transduced with 53RPK PGK GFP
transduced with FIV CMV GFP VSVG

Figure 4

10. ACKNOWLEDGEMENTS

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11. CURRICULUM VITAE AND PRESENTATION

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Education

1995	Matura Typ C, Gymnasium Sursee, Luzern
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1997-1999	University of Basel, Faculty of Science, Biology (Vordiplom 1 und 2)
1999-2000	University of Basel, Neurobiology and Ecomomical Sciences
2000-2001	James Cook University, Australia (Graduate Diploma of Science)
2000-2001	Honours thesis, Department of Molecular Biology and Biochemistry, James Cook University, Townsville, Australia, supervisor Dr. David Miller (BSc. Hons IIA, accepted as Diploma by the University of Basel) Title: Characterisation of the head patterning genes *tailless* and *aristaless* of the staghorn coral *Acropora millepora*
2002-at the present	PhD at the University of Basel, Switzerland. The thesis study is carried out at the Dept. of Research of the University of Basel with Prof. Dr. Beat Müller Title: Hormokines: A novel concept of plasticity in Neuro-Endo-Immunology

Work experiences

1999	Codelab Software AG, Basel, software programming
1999	Research Project at the Department of Medical Microbiology, University of Basel with Prof. Dr. Klimkait.
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Languages

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Publications

In vitro and in vivo calcitonin-I gene expression in parenchymal cells: a novel product of human adipose tissue.
Linscheid P, Seboek D**,** Nylen ES, Langer I, Schlatter M, Keller U, Becker KL, Muller B 2003 **Endocrinology** 144(12):5578-5584

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Timper K*, Seboek D*, Eberhardt M, Linscheid P, Keller U, Müller B and Zulewski H 2006 **Biochem Biophys Res Commun** Mar 24;341(4):1135-40

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Multipotential nestin and Isl-1 positive mesenchymal stem cells isolated from human pancreatic islets
Eberhardt M, Salmon P, von Mach MA, Hengstler JG, Brulport M, Linscheid P, Seboek D, Oberholzer J, Barbero A, Martin I, Müller B, Trono D and Zulewski H 2006 **Biochem Biophys Res Commun** Jul 7;345(3):1167-76

Human bone marrow-derived mesenchymal stem cells from differentiate into insulin, somatostatin and glucagon expressing cells
Seboek D, Timper K, Eberhardt M, Linscheid P, Keller U, Müller B and Zulewski H (submitted)

Lentiviral vectors efficiently transduce human mesenchymal stem cell- and preadipocyte derived mature adipocytes
Seboek D, Linscheid P, Firm C, Zulewski H, Salmon P, Russo AF, Keller U, Müller B (submitted)

Awards
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PRESENTATIONS:

May 29 – June 1, 2003 ECO
12th European Congress on Obesity, Helsinki, Finland
Adipose tissue is a source of procalcitonin in sepsis and inflammation
Philippe Linscheid, Dalma Seboek, Gerhard Pierer, Ulrich Keller, Beat Müller

June 19 – 23, 2003 ENDO
The Endocrine socyiety's 85th annual meeting, Philadelphia, USA
Poster: Somatostatin receptor and somatostatin gene expression following inflammatory stimulation of human adipocytes.
Dalma Seboek, Philippe Linscheid, Gerhard Pierer, Michael Eberhardt, Henryk Zulewski, Ulrich Keller, Beat Müller

September 21 – 24, 2003 ELSO
European life scientist organisation, Dresden, Germany
Poster: Human adipose tissue expresses somatostatin and its receptors- new mediators of the immuno-endocrine activity
Dalma Seboek, Philippe Linscheid, Igor Langer, Henryk Zulewski, Ulrich Keller, Beat Müller

November 28 – 29, 2003 SGED
Schweizerische Gesellschaft für Endokrinologie und Diabetologie, Bern, Switzerland
Oral presentation: Human adipose tissue is a source of procalcitonin in human sepsis
Philippe Linscheid, Dalma Seboek, Igor Langer, Mirjam Schlatter, Ulrich Keller, Beat Müller
Poster: Human adipose tissue epxresses somatostatin and its receptors- new mediators of the immuno-endocrine activity
Dalma Seboek, Philippe Linscheid, Igor Langer, Henryk Zulewski, Ulrich Keller, Beat Müller

June 16 – 20, 2004 ENDO
The Endocrine socyiety's 86th annual meeting, New Orleans, USA
Poster: Inflammatatory stimulation induces somatostatin expression and secretion in human asipocytes
Dalma Seboek, Philippe Linscheid, Igor Langer, Mirjam Christ-Crain, Henryk Zulewski, Ulrich Keller, Beat Müller

September 4 – 8, 2004 ELSO
European life scientist organisation, Nice, France
Poster: Lentiviral vectors efficiently transduce mature adipocytes derived from human mesenchymal stem cells
Dalma Seboek, Christina Firm, Philippe Linscheid, Henryk Zulewski, Adrew Russo, Ulrich Keller, Beat Müller

November 26 - 27, 2004 SGED
Schweizerische Gesellschaft für Endokrinologie und Diabetologie, Bern, Switzerland
Poster: Lentiviral vectors efficiently transduce mature adipocytes derived from human mesenchymal stem cells
Dalma Seboek, Christina Firm, Philippe Linscheid, Henryk Zulewski, Adrew Russo, Ulrich Keller, Beat Müller
Oral Presentation: Isolation, immortalization and characterization of multipotent mesenchymal stem cells from human islets of Langerhans that express nestin, ABCG2 and islet-1
Michael Eberhardt, Patrick Salmon, Philippe Linscheid, Dalma Seboek, Marc von Mach, Beat Müller, Didier Trono, Henryk Zulewski

April 26, 2005 SGIM
Schweizerische Gesellschaft für Innere Medizin, Basel, Switzerland
Poster: Human Isl-1 positive mesenchymal stem cells differentiate into insulin, somatostatin and glucagon expressing cells
Dalma Seboek, Katharina Timper, Michael Eberhardt, Philippe Linscheid, Ulrich Keller, Beat Müller and Henryk Zulewski

June 4 – 7, 2005 ENDO
The Endocrine socyiety's 87th annual meeting, San Diego, USA
Poster: Lentiviral vectors efficiently transduce mature human adipocytes
Dalma Seboek, Christina Firm, Philippe Linscheid, Henryk Zulewski, Andrew Russo, Ulrich Keller, Beat Müller
Poster: Nitric oxide synthase activity in human adipocytes?
Philippe Linscheid, Dalma Seboek, Nenad Blau, Ulrich Keller and Beat Müller

June 10 – 14, 2005 ADA
65th Scientific Session of the American Diabetes Association, San Diego, USA
Poster: Differentiation of human bone marrow- derived stem cells into insulin, amylin, somatostatin and glucagon expressing islet-like clusters *in vitro*
Dalma Seboek, Katharina Timper, Michael Eberhardt, Philippe Linscheid, Ulrich Keller, Beat Müller and Henryk Zulewski
Poster: Differentiation of human preadipocytes into insulin, amylin, somatostatin and glucagon expressing islet like clusters in vitro
Katharina Timper, Dalma Seboek, Philippe Linscheid, Michael Eberhardt, Ulrich Keller, Beat Müller and Henryk Zulewski

August 20 – 24, 2005 Inflammation
7th World Congress on Inflammation, Melbourne, Australia
Poster: Calcitonin peptides are produced by human adipocytes during inflammation and exert autocrine/paracrine effects
Philippe Linscheid, Dalma Seboek, Ulrich Keller and Beat Müller

September 12-15, 2005 EASD
41st The European Association for the Study of Diabetes, Athen, Greece
Poster: Coordinated activation of pancreatic developmental genes during differentiation of human bone marrow- derived stem cells into insulin, amylin, glucagon and somatostatin expressing cells.
Dalma Seboek, Katharina Timper, Michael Eberhardt, Philippe Linscheid, Ulrich Keller, Beat Müller and Henryk Zulewski
Oral Presentation: Engineering of multipotent human stem cells from pancreatic islets of Langerhans
Michael Eberhardt, Patrick Salmon, Philippe Linscheid, Dalma Seboek, Marc von Mach, Andrea Barbero, Ivan Martin, Beat Müller, Didier Trono, Henryk Zulewski

November 18 – 19, 2005-12-21 SGED
Schweizerische Gesellschaft für Endokrinologie und Diabetologie, Bern, Switzerland
Oral presentation: Differentiation of human mesenchymal stem cells and preadipocytes into insulin, somatostatin and glucagon expressing islet like clusters *in vitro*
Dalma Seboek, Katharina Timper, Michael Eberhardt, Philippe Linscheid, Mirjam Christ-Crain, Ulrich Keller, Beat Müller and Henryk Zulewski
Poster: Bone marrow derived mesenchymal stem cells isolated from patients with diabetes mellitus type 1 are able to adopt a pancreatic endocrine phenotype in vitro
Michael Eberhardt, Dalma Seboek, Andrea Barbero, Philippe Linscheid, Katharina Timper, Ivan Martin, Ulrich Keller, Beat Müller and Henryk Zulewski

December 16, 2005 SSCNP
2nd annual meeting of the swiss stem cell network, Basel, Switzerland
Oral presentation: Differentiation of human mesenchymal stem cells and preadipocytes into insulin, somatostatin and glucagon expressing islet like clusters *in vitro*
Dalma Seboek, Katharina Timper, Michael Eberhardt, Philippe Linscheid, Mirjam Christ-Crain, Ulrich Keller, Beat Müller and Henryk Zulewski