Caveolar dysfunction leads to signal transduction defects that are critical for obesity-driven disorders

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(Dekan)

1. Foreword

To say that obesity and diabetes have reached epidemic proportions has become something of a cliché. This should not lead us to simply accept it as a byproduct of our changing lifestyle, or to overlook the socioeconomic importance of these conditions.

Our attempts at therapeutic intervention have been hindered by a lack of knowledge about the precise pathophysiological mechanisms via which obesity triggers secondary disorders such as diabetes and cardiovascular problems. Our approach to understanding the mechanisms have been largely focused on single molecules whose levels or activity are altered in obesity, and which are known independently to contribute to these secondary disorders. Although we have accumulated genetic and biochemical evidence for the potential role of these factors in these disorders, it must be noted that no single candidate or mechanism has yet given a satisfactory explanation for all the obesity-related disorders. This has led researchers to conclude that the underlying pathology is polygenic or multi-factorial. However, based on our experiments and the recent findings of a few other laboratories, we propose a unifying theory where obesity leads to a membrane microdomain disorder that will in turn lead to the corruption of multiple signaling pathways that are known to be implicated in insulin resistance and cardiovascular disorders. We also explore the possibility of pharmacologically modulating a nodal, but downstream, drug target that might retard or prevent these disorders. Having said that, I would like to clearly state that this theory is still in its infancy, and remains to be tested in both animal models and human subjects.

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Summary

Obesity is a major risk factor for the development of secondary disorders such as type-2 diabetes mellitus, cardiovascular complications and certain types of cancers. Quenttesentially, these secondary disorders results from the corruption of physiological signal transduction mechanisms. We know relatively well as to what these signal transduction disorders are, but how obesity leads to these signaling defects remains obscure. In this thesis we show that caveolar microdomains are a key signal transduction platform, whose perturbation can lead to a plethora of signaling defects that are known to elicit secondary-disorders of obesity. Elevated levels of PAI-1 represent a ‘nodal’ and ‘downstream’ mediator of these signaling defects. By using peptide antagonists of E2F-pRB interaction, we could inhibit PAI-1 gene expression in adipocytes without causing unwanted effects such as cell-cycle reentry. In effect, this study proposes a pathological mechanism and pharmacological intervention strategy for obesity-driven secondary disorders.

2. General introduction

2.1 Obesity and its medical complications

Obesity has reached epidemic proportions globally, with more than 1 billion adults overweight - at least 300 million of them clinically obese - and is a major contributor to the global burden of chronic disease and disability (1). Often coexisting in developing countries with under-nutrition, obesity is a complex condition, with serious social and psychological dimensions, affecting virtually all ages and socioeconomic groups.

Increased consumption of more energy-dense food, combined with reduced physical activity, have led to obesity rates that have risen three-fold or more since 1980 in some areas of North America, the United Kingdom, Eastern Europe, the Middle East, the Pacific Islands, Australasia, and China (2). The obesity epidemic is not restricted to industrialized societies; this increase is often faster in developing countries than in the developed world.

Benchmarking obesity

The prevalence of obesity and being overweight is commonly assessed by using the body mass index (BMI), defined as the weight of a person in kilograms divided by the square of the height in meters (kg/m^2). A BMI of over 25 kg/m^2 is defined as overweight, and a BMI of over 30 kg/m^2 as obese (3).

BMI (kg/m^2)	WHO classification	Popular description
< 18.5	Underweight	Thin
18.5–24.9	Normal range	'Healthy', 'normal' or 'acceptable' weight
25.0–29.9	Grade 1 overweight	Overweight
30.0–39.9	Grade 2 overweight	Obesity
≥ 40.0	Grade 3 overweight	Morbid obesity

Table. 2.1: WHO guidelines for classification of overweight and obesity

The medical problems posed by obesity

The problem posed by obesity is not limited to that of aesthetics. Being overweight or obese can lead to adverse metabolic effects on blood pressure, cholesterol, triglycerides, and insulin resistance (4). The non-fatal, but debilitating, health problems associated with obesity include respiratory difficulties, chronic musculoskeletal problems, skin problems, and infertility. The more life-threatening problems fall into three main categories: cardiovascular disorders (CVD); conditions associated with insulin resistance, such as type-2 diabetes mellitus (T2DM); and certain types of cancers, especially hormonally related and large-bowel cancers.

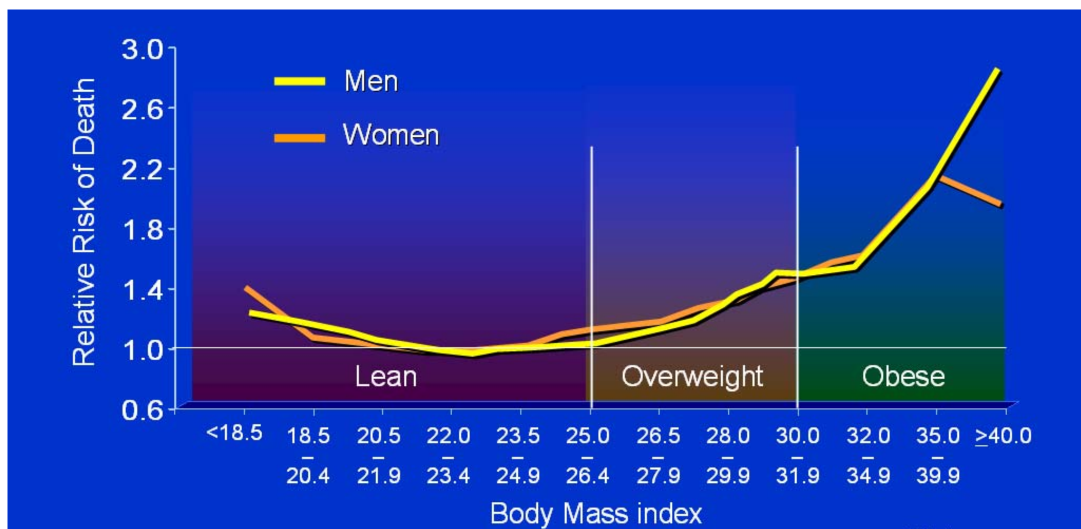

Fig. 2.1. Relationship between BMI and cardiovascular mortality. *BMI is a strong risk factor for the development of cardiovascular disorders.*

The effects of obesity on cardiovascular health are many. Risk estimates from population studies suggest that ≥75% of hypertension can be directly attributed to obesity (5). It is well documented that blood pressure increases with weight gain and decreases with weight loss. Apart from hypertension, the risk for other major cardiovascular disorders, including atherosclerosis, dyslipedimia, and coagulation problems, are strongly increased by obesity. As illustrated in Fig. 2.1, BMI strongly corresponds to cardiovascular mortality in both men and women.

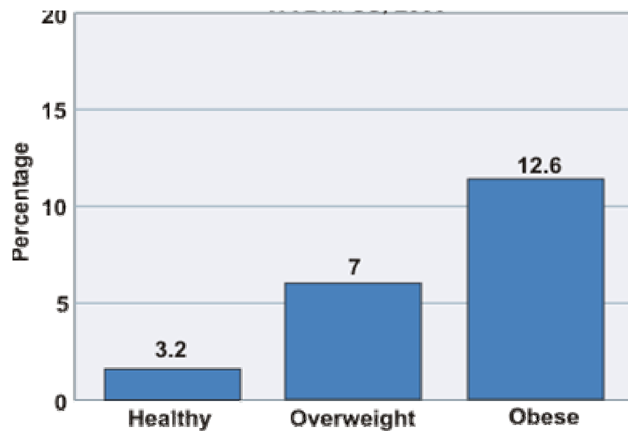

Fig. 2.2: Relationship between BMI and incidence of diabetes. *A dramatic increase in the incidence of diabetes in over-weight and obese people can be seen in this graph.*

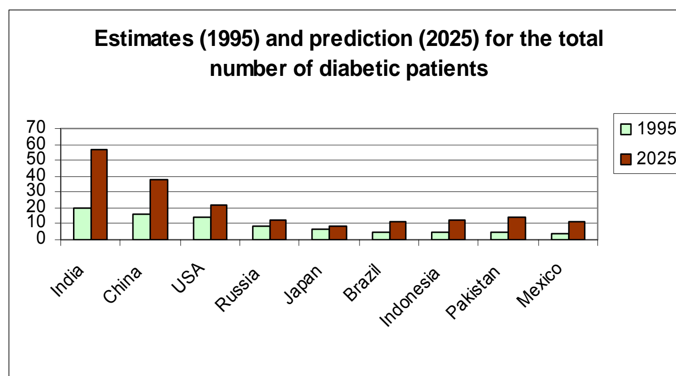

Fig. 2.3: The global epidemic of diabetes. *The incidence of diabetes is expected to increase globally. Y-axis represents patient number in millions. The major economic power houses (USA, China, and India), which are also among the most populous countries in the world, are supposed to be the worst affected by diabetes. Source: WHO-World Health Report, 1997.*

The likelihood of developing type-2 diabetes rises steeply with increasing levels of body fat. The close link between the two disorders has even led to the coining of new term 'diabesity' to collectively describe this condition. Confined to older adults for most of the 20th century, this disease now affects obese children even before puberty. Approximately 85% of people with diabetes are type-2 and, of these, 90% are obese or overweight. And this is increasingly becoming a developing world problem. In 1995, the emerging market economies had the highest number of diabetics. If current trends continue, India and the Middle Eastern crescent will have taken over by 2025. Large increases would also be observed in China, Latin America and the Caribbean, and the rest of Asia.

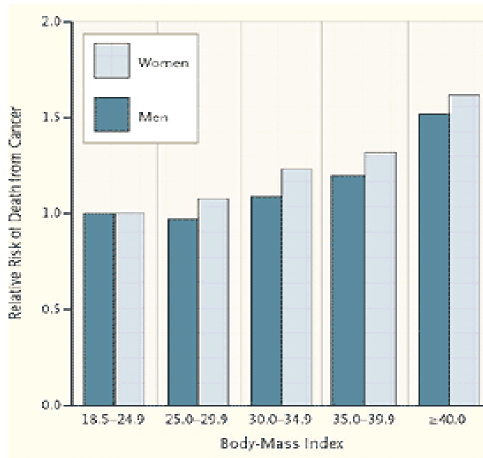

Fig. 2.4. Contribution of the overweight and obese to mortality from cancer in the United States. *Relative risk of cancer is positively correlated to the BMI.*

Raised BMI also increases the risk of cancer of the breast, colon, prostate, endometrium, kidney, and gall bladder (3). Although obesity should be considered a disease in its own right, it is also one of the key risk factors for other chronic diseases, together with smoking, high blood pressure, and high blood cholesterol. In the analyses carried out for the WHO World Health Report 2002, approximately 58% of type-2 diabetes and 21% of ischemic heart disease and 8-42% of certain cancers globally were attributable to a BMI above 21 kg/m^2 (2). In the later parts of this thesis, we discuss the possibility of how the dysfunction of a key cellular signal-transduction platform known as caveolae can activate signal transduction defects that are characteristic of obesity-driven disorders.

2.2 Caveolae

Structure

Caveolae are morphologically identifiable plasma membrane invaginations that are distinct from the larger electron-dense clathrin-coated pits. Originally identified in the 1950s by electron microscopists investigating cellular ultrastructure, caveolae appear as smooth, uncoated pits or vesicles at the plasma membrane, typically observed using conventional resin-embedded techniques. In general, caveolae are 50- to 100-nm flask-shaped invaginations of the plasma membrane that can be singular or found in detached grape-like clusters (rosette formation), or long, tubular structures

thought to evolve from the fusion of individual caveolae (Fig. 2.5) While the overall function of the prototypical caveolae organelle has been an area of intense exploration, little attention has been focused on the specialized function, if any, of the various morphological subsets of caveolae. Consequently, the functional significance of these caveolae-related organelles remains unknown.

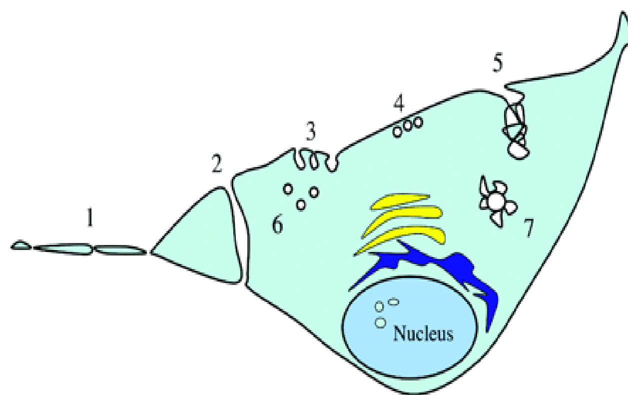

Fig. 2.5. Stylized view of the cell, showing the morphological variants of caveolae and selected subcellular compartments: *(1) fenstra, (2) a transcellular channel, (3) traditional caveolae, (4) plasmalemmal vesicles (fully invaginated, static caveolae), (5) a vesiculo-vacuolar organelle (a grape-like cluster of interconnected caveolae and vacuoles), (6) cavicles (mobile, internalized caveolae not associated with the plasma membrane), and (7) a caveosome (a slow-moving, irregularly shaped, cytoplasmic organelle).*

Tissue distribution

Caveolae were first identified in capillary endothelial cells and epithelial cells from the mouse gall bladder (6). Since then, caveolae have been identified in a wide variety of tissues and cell types (7). While no all-encompassing ultrastructural study has been undertaken, a review of published literature reveals that caveolae are present to some degree in most differentiated cell types. In particular, caveolae have been well-described in adipocytes, where they are extremely abundant, endothelial cells, type I pneumocytes of the lung, and striated and smooth muscle cells. Because of their relative abundance in endothelial cells and type I pneumocytes, the two major constituents of lung alveoli, the lung stands out as one of the most abundant sources of identifiable caveolae, second only to adipocytes. Ultrastructural

analysis of adipocytes has shown that as much as 20% of the total plasma membrane is occupied by caveolae (8). Thus, caveolae can greatly increase the surface area of numerous cell types, an observation that lends credence to the original speculation that caveolae are involved in macromolecular transport and mechanotransduction events.

Biochemical properties

Unlike earlier views of the plasma membrane as a "fluid mosaic" (9), where integral membrane proteins were thought to float and diffuse freely through a sea of homogeneous lipids, a more contemporary view of the plasma membrane is that proteins are much more heterogeneously distributed and can be found clustered within specialized microdomains, termed lipid rafts. These lipid rafts are thought to form via the aggregation of glycosphingolipids and sphingomyelin in the Golgi apparatus (held together by transient and weak molecular interactions) and are then delivered to the plasma membrane as concentrated units (10) (11). These lipid rafts are also enriched with cholesterol and several resident proteins, including glycophosphatidylinositol (GPI)-linked proteins. Relative to the plasma membrane proper, which contains an abundance of cis-unsaturated phospholipids, the sphingolipids in lipid rafts contain primarily saturated fatty acyl chains, allowing tighter molecular packing that results in a higher melting temperature ($T_m \sim 41$°C vs. $T_m < 0$°C for phospholipids) (12). The high cholesterol and sphingolipid content of lipid rafts imparts a resistance to extraction in non-ionic detergents such as Triton X-100 at 4°C, and a light buoyant-density in sucrose gradients, properties instrumental for their purification and biochemical characterization. It should be mentioned that the exact nature and defining characteristics of lipid rafts, as well as the techniques involved in isolating them, are now quite well-developed (13).

Caveolae represent a morphologically identifiable subset of lipid rafts. They contain the coat protein caveolin, which is essential for the invagination of the plasma membrane through a largely unknown process, giving them their

characteristic flask-like appearance. While the overall biochemical composition of lipid rafts and caveolae is thought to overlap, these microdomains are not completely equivalent. In addition to the caveolins, several proteins have been shown to preferentially localize to either caveolae or lipid rafts (14).

Caveolins and cholesterol: integral components of caveolae

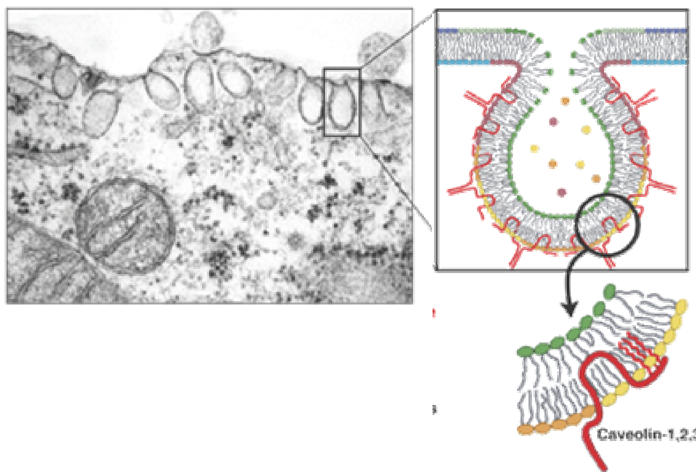

Fig.2.6. The structure of caveolae. *Deep-freeze electron microscopy photograph of caveolae (left) and their graphical representation (right).*

Caveolae are sphingolipid- and cholesterol-rich invaginations of the plasma membrane, and are decorated intracellularly with the protein caveolin.

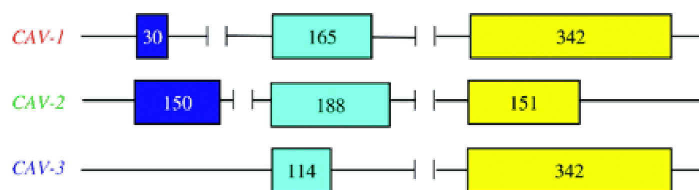

Fig. 2.7. Schematic depiction of the caveolin gene family. *Color-coded boxes indicate the exon arrangement of each caveolin family member. The numbers within each box refer to the number of nucleotides in each exon.*

To date, three members of the caveolin (CAV) gene family have been identified (15). Caveolin-1 is composed of three exons that are highly conserved in sequence and structure across species. Several key conserved caveolin-1 residues are absent or altered in caveolin-2. Human caveolin-2 is ~38% identical and ~58% similar to human caveolin-1, while caveolin-3 is ~65% identical and ~85% similar to caveolin-1. Moreover, a short stretch of eight amino acids has been identified (FEDVIAEP) that constitutes the

"caveolin signature sequence," a motif that is identical in all three caveolin proteins.

Caveolin (Cav)-1 and Cav-3 form ~350-kd homo-oligomers made up of 14–16 caveolin monomers. These homo-oligomers serve as the basic structural units that drive the formation of caveolae membranes. In contrast, Cav-2 either homodimerizes or forms high molecular mass hetero-oligomers with Cav-1 (16) (17) (18). Cav-1 and Cav-3 are both independently necessary and sufficient to drive caveolae formation in heterologous expression systems, while Cav-2 requires the presence of Cav-1 for proper membrane targeting and stabilization. In the absence of Cav-1, Cav-2 localizes to the Golgi complex where it is degraded by the proteasome (19) (20). Caveolins 1 and 2 are co-expressed in many cell types, such as endothelial cells, fibroblasts, smooth muscle cells, and adipocytes, where they form a heteroligomeric complex. In contrast, the expression of caveolin-3 is muscle-specific.

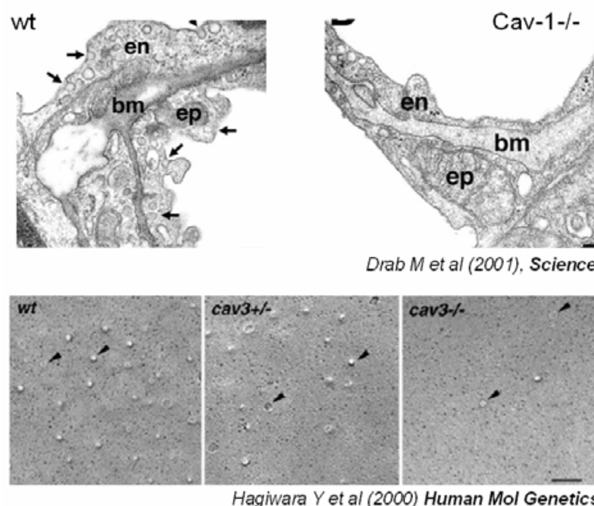

Fig. 2.8. Caveolin knock-out mice lack caveolae. *Mice lacking caveolin-1 are shown to lack caveolar structures in alveolar epithelium (upper panel); Knock-out of caveolin-3 leads to a dramatic reduction in caveolar structures in skeletal muscle (lower panel).*

Studies performed on knock-out mice have indicated that Cav-1 expression is required for caveolae formation in non-muscle cells (21), while the expression of Cav-3 drives caveolae formation in striated muscle cell types (cardiac and skeletal) (22).

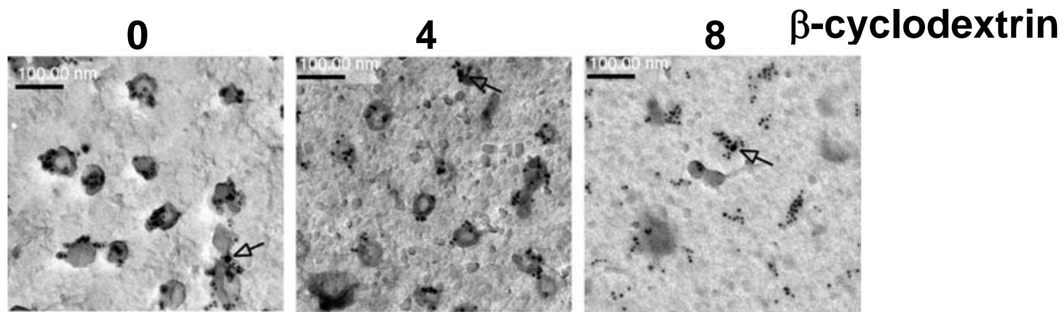

Fig. 2.9. Depletion of cholesterol leads to caveolar dysfunction*. Electron-microscopic analysis shows that cyclodextrin treatment dose-dependently leads to loss of caveolae (patches), even if the caveolin (black spots) are unaffected.*

Moreover, it has been shown that depletion of cholesterol using cholesterol-depleting agents such as cyclodextrin and filipin leads to perturbation of caveolar structure (23). Taken together, both Cav-1/3 and plasma membrane cholesterol appear to be critical for the structural integrity of caveolar microdomains.

Physiological functions of caveolae

Caveolins bind to many different molecules involved in signal transduction, including transmembrane receptors, G-proteins, kinases, adaptor proteins, and enzymes. Thus, caveolae act as a major cellular signal transduction platform. A short list of molecules that are known to bind to caveolae is given in the following table.

Class of molecules	**Name of molecule**	**Biochemical localization**	**Morphological localization**
Lipid	Ganglioside	√	√
	Sphingomyelin	√	—
	Ceramide	√	—
	Diacylglycerol (DAG)	√	—

	Cholesterol	√	√
Acylated protein	Hetero-trimeric G-proteins	√	√
	Src, Fyn, Hck, Lck	√	—
	E-NOS	√	√
	CD-36	√	—
	Caveolin	√	√
Glycosylphospha-tidylinositol (GPI)-anchored protein	Folate receptor	√	√
	Thy 1	√	√
	Alkaline phosphatase	√	√
	Prion	√	√
	Urokinase Rec	√	√
	Multiple GPI proteins	√	—
	5'-nucleotidase	√	√
	CD14	√	—
Prenylated protein	Rap1A	√	√
	Ras	√	—
Membrane receptor	Platelet-derived growth factor (PDGF)	√	√
	Insulin growth factor (IGF)	√	√
	Insulin	—	√
	Epidermal growth factor (EGF)	√	—
	Receptor for advanced glycation end product (RAGE)	√	—
	Cholecystokinin (CCK) receptor	√	√
	m2 acetylcholine	√	
	Tissue factor	√	—
	β adrenergic	—	√
	Bradykinin	√	—

	Endothelin	√	—
	SR-B1	√	√
Signal transducer	PKC	√	√
	SHC	√	—
	SOS	√	—
	GRB_2	√	—
	MAP kinase	√	√
	Adenylyl cyclase	√	√
	SYP	√	—
	PI3 kinase	√	—
	Raf1	√	—
	Calmodulin	√	—
	Phosphoinositides	√	
	Polyphosphoinositide phosphatase	√	—
	Engrailed	√	—
Membrane transporter	Porin	√	—
	IP_3 receptor	√	√
	Ca^{2+} ATPase	√	√
	Aquaporin-1	√	—
	H^+ ATPase	√	—
Structural molecules	Annexin II	√	—
	Ezerin	√	—
	Myosin	√	—
	VAMP	√	—
	NSF	√	—
	MAL	√	—
	Actin	√	√
Miscellaneous	Atrial natriuretic	—	√
	Peptide		
	Flotillin	√	—

Table 2.2: Partial list of caveolin binding proteins [adapted from Anderson et al. (24)]

The three well-described functions of caveolae are vesicular transport, cholesterol homeostasis, and, more importantly, signal transduction. It is important to note that, while caveolin seems to be a negative regulator of the vast majority of signaling proteins with which it interacts, at least one protein, the insulin receptor, is positively regulated by an interaction with caveolin-1 (25) (26) (27) (28).

In the later parts of this thesis, we will show that caveolar-dysfunction leads to increased gene-expression of plasminogen activator inhibitor-1 (PAI-1), whose high plasma levels are implicated in obesity associated disorders. An introduction to PAI-1 is given below.

2.3 Plasminogen Activator System

The plasminogen activator system represents an enzymatic cascade involved in the control of fibrin degradation, matrix turnover, and cell invasion. Extracellular proteolysis mediated by the plasminogen activator system plays an important role in various physiological processes such as angiogenesis, wound healing, inflammation, and cell proliferation (29).

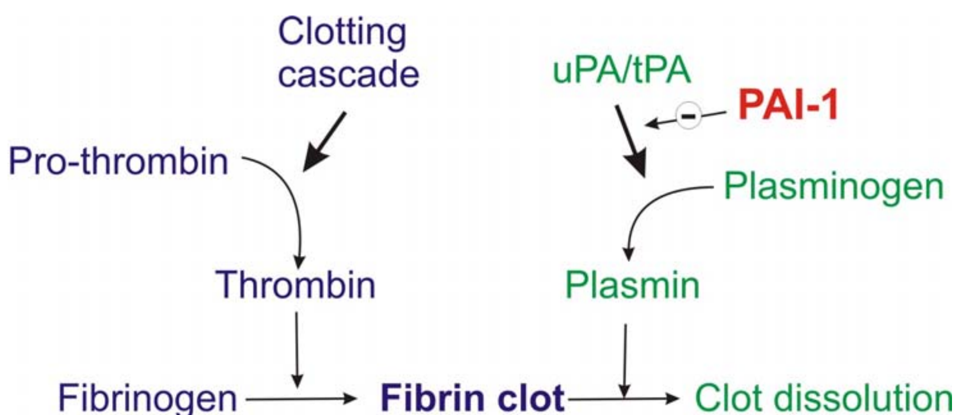

***Fig. 2.10. Schematic diagram of the plasminogen activator system and its role in clot lysis**. PAI-1 acts as the physiological inhibitor of plasminogen activators, uPA, and tPA.*

The plasminogen activation system consists of several components including plasminogen, plasmin, activators, inhibitors, and a receptor (see Appendix-1). Plasminogen, the main component of the plasminogen system, is an inactive proenzyme that can be converted to the proteolytically active enzyme plasmin. This trypsin-like protease degrades a wide range of substrates, including various extracellular matrix (ECM) proteins such as fibronectin, vitronectin, and fibrin. Plasmin also activates matrix metaloproteinases and collagenases that in turn degrade ECM components (30).

There are two physiological plasminogen activators (PA): tissue-type (tPA) and urokinase-type (uPA) (31). These secreted serine proteases convert the ubiquitous zymogen plasminogen to plasmin. The primary role of tPA is the generation of plasmin for fibrinolysis in blood vessels. uPA, on the other hand, activates plasmin for degradation of components of ECM during cell migration and tumor invasion.

The activities of both tPA and uPA are negatively regulated by the binding of PA inhibitors (PAI-1 and PAI-2), protein nexin-1 (PN-1), and protein C inactivator (PCI). These inhibitors belong to the serine protease inhibitor (SERPIN) superfamily. All members of this family have in their COOH-terminal region a specific reactive-site peptide bond (Arg-X or Lys-X), which is cleaved by their target enzyme, resulting in the formation of an inactive enzyme-inhibitor complex. The PA system also includes the uPA receptor (uPAR), a cell-membrane anchored uPA-binding protein. This receptor plays a key role in the regulation of localization, activity, function, and interactions among PA system components. Except for its well established role in proteolysis, the plasminogen activator system also supports cell migration and invasion by plasmin-independent mechanisms as explained earlier.

Physiology and regulation of PAI-1

Plasma PAI-1 reflects the output of several sources, including the adipose tissue, vascular endothelium, and liver. Large quantities of PAI-1 are stored in platelets and render platelet-rich clots resistant to thrombolysis. Recent data indicate that there is a continuous production of large amounts of active PAI-1 in platelets (32), which could be a mechanism by which platelets contribute to the stabilization of blood clots. Endothelial cells in culture synthesize PAI-1 at relatively high rates, although in the absence of hormonal, metabolic, or inflammatory stimuli, the vascular endothelium is not likely to be a major contributor to circulating PAI-1 levels (33). Several different fates are possible for PAI-1 after it is synthesized and secreted. The majority of PAI-1 probably circulates briefly in the plasma, and only a fraction of the secreted, active PAI-1 has the opportunity to react with plasma t-PA, and form inert, covalent complexes. There appears to be no endogenous mechanism for recycling PA-PAI-1 complexes, which are cleared through the low-density lipoprotein-related (LRP) receptor (34) and the VLDL receptor (35). Active PAI-1 in plasma can also bind to vitronectin (36), which actually stabilizes PAI-1 in the active conformation. The relative abundance of vitronectin in the subendothelial matrix provides a mechanism for preserving PAI-1 activity, and it is likely that vitronectin-bound PAI-1 represents the physiologically relevant form of the inhibitor in the extracellular matrix (37).

A number of cytokines have been found to stimulate endothelial PAI-1 production. PAI-1 is classified as an acute phase reactant, and pro-inflammatory cytokines such as interleukin-1 (38) and tumor necrosis factor α (TNFα) (39) can induce PAI-1 production. These same factors are thought to promote vascular inflammation and atherosclerosis. Growth factors and hormones also regulate PAI-1 production, including transforming growth factor β (TGFβ)], angiotensin-II, estrogen, thrombin, and insulin (31). Of these factors, insulin is of particular importance since it plays an important

role in hyperinsulinemic conditions associated with T2DM. The role of insulin in PAI-1 gene expression is discussed in the later sections of this thesis.

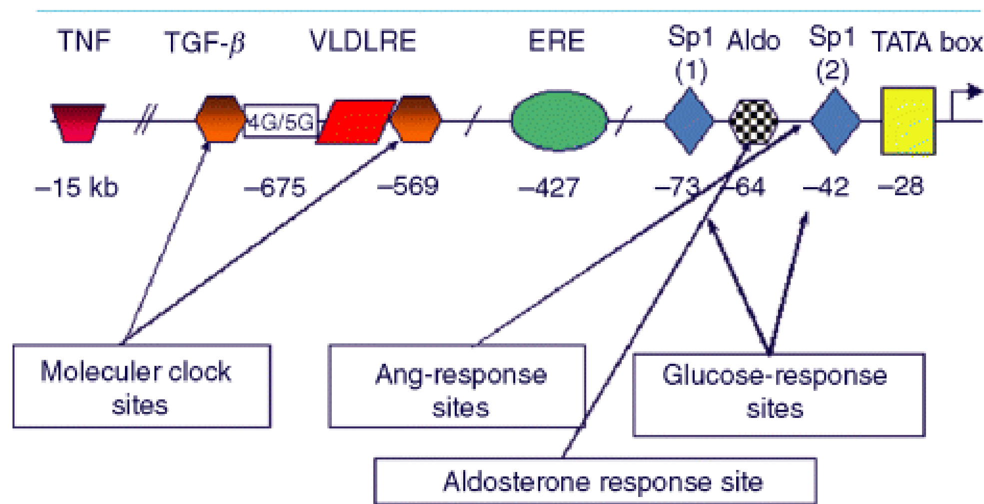

Fig. 2.11. Schematic localization of the promoter region of the human PAI-1 gene

The gene for PAI-1 is located on chromosome seven, spans approximately 12 kb, and is composed of nine exons and eight introns (40). The PAI-1 promoter has been exhaustively characterized, yielding numerous insights into the molecular physiology of PAI-1. Some of the most important regulatory elements have been identified in the PAI-1 gene (Fig. 2.7), including a glucocorticoid response element that also mediates aldosterone responsiveness, a VLDL response site adjacent to a common diallelic polymorphism at −675, and two Sp1 sites that appear to mediate glucose and glucosamine responsiveness (33). Using cross-species sequence homology analysis and DNase I hypersensitive site analysis, a TNF-responsive enhancer element was located approximately 15 kb upstream of the transcription start site (41). This region contains a conserved NFκB binding site that mediates the response to TNFα and is capable of binding NFκB subunit p50 and p65 *in vitro*.

Pathological role of PAI-1

PAI-1 and vascular diseases

There is substantial experimental and epidemiological evidence that PAI-1 might contribute to the development of ischaemic cardiovascular disease (42). There is also experimental evidence that these epidemiological links are more than just a casual association. Transgenic mice that overexpress a stable form of human PAI-1 driven by the murine preproendothelin promoter develop spontaneous macrovascular coronary thrombosis and subendocardial myocardial infarction in the absence of hyperlipidemia or hypertension (43). These animals have essentially no tissue plasminogen activator (t-PA) activity in plasma, and also exhibit significant reductions in plasma levels of activated protein C. Thus, the spontaneous coronary thrombosis seen in these transgenic mice appears to be explained by the simultaneous loss of PA function and reduced protein C activity, the two critical pathways implicated in the defense against clotting in the coronary circulation (44).

Apart from its role in thrombosis, PAI-1 also plays an important role in vascular and tissue remodeling. Several groups have reported excess PAI-1 in atherosclerotic plaques in humans, a finding that is exaggerated in patients with T2DM (45). Metabolic derangements alone are sufficient to increase the arterial vascular content of PAI-1 even in the absence of atherosclerosis, as described in internal mammary artery sections obtained from patients with T2DM undergoing coronary bypass grafting (46). In tissues where PAI-1 is overproduced, local PA is impaired, which in turn has profound effects on vascular housekeeping and remodeling capacity. Indeed, it has been shown that PAI-1 deficiency effectively prevents the development of arteriosclerosis and hypertension in mice treated with the nitric oxide synthase inhibitor L-NAME for periods of 8–16 weeks (47). It seems that the increased vascular PAI-1 production and accumulation plays

a major role in the arterial remodeling that contributes to the development of hypertension in obesity and the metabolic syndrome

PAI-1 and cancer

The PA system containing serine proteases and their inhibitors, as well as a number of metalloproteases, is involved in extracellular proteolysis and tissue remodeling. Changes in the expression of components of these systems might reflect uncontrolled tissue remodeling and therefore promote cell invasion, cell migration, and proliferation (48). PAI-1 may contribute to the degradation of the extracellular matrix during cancer invasion by decreasing the adhesive strength of cells on their substratum, and detaching cells from extracellular matrices by inactivating integrins (49). A binding site for p53 in the region of the PAI-1 promoter leading to PAI-1 transcription has been identified (50). Strong expression of PAI-1 in tumor cells like ductal carcinoma in situ cells (51) or tumor tissue of squamous cell cancer (52) has been found while angiogenesis and tumor invasion of specific cancer cell lines are strongly reduced in PAI-1-deficient mice (53). Myofibroblasts located at the invasive front of colon cancer and malignant melanoma cells have been found to produce significantly higher amounts of PAI-1 than normal surrounding tissue (54). Inhibition of PAI-1 activity by antibodies or modulators, on the other hand, has been shown to suppress tumor cell invasion and angiogenesis in vitro (55). Elevated levels of both u-PA and PAI-1 are associated with poor prognosis in a variety of solid tumors. The elevation of u-PA and PAI-1 have been shown to have a significant impact on relapse-free and overall survival in patients with primary breast cancer (56). In addition, high levels of PAI-1 seem to be associated with preferential response to adjuvant chemotherapy, but relatively high resistance to hormone therapy in these patients (57). Expression of PAI-1 protein in tumor cells has also been described as a strong independent prognostic factor in cervical cancer (58) and in non-small cell lung carcinoma (59), and is also associated with a higher risk of relapse in squamous cell cancer of the oral cavity.

PAI-1 and type-2 diabetes

Diabetes is a chronic metabolic disorder characterized by poor blood glucose control due to insulin deficiency (type 1) and/or insulin resistance (type-2) (60). Glucose is the primary fuel of cells. In healthy individuals, two principal glucose-regulating hormones, insulin and glucagon, maintain a constant glucose concentration in both the fasting and post-meal (post-prandial) state. When blood glucose levels are high, such as after eating a meal, insulin is released. Produced by cells in the pancreas, called beta cells, it acts to encourage the uptake, utilization, and storage of glucose in muscle and fat tissues (adipose tissue), but mainly in the liver. During fasting, insulin output falls and a counter regulatory enzyme, glucagon, is released. Also produced in the pancreas, but by alpha cells, glucagon stimulates the release of glucose into the blood from the liver by breaking down glucose stores (called glycogen) and converting other fuel sources such as fats and proteins. In a healthy body, blood glucose levels rise and fall within a fairly tight range of 70-110 mg/dl. However, with diabetic insulin deficiency and/or resistance, blood glucose can rise to substantially higher concentrations, resulting in hyperglycaemia and, in the long term, causing blindness and damage to the kidney and heart, among other organs. There are two types of diabetes mellitus:

A) In type 1 diabetes, or insulin-dependent diabetes, there is an absolute shortage of insulin resulting from destruction of the insulin-producing beta cells by the patient’s own immune system. Accounting for roughly 10% of cases, type 1 diabetes sufferers are typically young. Given type 1 patients’ inability to produce insulin, treatment inevitably includes supply of exogenous insulin.

B) Type 2 diabetes accounts for roughly 90% of cases and occurs predominantly in people over 40. In over 60% of reported cases, patients are overweight. The disease is most often characterized by a resistance of the peripheral tissues to insulin, and impaired regulation of insulin secretion. Consequently, type 2 diabetes is also known as non-insulin dependent

diabetes mellitus (NIDDM); that is, insulin is produced but the body's insulin receptors are insufficiently sensitive to its presence. As a result, the insulin-producing beta cells over-compensate for this poor sensitivity, with the frequent result that, over time, they 'burn out'. Hence, insulin production gradually deteriorates, with around 30% of patients eventually becoming insulin-dependent.

In line with the observation by us and others that PAI-1 can induce insulin resistance by inhibiting the interaction between vitronectin and $\alpha v\beta 3$ integrin (61, 62), it has recently been show that PAI-1 knock-out mice are resistant to high-fat-diet-induced insulin resistance (63, 64), indicating a role for PAI-1 in the pathogenesis of obesity-driven insulin resistance and hence T2DM. Moreover, levels of plasma PAI-1 have been shown to be a prognostic marker of T2DM independent of insulin resistance and other known risk factors for diabetes (65). In addition, plasma PAI-1 levels are elevated throughout the spectrum of insulin resistance, from the metabolic syndrome to prediabetes (period of impaired glucose tolerance) and to diabetes (66) (67) (68). Indeed, strategies that have been demonstrated to prevent diabetes, such as diet and exercise or administration of metformin or thiazolidinediones (TZDs) have also been shown to decrease plasma PAI-1 concentrations (69) (70) (71) (72) (73).

2.4 E2F transcription factor

The E2F transcription factor is one of the key regulators of cell cycle events at the boundary of G0/G1 and S phases. E2F was originally defined as a cellular factor required for the adenovirus early region 1A (E1A)-transforming protein to mediate the transcriptional activation of the viral E2A promoter (74). The E2F consensus binding site is "TTTCGCGC," present in the adenovirus E2A promoter (74), and predominantly within the promoters of cellular genes required for cell division and apoptosis (75). Identified E2F target genes include cell cycle regulators such as cyclin E, cyclin A, cyclin

D1, Cdc2, and Cdc25A, enzymes involved in DNA synthesis such as dihydrofolate reductase (DHFR), DNA polymerase α, and thymidine kinase, and proteins essential for DNA replication, including Cdc6, ORC1, and the minichromosome maintenance (MCM) proteins (75). Apoptotic E2F target genes include apoptosis protease-activating factor 1 (Apaf1), p73, and ARF (76).

E2F family

In cells, E2F activity arises from a family of heterodimeric transcription factors, where each heterodimer consists of one member of the E2F family bound to a member of the DP family (75). In mammalian cells, six E2F family members have been identified (E2F-1 to E2F-6), while two members of the DP family, DP-1 and DP-2, have been characterized (Fig. 2.12). All possible combinations of E2F/DP complexes can exist in vitro, potentially allowing for the formation of an array of E2F complexes in cells (75).

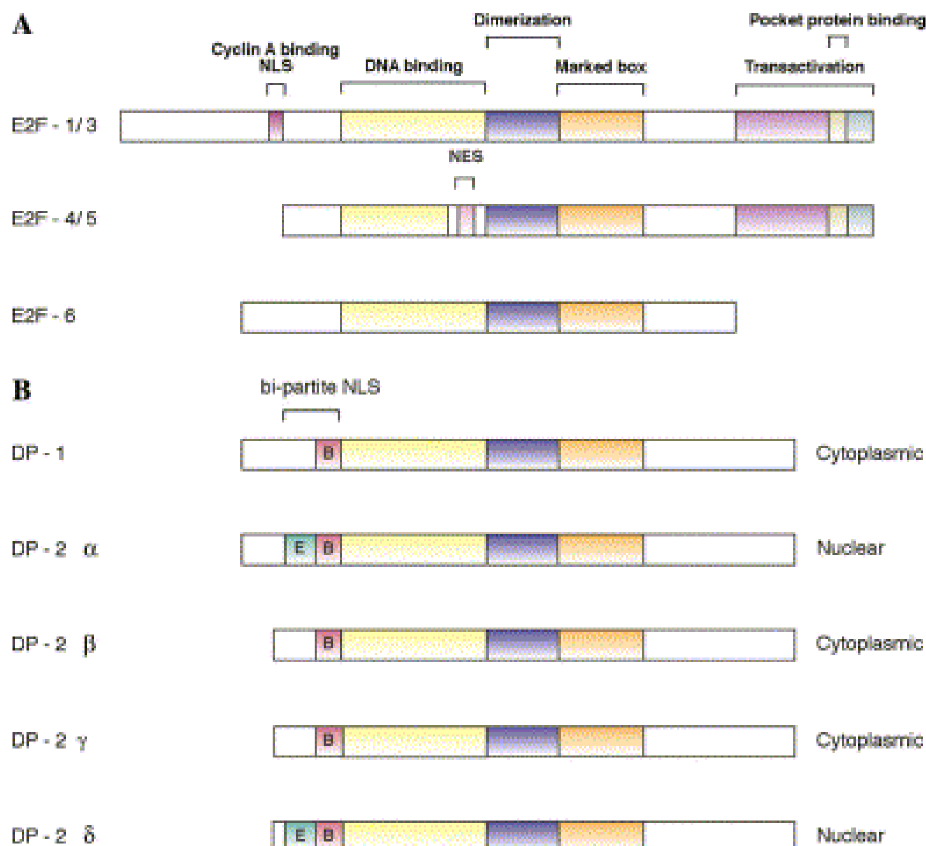

Fig. 2.12. Structural comparison of E2F and DP family members. *(A) The E2F family can be divided into three subgroups based on sequence homology. E2F-1, E2F-2, and E2F-3 represent one subgroup; E2F-4 and E2F-5 represent a second subgroup; and E2F-6 represents a third subgroup. The E2F-1 subgroup has an N-terminal cyclin/cdk binding site and a nuclear localization signal (NLS). E2F-4 and E2F-5 have truncated N-terminal regions and lack a cyclin/cdk binding site and an NLS, instead possessing a nuclear export signal (NES). The positions of pocket protein binding and transactivation domains are indicated. (B) Summary of DP-1 and DP-2 family members. E2F and DP proteins share a conserved DNA binding and dimerization domain. Four alternatively spliced forms of DP-2, which are either cytoplasmic (β and γ) or nuclear (α and δ), have been described. The expression of specific DP proteins may influence E2F localization.*

The E2F family can be further divided into three subgroups based on sequence homology: E2F-1, E2F-2, and E2F-3 represent one subgroup; E2F-4 and E2F-5 represent a second subgroup; and E2F-6 is the third. Functional similarities are evident within each subgroup: E2F-1, E2F-2, and E2F-3 share an N-terminal cyclinA/cdk binding domain and a canonical basic nuclear localization signal (NLS), while both are absent in E2F-4 and E2F-5 (77). Moreover, E2F/DP heterodimers interact with pocket proteins with a specificity that is largely determined by the E2F component (75). E2F-1, E2F-2, and E2F-3 preferentially associate with pRb, whereas E2F-4 and E2F-5 predominantly interact with p107 and p130 (75). E2F-6 acts principally as a transcriptional repressor in a distinct pocket protein-independent manner (78). In fact, E2F-6 diverges considerably from the other E2F family members, sharing almost no homology outside the core DNA binding and dimerization domains, and possesses truncated C- and N-terminal regions relative to those of the other E2F subgroups (78).

E2F-1, E2F-2, and E2F-3

E2F-1, E2F-2, and E2F-3 activate E2F-responsive genes and drive cellular proliferation (79). Overexpression of each protein is sufficient to induce quiescent cells to re-enter the cell cycle (80-83), and dominant-negative mutants block S-phase entry (84). Furthermore, the combined ablation of E2F-1, E2F-2, and E2F-3 prevents entry into S phase (85), and their overexpression overrides the effects of growth inhibitory proteins, such as p16, p21, and p27, and cell cycle arrest induced by γ-irradiation, TGFβ, or dominant-negative cdk2 (82), (86). This ability is dependent on its dimerization, DNA-binding, and transactivation domains, suggesting that the induction of transcription of E2F target genes is required for S-phase entry (80, 87).

E2F can become inactivated through pocket protein binding, which prevents the expression of target genes. This inactivation is primarily caused by masking the E2F activation domain, since the pocket protein binding domain is integrated with the activation domain, thereby making E2F unable to communicate with the basal transcriptional machinery. Using in vitro transcription and DNase footprinting assays, pRb has been shown to hinder the assembly of the transcription initiation complex (88). In addition, pRb is able to mediate active repression of E2F targets through nucleating the assembly of a dominantly acting repressor complex (89, 90). Once targeted to the promoter by E2F, pRb recruits proteins endowed with chromatin-modifying activity, including HDACs, the histone methyltransferase (MTase) SUV39H1, human brahma (HBRM), and human brahma-related gene 1 (BRG1): human homologues of the yeast SWI2/SNF2 proteins that possess nucleosome-remodeling activities (86).

Contrary to the earlier belief that E2F1-3 are transcriptional activators and E2F4-5 are transcriptional repressors (91), our laboratory have previously demonstrated that E2F1-3 act as transcriptional repressors of the PAI-1 gene in a cell type-independent manner (92), (93). Subsequently, other

groups have shown repressive effects of E2F1 (94), E2F2 (95), and E2F3 (96) on other target genes.

Role of E2F in differentiation

E2F family members have also been implicated in the regulation of differentiation. A recent study demonstrated that repression of E2F-1/DP-1-dependent transcription by the C/EBPα transcription factor is essential for cell cycle exit and the differentiation of adipocytes and neutrophil granulocytes in vivo (97). The importance of repressing E2F activity for the initiation of differentiation has also been observed in myogenic cells and in keratinocytes (98, 99), and loss of E2F repression through Rb mutation leads to uncontrolled proliferation and apoptosis in vivo (100, 101). While this highlights the role of both Rb and C/EBPα in the repression of E2F activity during development, C/EBPα can repress E2F in the absence of functional pRb protein (102, 103).This indicates that the C/EBPα and pRb pathways of E2F repression act independently. Moreover, in differentiating granulocytic cells p130, rather than pRb, has been found to be the critical pocket protein (103, 104), indicating that, in addition to E2F-1, repression of E2F4-5 is critical for proper differentiation of granulocytes (97).

The differentiation of preadipocytes into adipocytes requires that growth-arrested preadipocytes re-enter the cell cycle before undergoing terminal differentiation (105). In confluent preadipocytes, the E2F-4/p130 complex acts to repress the transcription of E2F target genes, including PPARγ, the master regulator of adipogenesis. Hormonal stimulation results in the loss of this repressive complex and the induction of activating E2Fs, particularly E2F-1. E2F-1 can directly activate PPARγ transcription, and thereby activate differentiation. Interestingly, during the late stages of differentiation the E2F-4/p130 complex reforms, possibly to switch off PPARγ transcription in terminally differentiated adipocytes.

2.5 References for general introduction

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3. Research objective

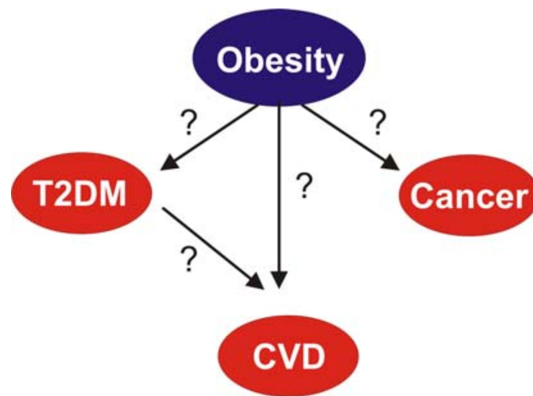

Fig. 3.1. Obesity and its secondary disorders. *Obesity is known to lead to type-2 diabetes mellitus (T2DM), cardiovascular disorders (CVDs), and certain types of cancer. T2DM is also known to independently contribute to the development of CVD.*

The signaling defects that underlie the secondary complications of obesity, such as diabetes and cardiovascular complications, are well characterized. **The 'molecular signatures' of diabetes and diabesity-induced cardiovascular complications are insulin resistance and inhibition of the glycolytic pathway, respectively**. Now that the strong link between obesity and its secondary complications are well recognized, this leads to the key question of how does obesity lead to these signal transduction defects? Numerous molecules, including TNF-α, PAI-1, adiponectin, resistin, leptin, klotho, PPARγ, RBP4, MCP-1, IL-6, IL-1β, JNK, and superoxide anions, etc. have been reported to contribute to the obesity-driven pathologies. Although their levels are altered in obesity, none of these factors can account for all of the diverse signaling defects, nor has restoring the physiological status of any one of these factors been shown to be sufficient to prevent (although it may retard) secondary disorders in the obese. Taken together, it appears that obesity leads to multiple signaling disorders, although no convincing unifying mechanism as to how this happens has been proposed.

In the <u>first</u> and <u>second</u> part of this study, we looked for nodal signal transduction points, whose potential dysfunction under conditions of obesity can activate pathological signaling implicated in T2DM and

cardiovascular complications. In the third part of our study, we studied the physiological regulation and pharmacological modulation of a common downstream mediator of these signal transduction defects.

PART 1

4. Perturbation of caveolae leads to insulin resistance and PAI-1 upregulation

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Abbreviations: PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; S6K1, p70 ribosomal S6 kinase 1; pRB, retinoblastoma protein; E2F, E2 (adenoviral protein) factor; MBCD, methyl-β-cyclodextrin; IRS, insulin receptor substrate; Erk, extracellular regulated kinase

4.1 Specific research objectives

We looked for a unifying mechanism via which obesity would lead to the characteristic signaling defects that are associated with obesity-driven secondary disorders.

It has been shown that obesity is accompanied by the reduction of two essential components of caveolae: cholesterol and caveolin-1/3. In this section, we investigated whether perturbation of a caveolar signal transduction platform would upset the physiological signaling and could lead to insulin resistance, the central feature of T2DM. We also sought to look at how insulin resistance regulates the gene expression of PAI-1, a critical mediator of cardiovascular complications.

4.2 Abstract

The levels of three integral components of caveolar microdomains, caveolin-1, caveolin-3, and plasma membrane cholesterol, are reduced during conditions of obesity. Here we show that perturbation of caveolar microdomains leads to insulin resistance in cultured 3T3L1 adipocytes. This was accompanied by the upregulation of PAI-1 in a manner that correlates with the degree of insulin resistance. We present several lines of evidence showing that the phosphatidylinositol 3-kinase (PI3K) pathway negatively regulates PAI-1 gene expression. Insulin-induced PAI-1 gene expression is upregulated by a specific inhibitor of PI3K. In addition, serum PAI-1 levels are elevated in protein kinase Bα (PKBα)-deficient mice, while they are reduced in p70 ribosomal S6 kinase 1 (S6K1)-deficient mice. Using cell-penetrating peptides that are homologous to the pRB binding region of E2F-1, we could disrupt pRB-E2F interaction, and thereby release free E2F, a known transcriptional repressor of PAI-1. These peptides were able to suppress PAI-1 levels that are elevated during insulin-resistant conditions. This study identifies a caveolar-dependent signal pathway that upregulates

PAI-1 in insulin-resistant adipocytes, and proposes a novel pharmacological paradigm of disrupting pRB-E2F interaction to suppress PAI-1 levels.

4.3 Introduction

Type-2 diabetes mellitus (T2DM) is characterized by insulin resistance, where the insulin receptor fails to elicit the metabolic signaling that is required for glucose metabolism and energy homeostasis. In insulin-sensitive tissues, the insulin receptor transduces two main signaling cascades: a metabolic signaling that is responsible for glucose uptake and glycogen synthesis; and a mitogenic signaling that is responsible for cell proliferation and growth. The IRS-PI3K-PKB and Cbl-CAP-Flotillin pathways represent the major metabolic signaling, while the Shc-Ras-Erk pathway represents the major mitogenic signaling (1). Both in animal models and clinical T2DM subjects, a selective impairment of metabolic signaling has been observed, while mitogenic signaling is more or less unaffected (2-4).

Obesity is prominent among the plethora of factors that leads to the development of T2DM, although the molecular mechanism underlying the pathogenesis of obesity-driven T2DM is not well understood. Comparative analysis of large and small fat cells within the same fat pad reveals a two-fold reduction in the levels of plasma membrane cholesterol in large fat cells, suggesting that a decrease in membrane cholesterol is characteristic of adipocyte hypertrophy per se (5). Recently, it has been proposed that the protein levels of caveolin-1 and caveolin-3 are inversely correlated to the body mass index (6). Plasma membrane cholesterol (7) and caveolins (8) are indispensable for the structural and functional integrity of caveolar microdomains. We therefore reasoned that obesity might lead to caveolar dysfunction. Since the insulin receptor and several of its downstream signal transducers are localized in caveolae (9), it was intriguing to investigate whether caveolar dysfunction could lead to insulin resistance.

There is compelling evidence that plasminogen activator inhibitor-1 (PAI-1), whose levels are elevated in both obesity and T2DM, plays an important role in the development of cardiovascular disorders (10). PAI-1, a primary physiological inhibitor of plasminogen activators (uPA and tPA), inhibits both fibrinolysis and proteolysis, and plays an important role in mediating the cardiovascular complications associated with T2DM, such as nephropathy (11), retinopathy (12), coronary artery disorders (13), and hypertension (14). Consequently, it is believed that normalizing plasma PAI-1 levels will retard the progression of cardiovascular complications (15). Insulin-induced Erk phosphorylation, followed by the activation of transcription factors of the AP-1 family, is considered to be partly, if not wholly, responsible for the insulin-induced PAI-1 upregulation in insulin-sensitive tissues (16, 17). However, because the mitogenic MAP kinase pathway is not affected during hyperinsulinemic conditions, such as insulin resistance or T2DM, this pathway is unlikely to be the primary cause of PAI-1 elevation during these pathological conditions. It is, therefore, intriguing to investigate whether insulin induction of PAI-1 during T2DM can be explained by directly linking the compromised PI3K pathway to PAI-1 upregulation. Interestingly, it has been shown that insulin receptor-mediated activation of metabolic signaling (PI3K pathway) induces the phosphorylation of retinoblastoma protein (pRB) in adipocytes (18). pRB phosphorylation is known to lead to the release of free E2F (19). We have previously demonstrated that E2F transcription factors can negatively regulate PAI-1 gene expression by repressing PAI-1 promoter activity independently of its binding to pocket proteins, revealing a novel mechanism for the E2F-mediated repression of gene expression (20). In this study, we investigate whether caveolar dysfunction can lead to insulin resistance, and if the resulting impairment of the PI3K-PKB-E2F pathway can by itself lead to the upregulation of PAI-1. We also explore the pharmacological disruption of the E2F-pRB interaction in order to release free E2F, which we hypothesize will attenuate PAI-1 transcription and hence its plasma level. Adipocytes are chosen for this study as they are responsible for obesity, abundant in caveolae (21), highly sensitive to insulin

(over 200,000 receptors per cell) (22), the primary site for insulin resistance (23), and a major contributor of plasma PAI-1 in the obese (24).

4.4 Materials and methods

Reagents. MBCD and filipin III were obtained from Sigma. Monoclonal antibodies against E2F1 (KH-95) and E2F2 (TFE-25), and rabbit polyclonal antibodies against E2F3 (C-18), E2F4 (C-20), E2F5 (C-20), pRB (M-153), p130 (C-20), p107 (C-18), IRS-1 (C-20), Erk, and PAI-1 (H-135) were from Santa Cruz Biotechnology. Rabbit polyclonal antibodies against phospho-pRB (Ser-795) and phospho-Erk were from Cell Signaling. Rabbit polyclonal antibodies against Shc and phospho-tyrosine (G410) were from Transduction Laboratories. Sheep polyclonal antibody against PAI-1 was from American Diagnostics. All reagents for real-time PCR were from Applied Biosystems. The oligonucleotide E2pro, the sequence of which corresponded to nucleotides -72 to -32 of the adenovirus E2 promoter and contained E2F binding sites, had the following sequence (only the upper strand is given) and was used for gel-shift assays: 5'-GAT CAG TTT TCG CGC TTA AAT TTG AGA AAG GGC GCG AAA CTA G-3'.

Adipocyte differentiation. 3T3-L1 preadipocytes were cultured in DMEM containing 10% FCS, and 2 days after cells reached confluency the medium was changed to DMEM containing 10% FCS, 10 μg/ml insulin, 1 μM dexamethasone, and 0.5 mM isobutylmethylxanthine. 2-3 days later, this medium was replaced with DMEM supplemented only with 10 μg/ml insulin and cells were kept for 2 days. The medium was then replaced with DMEM containing 10% FCS every 2 days. The cells were serum starved overnight prior to experiments.

Immunoprecipitation and Western blotting. Immunoprecipitation and Western blotting were performed as previously described (25).

Glucose uptake assay. Measurements of 2-deoxyglucose uptake into adipocytes were carried out as previously described (26).

RNA isolation and Northern blot analysis. Total RNA (12 μg) was isolated and subjected to Northern blot analysis as described (27). The cDNA clone for mouse PAI-1 was provided by A. Riccio (University of Naples).

Quantitative real-time PCR. One microgram of total RNA was reverse transcribed and 1 ul RT reaction was added to 24 ul PAI-1 PCR reaction (1×universal master mix, 900 nM forward primer [5'-CCTGGCCGACTTCACAAGTC-3'], 900 nM reverse primer [5’-TTGCAGTGCCTGTGCTACAGA-3'] and 200 nM TaqMan probe [5'-FAM-TCCGACCAAGAGC-MGB-3']). Thermal cycling was done as follows: 50°C for 2 min, followed by 95°C for 10 min, and then 45 cycles of 95°C for 1 min and 60°C for 1 min. The fluorophor dyes for the PAI-1 probe and 18S rRNA (internal control) probe were FAM and VIC, respectively. The quencher in both probes was TAMRA. The reaction was carried out in an ABI Prism 7700. The output raw data was normalized with internal control, and statistically analyzed using MS Excel.

siRNA nucleofection. siRNA used for targeting caveolin-1 mRNA has the following sequence: sense, 5'-GAGCUUCCUGAUUGAGAUU-3' and antisense, 5'-AAUCUCAAUCAGGAAGCUC-3'. Control siRNA sequences: sense, 5'-GUACCUGACUAGUCGCAGAAG-3' and antisense, 5'-UCUGCGACUAGUCAGGUACGG-3'. These sequences contain 3' UU overhangs. Specificities of these sequences were confirmed by performing a BLAST search against the GenBank/EMBL database. Each siRNA (final concentration 1 μM) was mixed with NIH-IR cell suspension (2x106 cells in 0.1 ml buffer-T/transfection), transferred to a 2-mm electroporation cuvette, and electroporated using an Amaxa Nucleofector™ (Amaxa, Germany) using program A-23. After electroporation, cells were immediately transferred to 1 ml growth medium, and cultured in 6-well plates at 37°C until analysis.

S6K1-/- and PKBβ-/- mice. PKBα knock-out mice were generated as previously described (28). S6K1 knock-out mice (29) were kindly provided by G. Thomas (FMI, Switzerland). The S6K1-/- mice and their wildtype counterparts were fed with a high-fat diet for 5-6 months (required for hyperactivity of the PI3K pathway) before their blood was taken using tail punctures. PKBα-/- mice were fed with normal chow diet.

Cell-penetrating peptide treatment. The sequence of the interfering peptide of 18 aa-length was derived from the pRB-binding region of E2F1 (aa 402-419: LDYHFGLEEGEGIRDLFD) (30). A control peptide with the same amino acid composition, GEELEGFHDGLLDFDIR, was prepared by randomly shuffling the sequence of this peptide. Cell-penetrating peptides were prepared by coupling these peptides to the carboxyl terminal of the cell-penetrating region of the HIV tat protein (aa 47-57: RRRQRRKKR) (31) via hinge peptide G. Differentiated adipocytes were separated from undifferentiated cells using a Percoll density gradient as previously described (32), with a slight modification. Adipocytes were pre-treated with collagenase (2 mg/ml) for 30 min, centrifuged at 1,500 rpm for 5 min at 4°C, and mixed with Percoll solution (1.025 g/ml) to form a homogenous suspension. The cell suspension was then layered on preformed Percoll solution (1.035 g/ml) and centrifuged at 3,000 rpm for 20 min at 4°C. The cells collected from the upper layer were resuspended in media and reseeded for the experiment. The penetrating peptide was then added to cells, incubated for 16 h, and then subjected to various treatments.

Nuclear extracts and electromobility shift assays. Nuclear extracts (5 μg) were first incubated at room temperature for 15 min in 20 μl binding reaction mixture containing 50 mM KCl, 20 mM HEPES (pH 7.9), 0.2 mM EDTA, 6% glycerol, 0.5% Ficoll 400, 1 μg salmon sperm DNA, 6 μg bovine serum albumin, and 1 mM DTT with or without penetrating peptide and antibodies, followed by a further 15-min incubation after addition of 0.3 ng radiolabeled oligonucleotide probes. Oligonucleotide probes were radiolabeled using *E. coli* polynucleotide kinase and [γ-32P]ATP. Aliquots (5 μl) of reaction mixture

were separated in a 4.5% polyacrylamide gel run in 0.25×TBE buffer at room temperature. The gel was dried and analyzed in a PhosphorImager.

4.5 Results

Perturbation of caveolar function mimics insulin resistance in 3T3L1 adipocytes

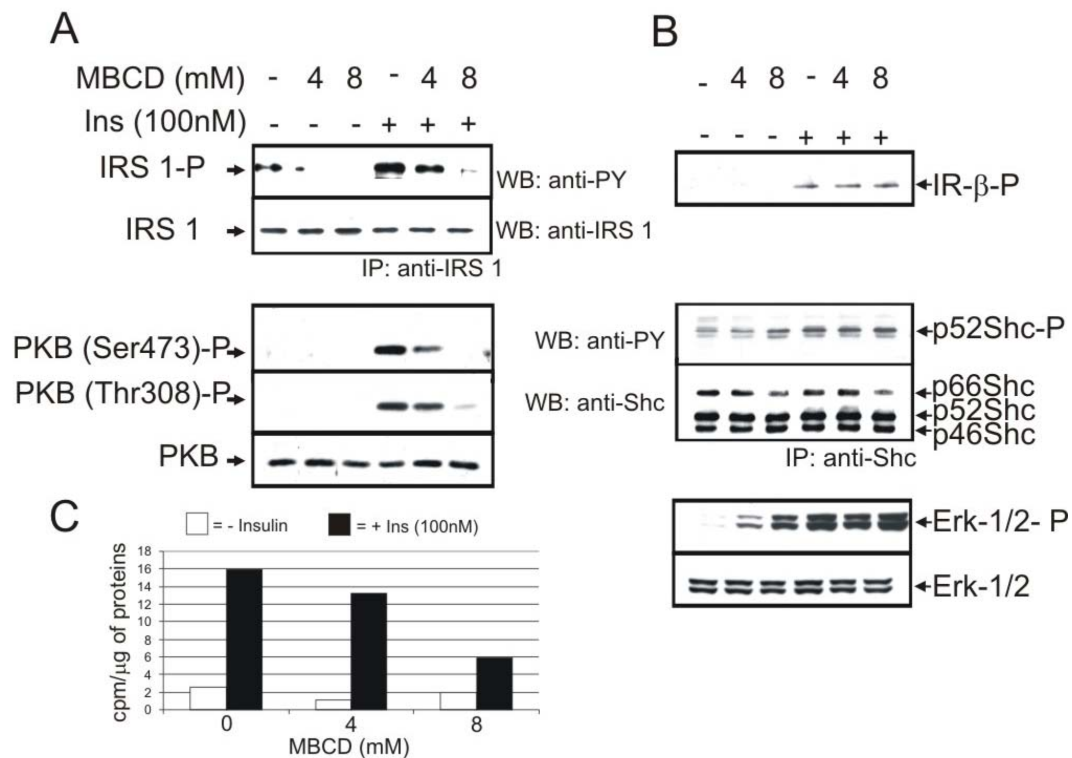


__Fig. 4.1. Effect of cholesterol depletion on insulin signaling.__ 3T3L1 adipocytes were treated with 0, 4 or 8 mM MBCD for 40 min, followed by insulin treatment (100 nM) for 10 min, and then whole cell extracts were prepared. The cell lysates were fractionated by SDS-PAGE, followed by Western blotting analysis using the specific antibodies indicated. For the analysis of IRS-1 and Shc, the lysates were first immunoprecipitated using IRS-1 and Shc antibodies, respectively, before Western blotting. (A) Analysis of PKB and IRS-1, molecules involved in the metabolic signal pathway. (B) Analysis of the insulin receptor (IR) and its mitogenic signal transducers Shc and Erk. (C) Effect of MBCD on insulin-induced glucose uptake. Adipocytes were treated similarly as above and then subjected to a glucose uptake

assay. All data shown here are representative of at least three independent experiments.

To find out if caveolar dysfunction can cause insulin resistance, we perturbed the integrity of caveolar microdomains and examined the subsequent effects on insulin signaling. Cholesterol depletion using methyl-β cyclodextrin (MBCD), a reagent widely used to perturb the structural integrity of caveolae, was employed. MBCD pretreatment dose-dependently inhibited insulin-induced IRS-1 phosphorylation, PKB phosphorylation (Fig. 1A), and 2-deoxyglucose uptake (Fig. 1C). On the other hand, MBCD pretreatment did not affect insulin-induced phosphorylation of the β-subunit of the insulin receptor, Shc, or Erk (Fig. 4.1B). MBCD treatment alone (in the absence of insulin) was also found to increase the phosphorylation of p52 Shc and Erk-1/2, although to a lesser extent (Fig. 4.1B).

Induction of insulin resistance leads to concomitant increase in PAI-1 gene expression

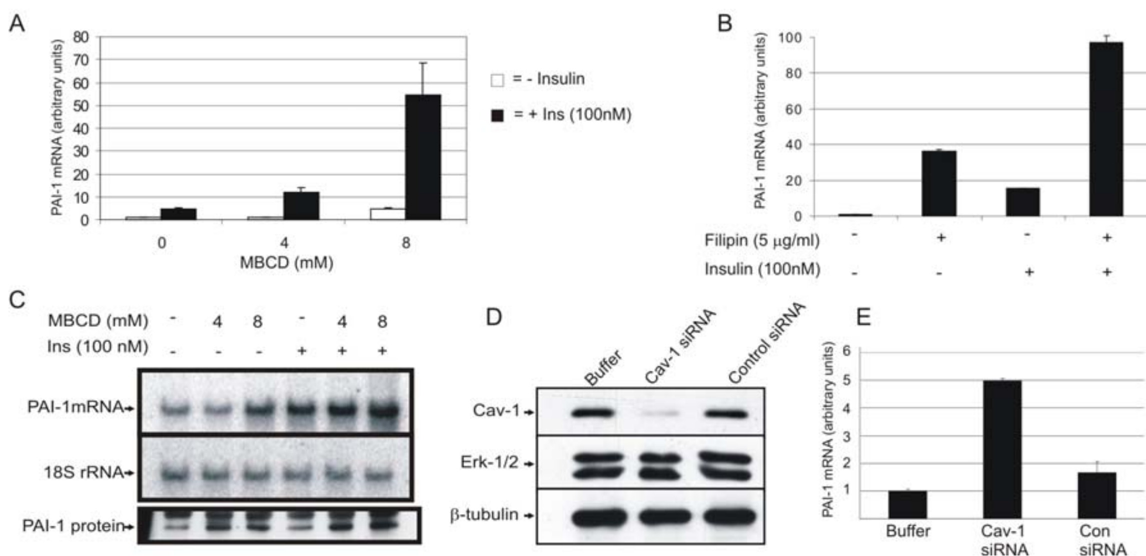


Fig. 4.2. Effect of caveolar dysfunction on insulin-induced PAI-1 mRNA levels. *(A) Effect of MBCD on insulin-induced PAI-1 mRNA levels. 3T3L1 adipocyte cells were treated with 0, 4, or 8 mM MBCD for 40 min, with or without subsequent insulin treatment (100 nM) for 2 h. The total RNA was prepared and PAI-1 mRNA levels were measured using real-time PCR with*

18S rRNA as an internal control. (B) Effect of filipin on insulin-induced PAI-1 mRNA levels. Adipocytes were treated with filipin (5 μg/ml) for 40 min, with or without subsequent 2 h insulin (100 nM) treatment. Total RNA was analysed as above. (C) Northern and Western blotting analysis. The RNA samples used in Fig. 4.2A were subjected to Northern blot hybridization analysis for PAI-1 mRNA levels. For the analysis of PAI-1 protein levels, cells were treated with 0, 4, or 8 mM MBCD for 40 min, followed by insulin (100 nM) treatment for 6 h. Then, PAI-1 protein was immunoprecipitated from the conditioned media (as PAI-1 is a secreted protein) using protein-G beads coupled to sheep anti-PAI-1 antibodies, and analyzed by Western blotting using rabbit anti-PAI-1 antibodies. (D, E) Effects of caveolin downregulation. NIH-IR cells were electroporated using buffer without siRNA, or with Cav-1 or control siRNA. After 24 h, the cells were treated with insulin (100 nM) for 2 h. Protein levels of caveolin-1, Erk, and β-tubulin (D) and mRNA levels of PAI-1 (E) were examined by Western blotting and RT-PCR, respectively.

To determine whether MBCD-induced insulin resistance leads to the upregulation of insulin-induced PAI-1 gene expression, we measured PAI-1 mRNA levels after insulin treatment with or without MBCD pretreatment. Results from both real-time PCR (Fig. 4.2A) and Northern blot hybridization (Fig. 4.2C) show that MBCD dose-dependently increased insulin-induced PAI-1 levels. To find out if this increase at the mRNA level is reflected at the protein level, PAI-1 protein levels were measured in the media. Corresponding to the mRNA levels, insulin-induced PAI-1 protein levels were dose-dependently upregulated by MBCD treatment. Filipin, a structurally distinct sterol-binding compound also augmented the insulin-induced PAI-1 levels (Fig. 4.2B), suggesting that the observed MBCD effects were through perturbation of caveolae function per se. Figs. 2A and 2B clearly show that the effect of cholesterol depletion synergistically upregulated insulin-induced PAI-1 gene expression. To ascertain that the upregulation of PAI-1 observed here is due to caveolar dysfunction and not due to an unspecific effect of cholesterol depletion, we depleted the

caveolin-1 protein using siRNA directed against caveolin-1 mRNA. In this experiment, NIH3T3 cells over-expressing the human insulin receptor (NIH-IR) were used instead of adipocytes, which proved difficult to transfect efficiently with siRNA. NIH-IR cells were chosen as the suitable alternative to adipocytes, as they are insulin-sensitive, rich in caveolae, share a common cell-lineage with adipocytes, and can be differentiated into adipocytes (33). This siRNA showed specific effects, as it significantly lowered the caveolin-1 protein levels but did not affect levels of other proteins such as Erk or β-tubulin (Fig. 4.2D). Furthermore, control siRNA had no effect on any of these proteins. Caveolin-1 siRNA treatment enhanced the levels of insulin-induced PAI-1 five-fold, whereas control siRNA had no significant effect (Fig. 4.2E).

Impairment of PI3K pathway leads to transcriptional upregulation of PAI-1

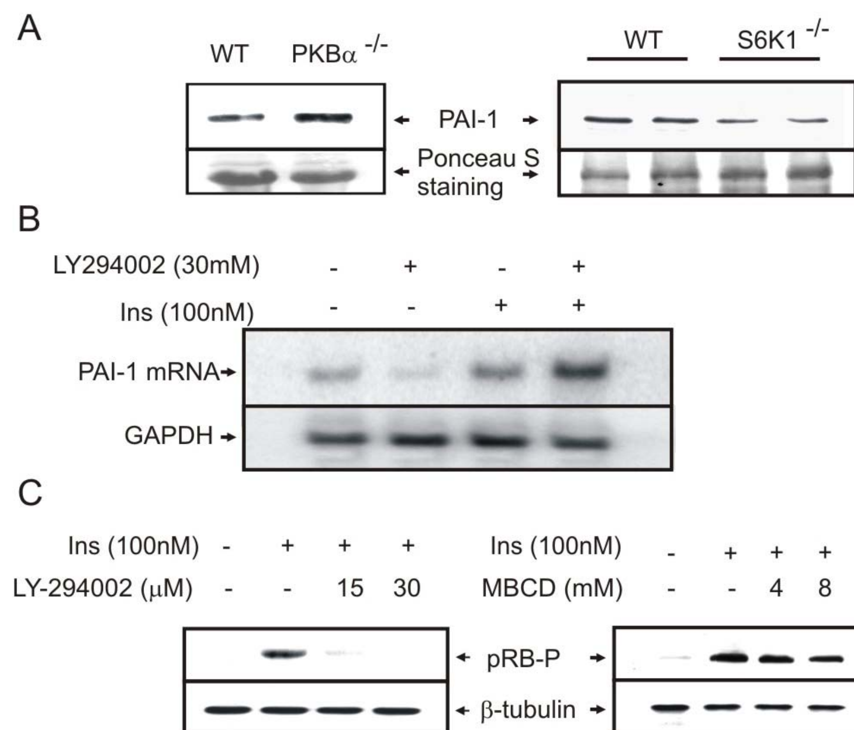


Fig. 4.3. The role of the PI3K pathway in PAI-1 gene expression. *(A) Serum PAI-1 levels of PKBα-/- and S6K1-/- mice, and their wildtype counterparts were analyzed by PAI-1 immunoprecipitation followed by*

Western blotting. The membranes were stained with Ponceau S for loading control. (B) 3T3L1 adipocytes were treated with LY294002 (30 μM) for 45 min, followed by insulin (100 nM) for 2 h. Total RNA was prepared and analyzed for levels of PAI-1 and GAPDH (loading control) mRNAs by Northern blot hybridization. (C) 3T3L1 adipocytes were treated with increasing concentrations of either LY294002 or MBCD for 45 min, followed by insulin (100 nM) for 2 h, and the phosphorylation status of pRB and the protein levels of β-tubulin (loading control) were measured by Western blotting.

To see if the upregulation of insulin-induced PAI-1 by caveolar dysfunction was a direct consequence of impaired metabolic signaling (PI3K pathway), we examined the levels of plasma PAI-1 in two different mouse models that are hallmarked by augmented (S6K1-/-) and attenuated (PKBα-/-) metabolic signaling. Activated S6K1 phosphorylates IRS-1 at serine residues and suppresses its tyrosine phosphorylation by the insulin receptor. Thus, deletion of S6K1 augments PI3K signaling in these mice (34). On the other hand, PKB is a critical mediator of PI3K signaling. Thus, deletion of PKB would attenuate the PI3K signal pathway. As shown in Fig. 4.3A, plasma PAI-1 levels were upregulated in PKBα-/- mice, while they were downregulated in S6K1-/- mice. The negative regulation of PAI-1 gene expression by the PI3K pathway was further confirmed in adipocyte cell culture, where the PI3K inhibitor (LY294002) enhanced insulin-induced PAI-1 mRNA levels (Fig. 4.3B). Treatment with LY294002 and MBCD, both shown to inhibit the PI3K pathway, dose-dependently inhibited insulin-induced pRB phosphorylation (Fig. 4.3C), thus providing a possible explanation of why PI3K pathway activation leads to the downregulation of PAI-1 gene expression (see below).

Disruption of pRB-E2F interaction using a cell-penetrating peptide

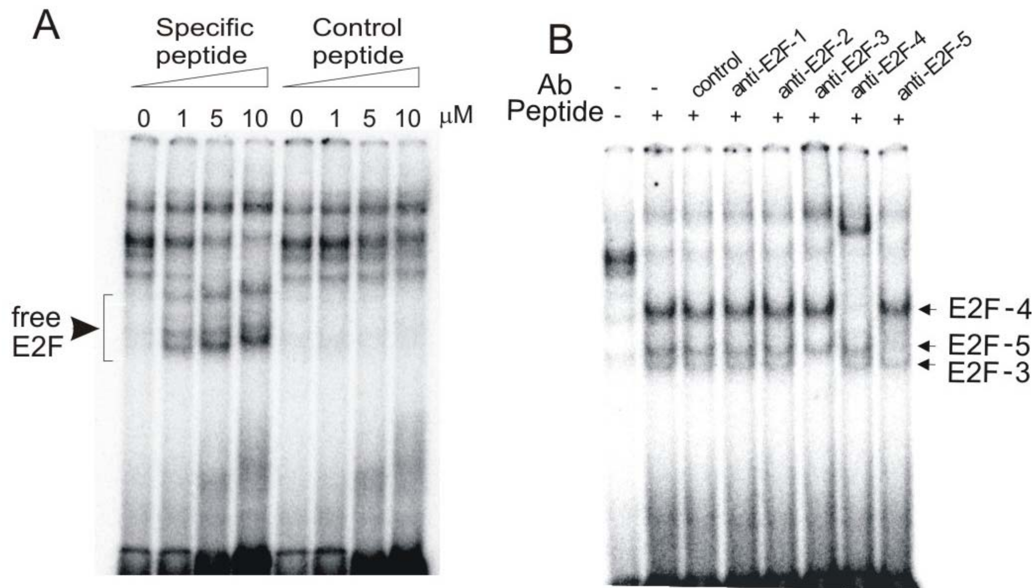


Fig. 4.4. Effect of cell-penetrating, interfering peptide on free E2F levels. *(A) In DNA gel-shift assays using ^{32}P-labelled E2pro oligonucleotide and nuclear extracts from adipocytes, cell-penetrating peptide (specific and control peptides) was added to binding reactions at increasing concentrations. (B) Nuclear extracts (day 8) were preincubated with the cell-penetrating, interfering peptide, together with specific antibodies against different E2F members, for 15 min, and then analyzed for E2F DNA-binding activity by gel-shift assays as above.*

Hypophosphorylated pRB is bound to E2F forming an inactive complex, while hyperphosphorylation of pRB leads to the release of free E2F (19). We have previously shown that overexpression of E2F isoforms can downregulate PAI-1 gene expression (20). It has also been shown that active pRB, which can bind to E2F, reverses this downregulation (27). Taken together, these results suggest that free E2F acts as a transcriptional repressor of the PAI-1 gene. We have observed that pRB phosphorylation is increased, while E2F-1 protein levels are reduced during adipogenesis (data not shown), suggesting that E2F activity (corresponding to free E2F) may be compromised in differentiated adipocytes. In order to restore the potential of E2F-mediated transcriptional repression of the PAI-1 gene in adipocytes, we sought to release free E2F by disrupting the E2F-pRB complex using an

interfering peptide that corresponds to amino acids 402-419 of E2F-1, the domain interacting with the pocket proteins (30). A BLAST search revealed that this domain is fairly conserved among members of the E2F family (excluding E2F-6) and, therefore, the peptide that we designed may disrupt the pocket protein-E2F interaction in general. As a control, we used a peptide with a randomly shuffled sequence. To render these peptides cell-penetrable, an HIV-1 Tat-derived peptide sequence was tagged to these peptides (31). Avidity of these peptides to disrupt the E2F-pRB complex was confirmed by DNA-gel shift assays using adipocyte nuclear extracts and a radioactive oligonucleotide, the sequence of which corresponds to the E2F-binding site of the adenovirus E2 promoter. As shown in Fig. 4.4A, DNA-protein complexes shifted to low-molecular-weight forms when increasing concentrations of the specific peptide were added to binding mixtures; the control peptide had no effect. Supershift assays using specific antibodies against E2F1-5 revealed that the main isoforms in the freed E2F fractions were E2F-3, -4, and -5 (Fig. 4.4B). These peptides thus serve as an effective tool to disrupt endogenous E2F-pRB interactions.

Elevation of PAI-1 levels in insulin-resistance is suppressed by the cell-penetrating peptide

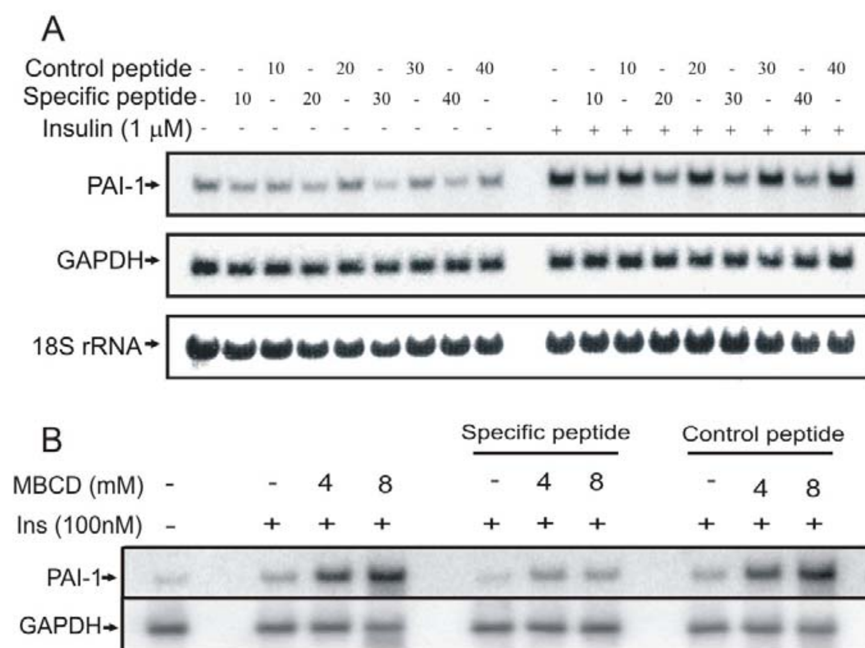


Fig. 4.5. Effect of cell-penetrating, interfering peptide on PAI-1 mRNA levels. *(A) Dose response of the peptides. 3T3L1 adipocytes (day 8) were treated with increasing concentrations (0-40 μM) of either specific or control peptide for 6 h, followed by treatment with or without 1 μM insulin for 2 h. Total RNA was prepared and levels of PAI-1 and GAPDH (loading control) mRNAs were measured by Northern blot hybridization. Ribosomal RNAs were stained with methylene blue to serve as an additional control for equal loading and blotting. (B) Effect of the peptides on PAI-1 mRNA levels, which are elevated under insulin-resistant conditions. Cells were serum starved and then treated with 40 μM of specific or control peptides for 12 h. Cells were then treated with MBCD for 40 min, followed by insulin (100 nM) for 2 h. Total RNA was prepared and analyzed for PAI-1 mRNA as above*.

To examine if interfering peptide treatment can compromise induction of PAI-1 by hyperinsulinemia and insulin resistance, we performed the following experiments. Adipocytes were prepared according to Materials and methods and were treated with either the specific or control peptide for 12 h, followed by 1 μM insulin for 2 h. As shown in Fig. 4.5A, the specific peptide, but not the control peptide, suppressed both basal and insulin-induced PAI-1 levels in a dose-dependent manner, the latter being more potently affected (Fig. 4.5A). Furthermore, the specific peptide also suppressed PAI-1 expression that was upregulated by MBCD-induced insulin resistance (Fig. 4.5B). Again, the control peptide had no effect.

4.6 Discussion

Obesity is characterized by increased adipocyte mass and altered adipocyte physiology. These traits contribute towards the progression from obesity to insulin resistance (35). Reductions in plasma membrane cholesterol and protein levels of caveolin-1/3 are associated with adipocyte hypertrophy and obesity, respectively (5, 6). It is thought that this could lead to caveolar

dysfunction, because plasma membrane cholesterol and caveolin are essential for the structural and functional integrity of caveolae (7, 8). To study the acute effects of caveolar dysfunction in adipocytes, we employed cholesterol-scavenging reagents, such as MBCD, which are known to perturb caveolae in adipocytes (36). Kinetic studies have suggested that the vast majority of cholesterol scavenged by cyclodextrins is from the plasma membrane (37), where more than 90% of cellular cholesterol is known to reside (38). Treatment with MBCD did not affect insulin-induced tyrosine phosphorylation of the insulin receptor and its downstream mitogenic signaling, but impaired the IRS-PKB signal pathway that leads to glucose uptake (Figs. 1A and B). This suggests that cholesterol depletion and, presumably, consequent caveolar dysfunction would lead to insulin resistance. Consistently, it was recently reported that caveolin-1$^{-/-}$ and caveolin-3$^{-/-}$ mice, both known to have a dramatic reduction in caveolae, exhibit insulin resistance (39, 40). Moreover, in adipocytes treated with TNFα, the insulin receptor accumulated less in the detergent-insoluble low-density membrane fractions (microdomains), and shifted to the high-density fractions, indicating a translocation of insulin receptor from caveolar to non-caveolar fractions (41). TNFα is a potent inducer of insulin resistance (42) and PAI-1 gene expression (43). It is chronically elevated in conditions of obesity and is a major culprit in the pathogenesis of obesity-driven insulin resistance (42). Thus, denying the caveolar platform for insulin receptor signaling could be a plausible mechanism through which obesity per se, and its secondary effectors, elicit insulin resistance. Our hypothesis warrants further investigations into the structural and functional integrity of caveolae in obese and T2DM patients.

Treatment of adipocytes with two structurally distinct cholesterol-depleting reagents (MBCD and filipin) leads to upregulation of insulin-induced expression of PAI-1 mRNA and protein levels (Figs. 2A, B, and C). Similar upregulation is also obtained in NIH-IR cells after RNAi-mediated caveolin-1 downregulation (Fig. 4.2E). Thus, perturbation of caveolae by depleting their integral components, either cholesterol or caveolin-1, leads to significant

upregulation of insulin-induced PAI-1 gene expression. MBCD alone induces PAI-1 mRNA to a certain extent (Fig. 4.2), but this level of induction can be explained by a slight activation of Erk, as Erk has been reported to upregulate the PAI-1 gene by activating the AP-1 transcription factor (44). However, the MBCD-mediated synergistic increase in insulin-induced PAI-1 gene expression cannot be explained by Erk activation, as the extent of insulin-induced Erk phosphorylation (Fig. 4.1B) was not affected by MBCD co-treatment. We therefore postulate an alternative mechanism for the upregulation of insulin-induced PAI-1 gene expression mediated by caveolar dysfunction (see below).

Insulin-induced PAI-1 mRNA levels are augmented by treatment with a specific PI3K inhibitor, suggesting that the PI3K pathway inhibits PAI-1 gene expression (Fig. 4.3B). We show that the level of serum PAI-1 is increased in PKB$\alpha^{-/-}$ mice, while it is reduced in S6K1$^{-/-}$ mice (Fig. 4.3A). The whole blood protein concentration in both PKB$\alpha^{-/-}$ and S6K1$^{-/-}$ is similar to that of their wildtype littermates. These results indicate that the functional state of the PI3K pathway plays an important role in the regulation of PAI-1 gene expression.

There are several reports of the PI3K pathway regulating pRB-E2F interactions in a cell-type independent manner. Activation of PKB by inhibition of its phosphatases reduced the pRB phosphorylation and E2F1 release in C141 cells (45). In T lymphocytes, expression of active PKB is sufficient to induce E2F activity (46). In C33A cells, both wortmannin (PI3K inhibitor) treatment and overexpression of PTEN (a negative regulator of the PI3K-PKB pathway) inhibited pRB phosphorylation, and this was reversed by co-expression of a catalytically active subunit of PI3K (47). In NIH3T3 fibroblasts, EGF-induced pRB phosphorylation, and hence G1 to S phase cell cycle progression, was inhibited by both LY294002 and wortmannin (48). In agreement with the finding of Usui et al. (18), we show that LY294002 inhibits insulin-induced phosphorylation of pRB in 3T3L1 adipocytes. MBCD also shows similar effects (Fig. 4.3C), implying that

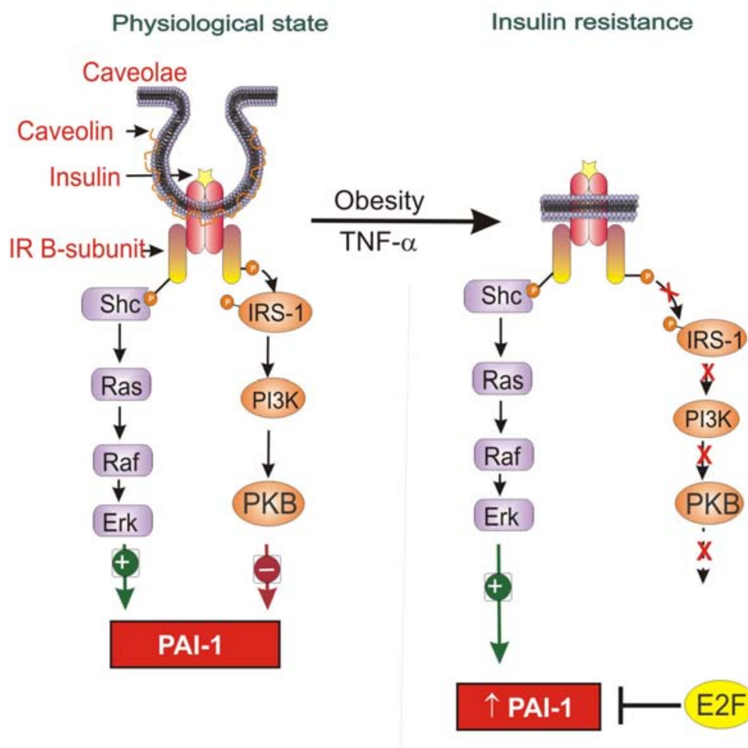
impaired metabolic signaling, characteristic of insulin resistance, may reduce the phosphorylation of pRB. Hypophosphorylated pRB is known to bind E2F1-3 proteins, and inhibits their activity, either by directly binding to and masking the transactivation domain of E2F, or by recruiting histone deacetylases (19). Reduction in pRB phosphorylation, therefore, will result in the reduction of free E2F levels. We have previously shown that free E2F can act as a repressor of PAI-1 gene expression in a variety of cell lines, including U2OS, T98G, SAOS2, LLC-PK1, and MEF cells, indicating that the E2F-mediated negative regulation of PAI-1 gene expression is a general phenomenon independent of cell type (27). Taking these observations together, we propose that the PI3K pathway in insulin signaling negatively regulates the PAI-1 gene, and phosphorylation of pRB and subsequent release of free E2F may play a role. This, in essence, is indicative of differential regulation of PAI-1 gene expression by insulin; positive regulation through the mitogenic pathway and negative regulation through the metabolic pathway. The compromised metabolic pathway may explain why insulin-induced PAI-1 levels are upregulated in states of caveolar dysfunction.

It is widely accepted that PAI-1 levels are raised in conditions of obesity and insulin resistance (10), and they play a key role in the development of cardiovascular complications in these patients. Recent studies show that PAI-1 knock-out mice are resistant to high-fat diet-induced obesity and insulin resistance, although the underlying molecular mechanisms are not well understood (49, 50). In line with the data from Vuori et al. (51) showing that vitronectin-$\alpha_v\beta_3$ integrin interaction facilitates insulin-induced IRS-1 activation, we had earlier proposed a model in which PAI-1 can induce insulin-resistance by binding to vitronectin and inhibiting its interaction with $\alpha_v\beta_3$ integrin (52). This suggests that PAI-1 can be a cause as well as a consequence of insulin resistance, and reducing its levels may offer immense therapeutic value. Both basal and insulin-induced PAI-1 levels could be dramatically reduced by treating adipocytes with a cell-penetrating peptide that physically disrupts the pRB-E2F interaction (Fig. 4.5A).

Furthermore, the interfering peptide was also able to suppress the elevation of PAI-1 mRNA levels in insulin-resistant adipocytes (Fig. 4.5B). These results strengthen our previous findings that E2F proteins can act as a transcriptional repressor of the PAI-1 gene.

In summary, we have demonstrated that caveolar dysfunction leads to the selective impairment of the PI3K pathway in adipocytes. This compromises E2F-mediated suppression of PAI-1 gene expression, resulting in the concomitant upregulation of PAI-1 levels. Our work thus establishes a direct link between an impaired PI3K pathway and elevated PAI-1 gene expression, both characteristics of insulin-resistant conditions. We also propose a novel pharmacological paradigm of disrupting pRB-E2F interaction to suppress PAI-1 levels that are elevated during insulin resistance. Recently, the crystal structure of E2F bound to pRB was solved (53), and this should facilitate the development of small molecule inhibitors of E2F-pRB interaction.

4.7 Working Model



Working model: *Under normal conditions, insulin receptor localized in the caveolar microdomains activates two major pathways. The mitogenic signaling (Shc-Erk pathway) upregulates PAI-1 gene expression, while the metabolic pathway (IRS-PKB) pathway downregulates PAI-1 gene expression. Destruction of caveolar microdomains selectively abrogates the metabolic signaling leading to insulin resistance. Based on other studies, we reason that under obese conditions and TNF-α treatment, the insulin receptor might not localize in caveolae, thereby indicating that caveolar dysfunction might be a natural cause of insulin resistance in obese conditions. Insulin resistance abolishes the inhibitory signal to the PAI-1 promoter, and thereby enhances PAI-1 gene expression. However, PAI-1 gene expression under insulin-resistant conditions can be effectively suppressed by the release of endogenous free E2F, previously demonstrated by us to be a transcriptional repressor of PAI-1 gene expression.*

4.8 Acknowledgements

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PART 2

5. Caveolar dysfunction may activate pathways that are critical for superoxide-induced vascular damage

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5.1 Specific research objectives

In the previous section, we showed that perturbation of caveolar microdomains leads to insulin resistance (characteristic feature of T2DM) in adipocytes. Obesity and T2DM are accompanied by increased oxidative stress mediated by enhanced mitochondrial superoxide anion production. It is now believed that the vascular complications in diabetic patients are a result of hyper-glycemica induced oxidative stress, which leads to the inhibition of glycolysis followed by activation of four pathways (Protein Kinase C pathway, Polyol pathway, AGE pathway and Hexosamine pathway). In this section we investigated if caveolar dysfunction can also lead to this central signaling defect considered to be responsible for the vascular complications associated with obesity and diabetes. This is particularly intriguing since the caveolin-1 deficient mice demonstrate severe vascular abnormalities.

5.2 Abstract

We show that three glycolytic enzymes, fructose-biphosphate aldolase, glyceraldehyde-3 phosphate and phosphoglycerate kinase-1, bind to caveolin-1. This novel finding is supportive of work from other laboratories that suggests that membrane-associated glycolysis may happen in the caveolar microdomain. Additionally, we show that the perturbation of caveolar microdomains using cyclodextrin leads to the activation of the PKC pathway, hexosamine pathway, and PAI-1 gene expression presumably by the inhibition of glycolysis and the diversion of glycolytic intermediates from these pathways.

5.3 Introduction

Obesity and diabetes are independent risk factors of cardiovascular complications. Diabetes-specific microvascular disease is a leading cause of blindness (retinopathy), renal failure (nephropathy) and nerve damage (neuropathy), cardiac problems (atherosclerosis, myocardial infarction), stroke and limb amputation [Vaughan, 2003 #552]. It is believed that the increased superoxide anion production is the main culprit in mediating these disorders. Diabetic hyperglycemia is the main inducer of superoxide anion production and current efforts towards the clinical management of diabetes is focused on the control of blood sugar level.

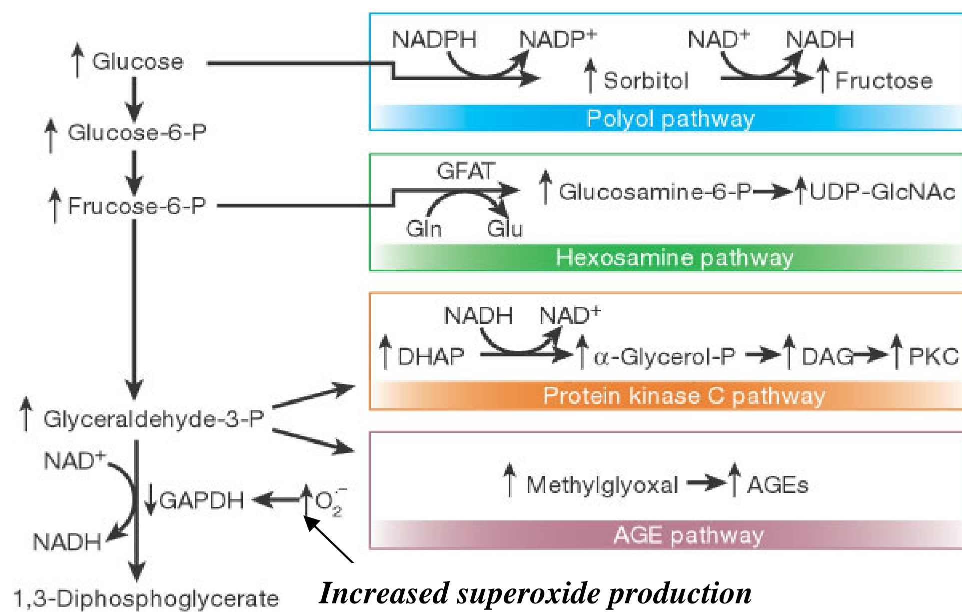

Fig. 5.1. Potential mechanism by which hyperglycaemia-induced mitochondrial superoxide overproduction activates four pathways of hyperglycaemic damage. *Increased oxidative stress leads to the inhibition of GAPDH, hence glycolysis. This then leads to the diversion of glycolytic intermediates to 4 pathways that are implicated in vascular damage. (Figure courtesy: Brownlee M, 2001, Nature)*

How does hyperglycemia lead to increased superoxide production?

Studies in cell-culture, animal models, and in clinical subjects have implicated four main molecular mechanisms mediating hyperglycemia-induced vascular damage [Brownlee, 2001 #571]. As shown in the Fig-1, the four pathways are the increased polyol pathway flux; increased advanced glycation end-product (AGE) formation; activation of protein kinase C (PKC) isoforms; and increased hexosamine pathway flux. All seem to reflect a single hyperglycaemia-induced process of overproduction of superoxide by the mitochondrial electron-transport chain.

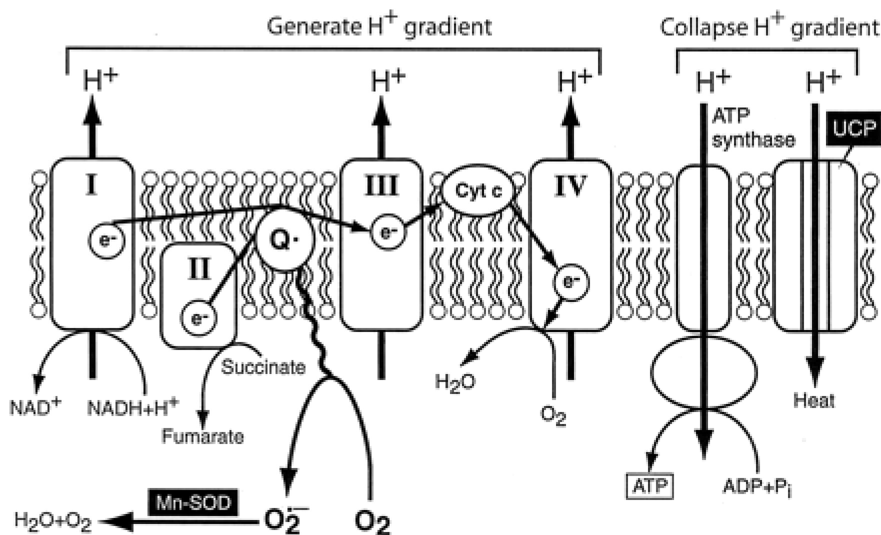

Fig. 5.2 Over production of superoxide by hyperglycemia *(Figure courtesy: Brownlee M, 2001, Nature)*

There are four protein complexes in the mitochondrial electron transport chain, called complex I, II, III, and IV (Fig. 5.2). When glucose is metabolized through the tricarboxylic acid (TCA) cycle, it generates electron donors. The main electron donor is NADH, which gives electrons to complex I. The other electron donor generated by the TCA cycle is $FADH_2$, formed by succinate dehydrogenase, which donates electrons to complex II. Electrons from both these complexes are passed to coenzyme Q, and then from coenzyme Q

they are transferred to complex III, cytochrome-C, complex IV, and finally to molecular oxygen, which they reduce to water. In normal cells, the electron transport system is organized in this way so that the level of ATP can be precisely regulated. As electrons are transported from left to right in Fig. 5.2, some of the energy of those electrons is used to pump protons across the membrane at complexes I, III, and IV. This generates a potential difference across the mitochondrial membrane. The energy from this voltage gradient drives the synthesis of ATP by ATP synthase [Mitchell, 1976 Biochem Soc Trans 4:399-430; Wallace, 1992 #2308; Trumpower, 1990 #2309]. Alternatively, uncoupling proteins (UCPs; Fig. 5.2) can divert the energy from the voltage gradient to heat production as a way of keeping the rate of ATP generation constant.

In contrast, in diabetic cells with high internal glucose concentrations, there is more glucose being oxidized by glycolysis [glucose is not a substrate of the TCA cycle, pyruvate/acetyl-CoA is], which pushes more NADH and $FADH_2$ into the electron transport chain. As a result, the potential difference across the mitochondrial membrane increases until a critical threshold is reached. At this point, electron transfer inside complex III is blocked [Korshunov, 1997 #2310], causing the electrons to back up to coenzyme Q, which donates the electrons one at a time to molecular oxygen, thereby generating superoxide (Fig. 5.2). The mitochondrial isoform of the enzyme superoxide dismutase degrades this oxygen free radical to hydrogen peroxide, which is then converted into H_2O and O_2 by hydrogen peroxidase.

This theory is supported by the following experiments. Intracellular hyperglycemia was found to increase the voltage across the mitochondrial membrane above the critical threshold necessary to increase superoxide formation [Du, 2001 #2311]. Not surprisingly, this led to a significant increase in production of ROS. In contrast, an identical level of hyperglycemia does not increase ROS at all in cells overexpressing UCP, where the mitochondrial voltage gradient is reduced [Nishikawa, 2000 #2312]. Similarly, hyperglycemia does not increase ROS in cells overexpressing superoxide dismutase (MnSOD), a scavenger of superoxide.

These data demonstrate two things. Firstly, the UCP effect shows that the mitochondrial electron transport chain is the source of the hyperglycemia-induced superoxide. Secondly, the MnSOD effect shows that the initial ROS formed is indeed superoxide. Furthermore, in $\rho 0$ endothelial cells (cells depleted of mitochondrial genes), which lack a functional mitochondrial electron transport chain the effect of hyperglycemia on ROS production as well as subsequent activation of the polyol pathway, AGE formation, PKC, or the hexosamine pathway is completely lost [Brownlee, 2005 #2313].

These results have been confirmed by *in vivo* experiments. When wild-type animals are made diabetic, all four of the pathways are activated in tissues where diabetic complications occur. In contrast, when MnSOD transgenic mice are made diabetic, there is no activation of any of the four pathways. Importantly, inhibition of hyperglycemia-induced superoxide overproduction using a transgenic approach (superoxide dismutase [SOD]) also prevents secondary complications of diabetes including long-term experimental diabetic nephropathy [DeRubertis, 2004 #2314].

How does obesity lead to increased superoxide production?

Recent studies have demonstrated that, in non-diabetic human subjects, fat accumulation is closely correlated with the markers of systemic oxidative stress [Furukawa, 2004 #2335]. These data are in good agreement with recent studies suggesting that systemic oxidative stress correlates with BMI [Keaney, 2003 #2347; Sinaiko, 2005 #2349]. Moreover, the production of reactive oxygen species was shown to be upregulated during 3T3L1 adipocyte differentiation. Studies using murine models of obesity indicate that the increased ROS production is restricted to white adipose tissue and is mediated by both NADPH oxidase upregulation and SOD downregulation [Furukawa, 2004 #2335; Sonta, 2004 #2351].

How does activation of the four pathways lead to the cardiovascular complications?

The precise mechanism via which the four pathways may contribute to cardiovascular events is discussed elsewhere [Brownlee, 2001 #571]. The graphical representations of the mechanisms are presented below. Notably, increase in the PAI-1 gene expression is a common mechanism via which at least two out of four pathways (glucosamine and PKC) elicit cardiovascular damage [Du, 2001 #2311] [Suzuki, 2002 #2350].

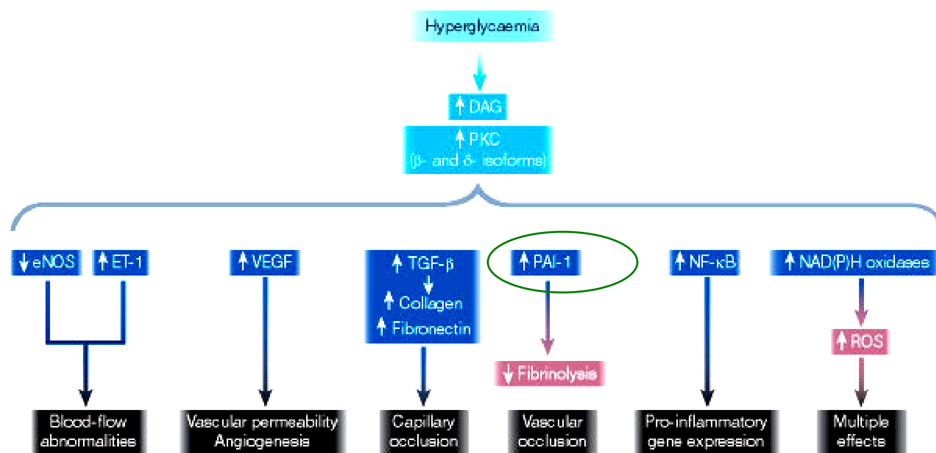

Fig. 5.3 Mechanisms contributing to the PKC-induced damage. *Upregulation of PAI-1 is contributing factor. (Figure courtesy: Brownlee M, 2001, Nature)*

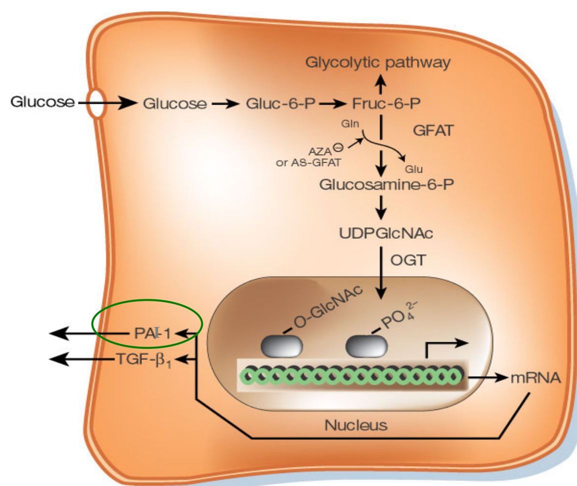

Fig. 5.4 Mechanisms contributing to the glucosamine-induced damage. *Glucosamine pathway can lead to an increase in glycosylation of SPI-1 transcription factors leading to enhanced expression of PAI-1and TGF-β. (Figure courtesy: Brownlee M, 2001, Nature)*

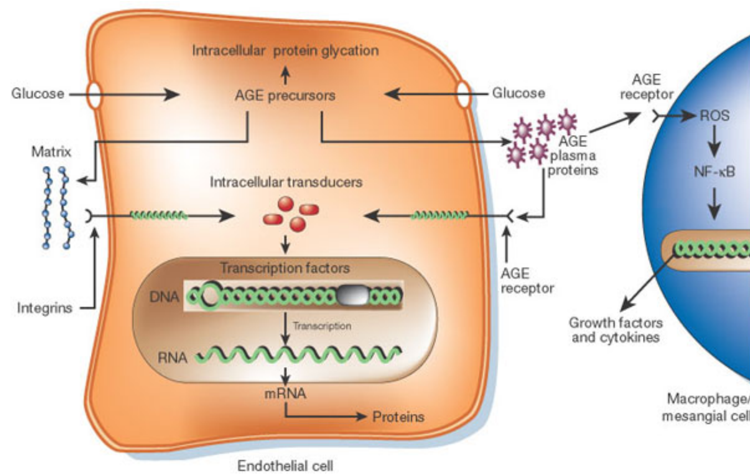

***Fig. 5.5 Mechanisms contributing to the AGE-induced damage**. (Figure courtesy: Brownlee M, 2001, Nature)*

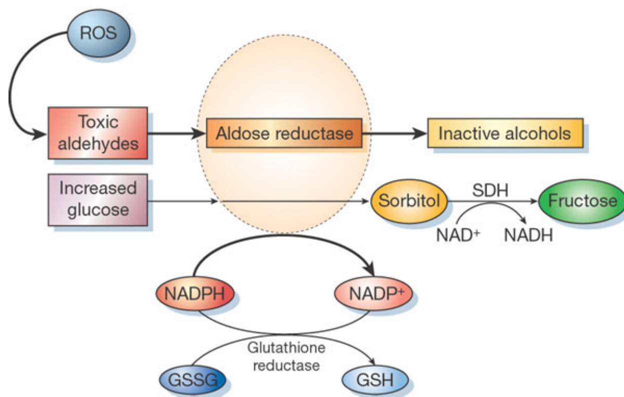

***Fig. 5.6 Mechanisms contributing to the polyolpathway-induced damage.** (Figure courtesy: Brownlee M, 2001, Nature)*

The hypothesis outlined above is considered by many to be the holy-grail of diabesity induced-vascular complications. Our studies propose caveolar dysfunction to be an inducer of insulin resistance in obese patients and recent studies implicate a role for caveolin down regulation in increased cell proliferation. Therefore it is intriguing to investigate if caveolar dysfunction can also activate pathways that are responsible for cardiovascular complications. If yes, then caveolar dysfunction could be considered to be the unifying mechanism by which obesity elicits its secondary disorders such as type-2 diabetes, cardiovascular complications and certain types of cancer.

5.4 Materials and methods

Adipocyte differentiation. 3T3-L1 pre-adipocytes were cultured in DMEM containing 10% FCS, and 2 days after cells reached confluency differentiation was induced by changing the culture medium to DMEM containing 10% FCS, 10 μg/ml insulin, 1 μM dexamethasone and 0.5 mM isobutylmethylxanthine. 2 or 3 days later, this medium was replaced with DMEM supplemented with only 10 μg/ml insulin, and cells were kept for 2 days. The medium was then replaced with DMEM containing 10% FCS every 2 days. Cellular morphology was observed using a Nikon Diaphot inverted microscope (10× objective with numerical aperture of 0.25).

2-D Gel electrophoresis and MALDI-TOF. Proteins were separated on a 4–12% Bis-Tris Mops-SDS gradient gel and stained with Silverquest silver stain. Three protein spots on the two-dimensional gel were excised, and in-gel digestion was carried out with a Montage in-gel digestion kit (Millipore). Digests of protein bands were first analyzed by MALDI-TOF mass spectrometry, and samples of adequate quality were then subjected to microliquid chromatography/electrospray ionization/tandem MS (micro-LC-ESI-MS/MS) using a QTOF Ultima system (Waters, Milford, MA). MS/MS fragmentation spectra were analyzed using the PROTEINLYNX software package.

RNA isolation and Northern blot analysis. Total RNA (12 μg) was isolated using the acid-guanidinium thiocyanate-phenol-chloroform method and subjected to Northern blot analysis as previously described [Koziczak, 2001 #568].

Quantitative real-time PCR. Total RNA extraction for RT-PCR was performed using RNeasy kits (Qiagen) according to the instructions from the manufacturer. RT-PCR for PAI-1 was carried out as described [Venugopal, 2004 #823].

Immunoprecipitation and Western blotting. Immunoprecipitation and Western blotting were performed as previously stated [Venugopal, 2004 #823].

Cell-penetrating peptide treatment. The sequence of the interfering peptide was derived from the pRB-binding region of E2F1 (aa 402-419: LDYHFGLEEGEGIRDLFD) [Helin, 1992 #620]. A control peptide with the same amino acid composition, GEELEGFHDGLLDFDIR, was prepared by randomly shuffling the sequence of this peptide. Cell-penetrating peptides were prepared by coupling these peptides to the carboxyl terminus of the cell-penetrating region of the HIV tat protein (aa 47-57: RRRQRRKKR) via hinge peptide G. Differentiated adipocytes were separated from undifferentiated cells using a Percoll density gradient as previously described [Venugopal, 2004 #823]. The penetrating peptide was then added to cells, incubated for 16 h and then subjected to various treatments.

5.5 Results

Key glycolytic enzymes bind to caveolin and perturbation of caveolar microdomains leads to modifications of these enzymes

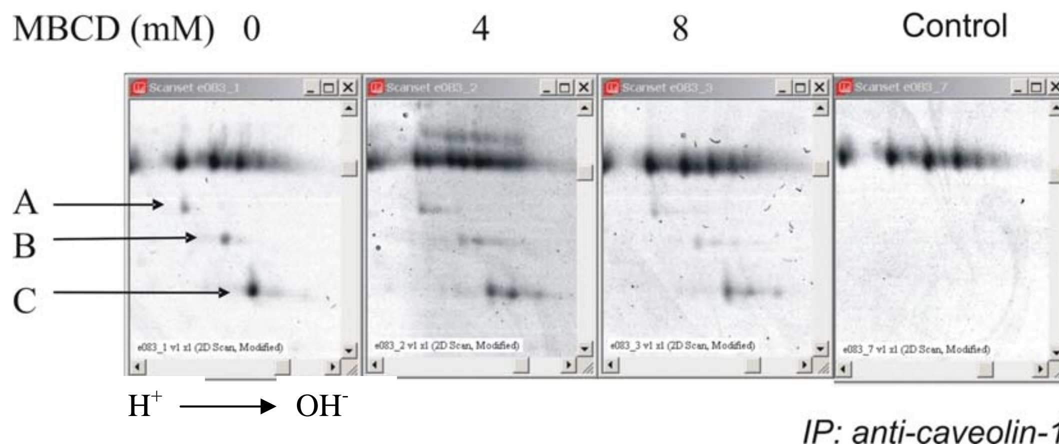

Fig. 5.7. Two-dimensional gel screening for changes in caveolin interactions upon cyclodextrin treatment. *(A) Total cell extracts were prepared from cells treated with or without cyclodextrin for 50 min. Caveolin-1 protein was immunoprecipitated using anti-caveolin-1 antibody. The*

protein complexes were eluted with Glycine elution buffer and analysed using two-dimesional gel electrophoresis. Specific spots that were altered upon cyclodextrin treatment were excised and digested. They were then identified by MALDI-TOF.

To screen the caveolin-1 interaction partners and to detect changes in their interactions upon cholesterol depletion, we treated the 3T3L1 adipocytes with increasing concentrations of MBCD and then subjected the total cell extract to two-dimensional gel electrophoresis. Three spots showed charecterestic shift towards the negative pH. This may indicate dephosphorylation, but needs to be examined. The spots that underwent characteristic changes upon MBCD treatment were analyzed using MALDI-TOF. As show in the Fig. 5.7, three spots that underwent characteristic change were identified as fructose-biphosphate aldolase, glyceraldehyde-3 phosphate and phosphoglycerate kinase-1, three key glycolytic enzymes.

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Perturbation of caveolar microdomains activates two pathways that are critical for hyperglycemia-induced damage

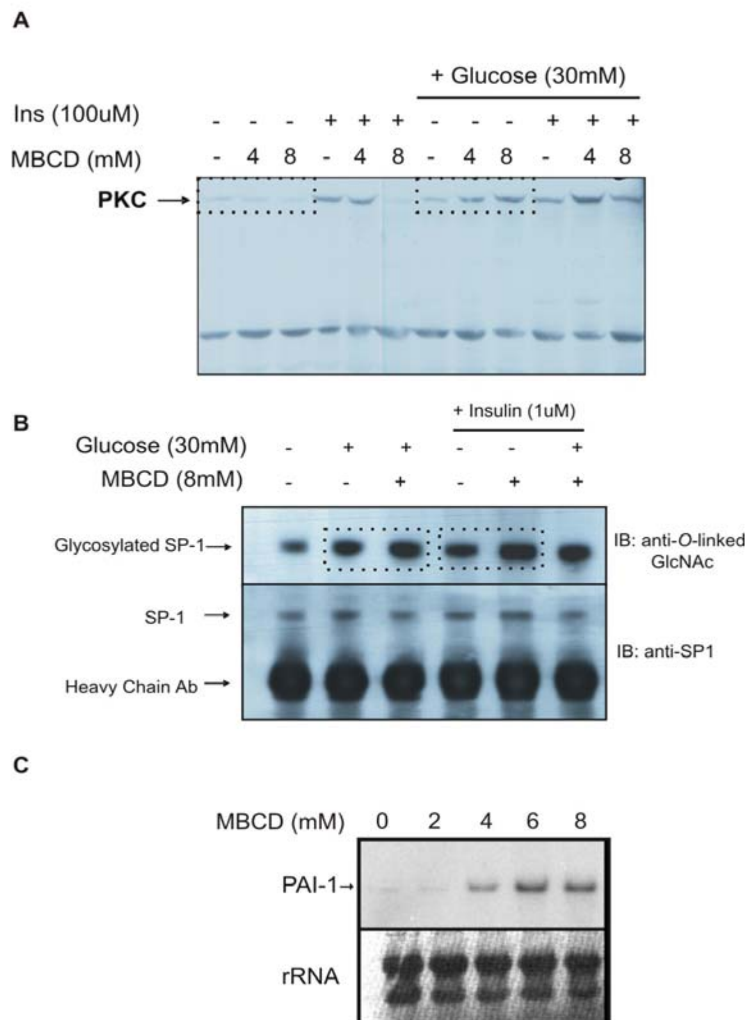

Fig. 5.8. Effect of cholesterol depletion on signal pathways that are critical for hyperglycemia-induced damage. *(A) Cells treated with or without glucose (30mM) for 48 h were treated with MBCD and insulin. The membrane fraction of the total cell lysate was prepared and run on an SDS-PAGE gel followed by blotting with anti-PKC antibodies. (B) The cells were subjected to similar treatment and the SPI-1 protein was immunoprecipiated before Western blot analysis using anti-O-linked GlcNAc antibody. (C) Cells were treated with increasing doses of MBCD. PAI-1 gene expression was analyzed by Northern blot.*

Since the 3 key glycolytic enzymes bind to caveolae, we investigated the potential effects that caveolar dysfunction might have on glycoysis and its associated pathways. We looked at PKC activation and glucosamine-induced SP-1 glycosylation. Both of these pathways are known to activate PAI-1 gene transcription. High glucose treatment has only a slight positive effect on PKC activation as measured by translocation of PKC to membrane

fractions (Fig. 5.8A). MBCD treatment by itself did not have any effect on PKC activation. Notably, MBCD could activate cells that were subjected to hyperglycemic conditions in a dose-dependent manner. Similarly, high-glucose as well as insulin increased the glycosylation of SPI-1 transcription factors, but were augmented by MBCD treatment (Fig. 5.8B). Subsequently MBCD treatment in hyperglycemic conditions upregulated the gene expression of PIA-1 in a dose-dependent manner.

5.6 Discussion

This study found three key glycolytic enzymes to be associated with caveolin. Previously, it has been shown in vascular smooth muscle cells that glycolysis and gluconeogenesis are compartmentalized [Hardin, 1995 #2315]. This makes sense if we take into account that glycolysis and gluconeogenesis are opposing pathways (the former consumes glucose, while the later produces it) despite the fact that they share eight common enzymes. It was later shown that glycolysis occurs in caveolar microdomains [Vallejo, 2004 #2316].

Recently, it was also shown that phosphofructokinase, a critical mediator of glycolysis, binds to caveolin-1 [Vallejo, 2005 #1184] and caveolin-3 [Vallejo, 2004 #1193] and this interaction is required for the membrane recruitment of this enzyme. Taken together, it is plausible to assume that caveolae might represent the cellular location of glycolytic activity. Perturbation of caveolar function therefore could lead to an inhibition of glycolysis and the subsequent activation the four pathways that are critical mediators of vascular complications associated with hyperglycemia. Upon treatment with cyclodextrin, we observe some shifts in the spots representing glycolytic enzymes (Fig. 5.8). This may indicate, but does not prove, that cyclodextrin treatment leads to post-translational modifications of these enzymes and in turn may affect their activities. The precise nature of modifications in these enzymes remains to be investigated.

We show that hyperglycaemic conditions in adipocytes lead to a slight activation of the 2 pathways implicated in hyperglycemia-induced vascular complications (Fig. 5.8). Interestingly, the hyperglycemia-induced activation was much stronger in the presence of cyclodextrin treatment. This led us to propose a working model, where caveolar dysfunction strengthens the glucose-induced inhibition of glycolysis and activation of the pathological pathways. Much of the work to test the validity of this hypothesis remains to be conducted. This includes reconfirming the interaction of caveolin and glycolytic enzymes by reverse immunoprecipitation, identifying the precise nature of the modifications that caveolar dysfunction imposes on glycolytic enzymes, testing the activity of these enzymes upon caveolar disruption, looking at the role of superoxide anions in this phenomenon, analyzing the effect of caveolar dysfunction on the remaining two pathways (polyol pathway and AGE pathway), and finally to test if the proposed four pathways are hyper-activated in caveolin-1 knock-out mice.

5.7 Working models

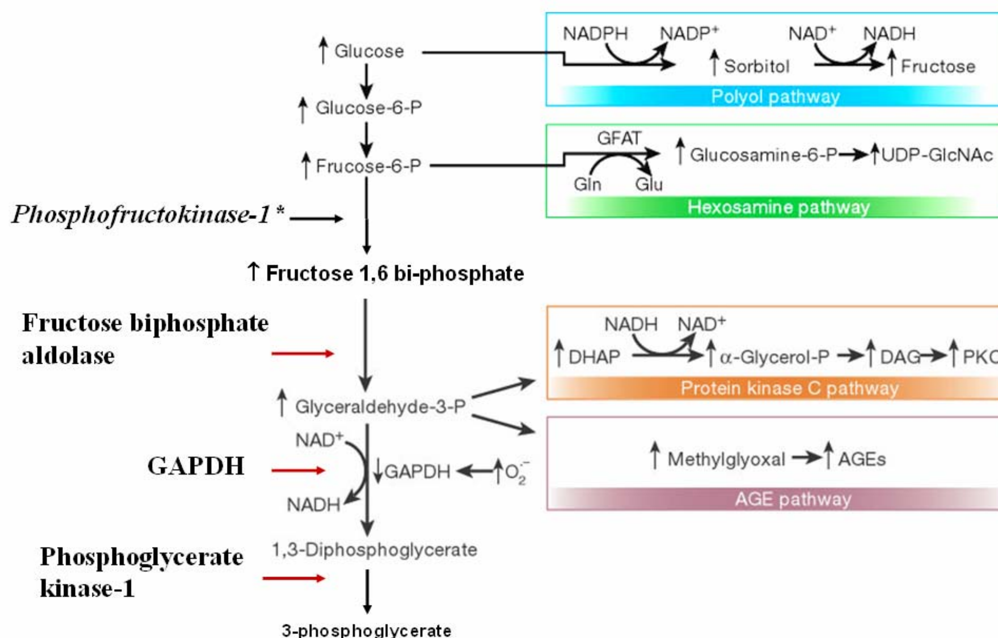

Figure 5.9. *Glycolytic enzymes bind to caveolin-1 and perform their reactions in caveolar microdomains.*

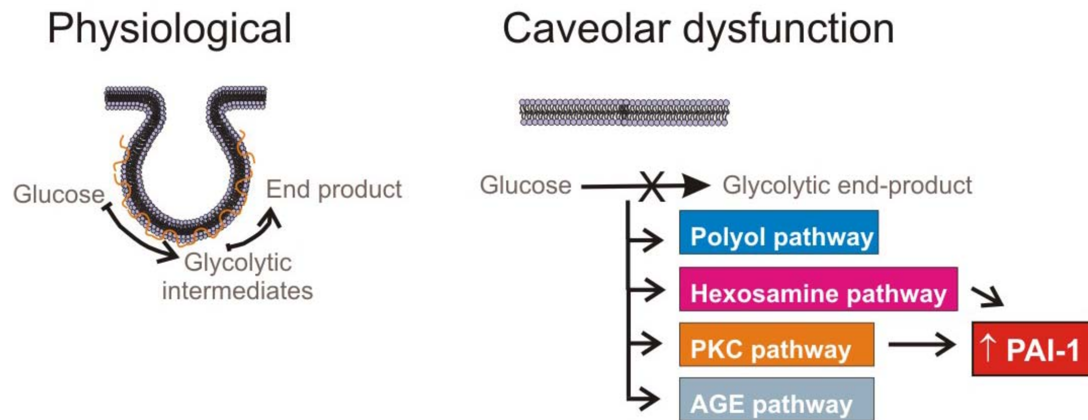

Figure 5.10. *Disruption of caveolar microdomains leads to the inhibition of membrane-associated glycolysis and subsequent shunting of glycolytic intermediates into four pathways that are critical for vascular abnormalities in obese and diabetic conditions. Activation of these pathways leads to the upregulation of PAI-1 gene expression.*

5.8 Acknowledgements

I would like to thank Sandra Kleiner, Lauren Smith and Janice Lai for experimental support.

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PART-3:

6. Physiological regulation and pharmacological modulation of PAI-1 gene expression in adipocytes

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* Both authors contributed equally. Abbreviations: pRB, retinoblastoma protein; E2F, E2 (adenoviral protein) factor; PPAR, peroxisome proliferator-activated receptor; TNF, tumor necrosis factor; TGF, transforming growth factor

6.1 Specific research objectives

In the earlier sections, we show that caveolar dysfunction-induced signaling defects lead to the upregulation of PAI-1 gene expression in adipocytes. Many studies have shown that adipose tissue-derived PAI-1 is primarily responsible for the elevated plasma PAI-1 levels in obese patients. This part of the study was aimed at understanding the molecular mechanisms which enable adipocytes to be a major PAI-1 producing organ. We further explored the pharmacological modulation strategy to inhibit PAI-1 production in adipocytes without causing unwanted effects such as cell-cycle re-entry.

6.2 Abstract

Obesity is characterized by elevated levels of circulating plasminogen activator inhibitor-1 (PAI-1), which contribute towards the development of obesity-driven secondary disorders such as type-2 diabetes mellitus, cardiovascular complications and certain types of cancer. This increase in plasma PAI-1 levels under obese conditions is attributed to an increase in PAI-1 derived from adipose tissue. Previous sections in this thesis show that caveolar dysfunction leads to signal transduction defects associated with secondary disorders of obesity, and also results in the upregulation of PAI-1 levels in cultured adipocytes. This study shows that adipose tissue evolved into a major PAI-1 producing organ by gaining capacity during adipocyte differentiation to respond to inducers of PAI-1 transcription. This is mediated by a decrease in E2F1 protein levels, an increase in retinoblastoma protein (pRB) levels and a decrease in pRB phosphorylation, all leading to a decrease in levels of free E2F, a known transcriptional repressor of PAI-1. Depletion of E2F1-3 was sufficient for inducers such as insulin to potently induce PAI-1 gene expression in pre-adipocytes. Conversely, forced release of pRB-bound endogenous E2F using cell-penetrating peptides can suppress PAI-1 gene expression in adipocytes without causing unwanted effects such as cell cycle re-entry. This study describes the novel paradigm of cellular differentiation-associated increase in PAI-1 gene expression which is mediated by a decrease in repressor activity, and describes a way

of desensitizing terminally differentiated cells to PAI-1 inducing agents by restoring endogenous repressor activity.

6.3 Introduction

Obesity has reached epidemic proportions globally, with more than 1 billion adults overweight – at least 300 million of them clinically obese – and is a major contributor to the global burden of chronic disease and disability. It is not obesity per se, but its chronic, life-threatening secondary disorders such as type 2 diabetes mellitus (T2DM), cardiovascular complications (1) and certain types of cancers (2) that burden healthcare systems worldwide. The precise molecular mechanisms that trigger these secondary disorders under obese conditions have been a subject of intense investigation, but remain inconclusive.

A simplified approach in this direction would be to identify proteins whose levels or activity are altered in obesity and are known independently to contribute to these secondary disorders. Plasminogen activator inhibitor-1 (PAI-1), a physiological inhibitor of plasminogen activators (uPA/tPA) and vitronectin, could be a viable candidate in this regard. Plasma PAI-1 levels are known to be elevated in obese patients (3). Congruently, a dramatic increase in plasma PAI-1 levels (~5-fold) has been observed in genetically obese (ob/ob) mice, in comparison with their lean counterparts (4). Conversely, weight loss has led to a subsequent reduction in plasma PAI-1 levels in obese patients (5, 6).

Elevated levels of PAI-1 are implicated in many chronic disorders that are secondary to obesity. PAI-1 is known to play important roles in eliciting thrombosis, fibrosis and vascular remodeling, thus contributing to a wide variety of cardiovascular disorders. The role of PAI-1 in cardiovascular complications has been reviewed elsewhere (7).

In line with the observation by us and others that PAI-1 can induce insulin resistance by inhibiting the interaction between vitronectin and $\alpha_v\beta_3$ integrin (8, 9), it has recently been show that PAI-1 knock-out mice are resistant to high-fat-diet-induced insulin resistance (10, 11), indicating a role for PAI-1 in the pathogenesis of obesity-driven insulin resistance and hence T2DM. Moreover, levels of plasma PAI-1 have been shown to be a prognostic marker of T2DM independent of insulin resistance and other known risk factors for diabetes (12).

The role of PAI-1 in cancer progression is rather controversial. Nevertheless, in almost all cancer types that are associated with obesity (2), a role for PAI-1 has been proposed. Possible mechanisms by which PAI-1 contributes to cancer dissemination include the prevention of excess degradation of the extra-cellular matrix, modulating cell adhesion (13), promoting angiogenesis (14), inhibiting apoptosis (15) and stimulating cell proliferation (16). Taken together, elevated levels of PAI-1 in obese patients may be an important factor contributing to obesity-driven chronic secondary disorders. Needless to say, understanding the molecular mechanisms that lead to PAI-1 upregulation in obesity assumes paramount importance.

The increase in plasma PAI-1 levels that is associated with obesity could be attributed to adipose tissue. Adipose tissue is one of the largest endocrine organs in obese patients (17). Weight loss in humans, either by exercise (18) or by surgical removal of fat (19), leads to a decrease in plasma PAI-1 levels, which rise again if weight is regained (20). Furthermore, comparative analysis of PAI-1 gene expression in various tissues of ob/ob mice and their wildtype counterparts shows that, in ob/ob mice, PAI-1 upregulation was significantly higher in the adipose tissue but only modestly increased in other tissues. These observations indicate that adipose tissue is a major, possibly the largest, contributor to plasma PAI-1 in conditions of obesity (4).

Adipose tissue is primarily composed of adipocytes that are formed by the differentiation of pre-adipocytes, a process commonly referred to as adipogenesis. Under serum-starved conditions, neither 3T3L1 pre-adipocytes nor differentiated adipocytes showed any significant PAI-1 gene expression (see results). But in the presence of serum (see results) or insulin (21), adipocytes, but not pre-adipocytes, showed significant PAI-1 gene expression. Taken together, this suggests that adipogenesis, as such, may not induce PAI-1; rather it enhances the potential of cells to respond to PAI-1 inducers (e.g. insulin) in serum.

Hyperinsulinemia has been shown to be associated with obesity and insulin resistance. The evidence for the pathophysiological role of insulin in the elevation of PAI-1 comes from the following observations. Conditions that increase endogenous plasma insulin levels (e.g. high calorie, high carbohydrate meals) were associated with increases in plasma PAI-1, whereas conditions that reduced endogenous insulin (e.g. fasting or treatment with metformin or troglitazone) were associated with decreases in plasma PAI-1 (22, 23). Moreover, administration of exogenous insulin in rabbits (24), mice (4) and human subjects (25) significantly increased plasma PAI-1 levels.

The E2F family of transcription factors are known to reversibly bind to the pocket proteins (pRB, p107 and p130) and are key regulators of the cell cycle. E2F1-3 complex with pRB, and the hyperphosphorylation of pRB leads to the release of E2F1-3 isoforms. Contrary to the earlier belief that E2F1-3 are transcriptional activators and E2F4-5 are transcriptional repressors (26), we have previously demonstrated that E2F1-3 act as transcriptional repressors of the PAI-1 gene in a cell type-independent manner (27, 28). Subsequently, other groups have shown repressive effects of E2F1 (29), E2F2 (30) and E2F3 (31) on other target genes. Using chromatin immunoprecipitation assays, it was shown by Wells J *et al* that E2F1-3 can directly bind to the PAI-1 promoter in a tissue specific-manner (32). As the compromise of E2F activity was shown to be required for

cellular differentiation (33), it would be intriguing to investigate if it can also be held responsible for the enhanced PAI-1 biosynthetic potential attained during adipogenesis.

As adipose tissue is the major contributor of plasma PAI-1, the crux of obesity-associated increase in plasma PAI-1 levels lies in the enhanced PAI-1 biosynthetic potential acquired during adipogenesis. It is this gain of function that enables the adipocytes to respond to various stimuli to express and secrete PAI-1. In this study, we investigated the mechanisms by which adipogenesis empowers the cells to respond to PAI-1 inducers such as insulin, which happens to be a major physiological stimulus in conditions of diabesity®. We also explored the potential of inhibiting PAI-1 gene expression in terminally differentiated adipocytes by the forced release of endogenous E2F (bound to pRB) without effectuating cell-cycle re-entry.

6.4 Experimental Procedures

Reagents. Insulin (I-5500) was obtained from Sigma. Monoclonal antibodies against E2F1 (KH-95) and E2F2 (TFE-25), and rabbit polyclonal antibodies against E2F3 (C-18), E2F4 (C-20), E2F5 (C-20), pRB (M-153), p130 (C-20), p107 (C-18), IRS-1 (C-20), Erk and PAI-1 (H-135) were from Santa Cruz Biotechnology. Rabbit polyclonal antibodies against phospho-pRB (Ser-795) and phospho-Erk were from Cell Signaling. Rabbit polyclonal antibodies against Shc and phospho-tyrosine (G410) were from Transduction Laboratories. The sheep polyclonal antibody against PAI-1 was from American Diagnostics. 5,6-dichloro-1-ß-D-ribofuranosylbenzimidazole (DRB) was from Fluka. Br-dU incorporation kit (Cat. No. 1296736) was from Roche Diagnostics.

Adipocyte differentiation. 3T3-L1 pre-adipocytes were cultured in DMEM containing 10% FCS, and 2 days after cells reached confluency differentiation was induced by changing the culture medium to DMEM containing 10% FCS, 10 µg/ml insulin, 1 µM dexamethasone and 0.5 mM

isobutylmethylxanthine. 2 or 3 days later, this medium was replaced with DMEM supplemented with only 10 µg/ml insulin, and cells were kept for 2 days. The medium was then replaced with DMEM containing 10% FCS every 2 days. Cellular morphology was observed using a Nikon Diaphot inverted microscope (10× objective with numerical aperture of 0.25).

Oil-red O staining. Cells were washed twice with phosphate-buffered saline (PBS) and fixed with 3.7% formaldehyde solution for 1 h. After washing twice with PBS, cells were stained with oil-red O [60:40 (v/v) dilution in water of 0.5% stock solution (v/v, in isopropanol)] for 1h. Cells were then washed twice with PBS and twice with water.

RNA isolation and Northern blot analysis. Total RNA (12 µg) was isolated using acid-guanidinium thiocyanate-phenol-chloroform method and subjected to Northern blot analysis as previously described (28).

Nuclear run-on transcription assay. Transcriptionally active nuclei from pre-adipocytes and adipocytes were extracted (34) and the amount of nuclei was equalized based on the 280 nm absorbance of the total nuclear lysate. A run-on assay was performed in the presence of [α-32P]UTP according to the basic protocol (35), with minor modifications. Purified mRNA from both samples was hybridized to nylon filters containing linearized and immobilized PAI-1 and GAPDH cDNA (1µg of each) and exposed to a phosphoimager.

Quantitative real-time PCR. Total RNA extraction for RT-PCR was done using RNeasy kits (Qiagen) according to the instructions from the manufacturer. RT-PCR for PAI-1 was carried out as described (34).

Immunoprecipitation and Western blotting. Immunoprecipitation and Western blotting were performed as previously stated (34).

siRNA nucleofection. The small interfering RNA (siRNA) used for targeting E2F mRNA has the following sequence: E2F1: sense, 5'-GAC UCC UCG

CAG AUC GUC AUC-3' and antisense, 5'-UGA CGA UCU GCG AGG AGU CGA-3'; E2F2/3: sense, 5'-ACA UCA CCA ACG UGC UGG AAG-3' and antisense, 5'-UCC AGC ACG UUG GUG AUG UCG-3'; control siRNA: sense, 5'-GUACCUGACUAGUCGFCAGAAG-3' and antisense, 5'-UCU GCG ACU AGU CAG GUA CGG-3'. Each siRNA (final concentration 1 µM) was mixed with 3T3L1 cell suspension ($1{\times}10^6$ cells in 0.1 ml buffer-V/transfection), transferred to a 2-mm electroporation cuvette, and electroporated using an Amaxa Nucleofector™ (Amaxa, Germany) the program T-20. After electroporation, cells were immediately transferred to 1 ml growth medium, and cultured in 6-well plates at 37°C until analysis.

Electromobility shift assays. Nuclear extracts (5 µg) were first incubated at room temperature for 15 min in 20 µl binding reaction mixture containing 50 mM KCl, 20 mM HEPES (pH 7.9), 0.2 mM EDTA, 6% glycerol, 0.5% Ficoll 400, 1 µg salmon sperm DNA, 6 µg bovine serum albumin and 1 mM DTT with or without penetrating peptide and antibodies, followed by a further 15-min incubation after addition of 0.3 ng radio-labeled oligonucleotide probes. Oligonucleotide probes were radio-labeled using *E. coli* polynucleotide kinase and [γ-32P]ATP. Aliquots (5 µl) of reaction mixture were separated in a 4.5% polyacrylamide gel run in 0.25×TBE buffer at room temperature. The gel was dried and analyzed on a PhosphorImager.

Cell-penetrating peptide treatment. The sequence of the interfering peptide was derived from the pRB-binding region of E2F1 (aa 402-419: LDYHFGLEEGEGIRDLFD) (36). A control peptide with the same amino acid composition, GEELEGFHDGLLDFDIR, was prepared by randomly shuffling the sequence of this peptide. Cell-penetrating peptides were prepared by coupling these peptides to the carboxyl terminal of the cell-penetrating region of the HIV tat protein (aa 47-57: RRRQRRKKR) via hinge peptide G. Differentiated adipocytes were separated from undifferentiated cells using a Percoll density gradient as previously described (34). The penetrating peptide was then added to cells, incubated for 16 h and then subjected to various treatments.

BrdU incorporation assay. Adipocytes were enriched using a Percol density gradient as described above. About 1×10^5 adipocytes were then seeded in a 6-well plate containing cover slips, incubated overnight in growth media. The media was then replaced with fresh media supplemented with bromodeoxyuridine (BrdU). The cells were subjected to various treatments for 48 h, washed with PBS^-, fixed with ethanol fixative (25 mM glycine in 100% ethanol; pH 2) at −20°C for at least 20 min and then subjected to immunofluorescence analysis according to the instruction from the manufacturers (BrdU Detection Kit-1, Roche). Cellular morphology was observed using a Nikon Diaphot inverted microscope (10× objective with numerical aperture of 0.25). Fluorescence was visualized with a Zeiss Axioplan fluroscence microscope (63× oil objective with numerical aperture of 1.4).

6.5 Results

PAI-1 levels are upregulated during adipogenesis

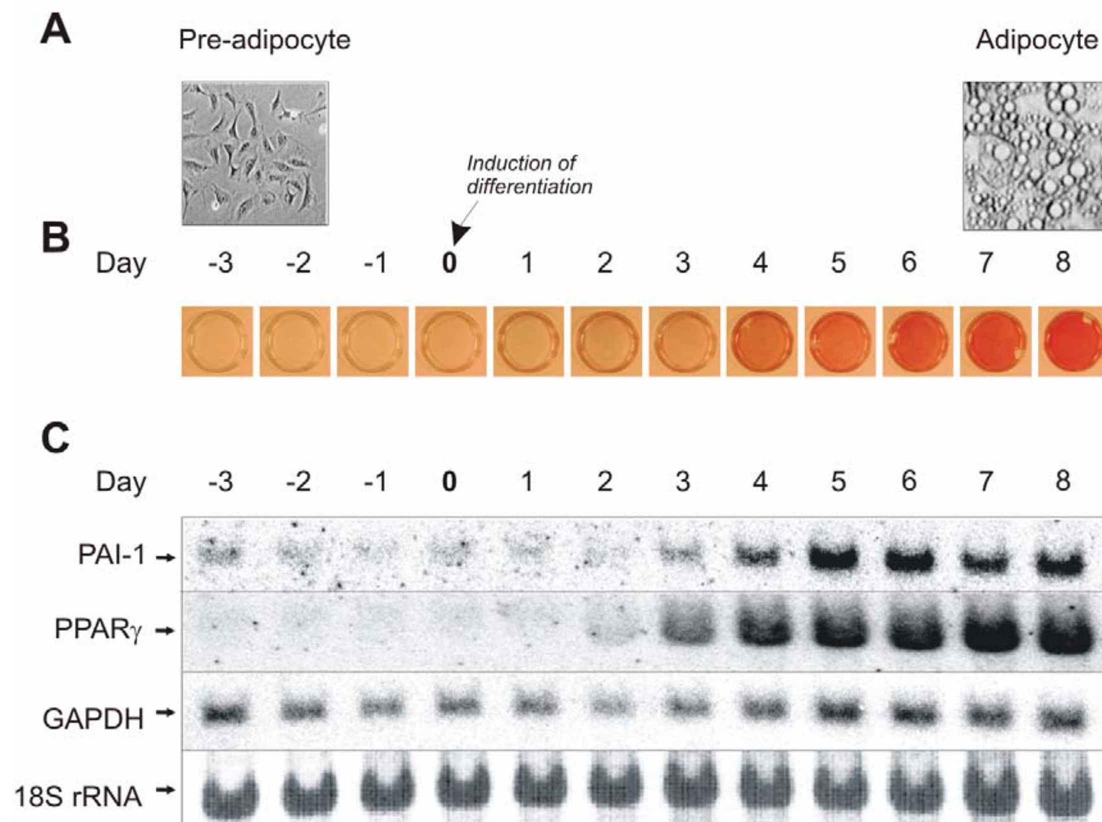


***Fig. 6.1. PAI-1 gene expression during adipogenesis.** 3T3L1 cells were differentiated and subjected to (A) microscopic examination and (B) oil-red O staining. (C) The total RNA was collected from cells on every day of adipogenesis, and mRNA levels of PAI-1, PPARγ and GAPDH (control) were measured using Northern blot analysis.*

The differentiation of 3T3L1 pre-adipocytes into adipocytes was verified by microscopic observation of cell morphology changes (Figure 6.1A) and lipid staining using Oil-Red O (Figure 6.1B). 8 days after the induction of differentiation, almost all cells contained vacuoles, a lipid storage compartment, and were positive for lipid staining. The differentiation process (adipogenesis) was accompanied by an increase in PAI-1 gene expression, which was comparable to the increase in the gene expression of PPARγ, a well-known adipocyte differentiation marker (Figure 6.1C).

Increase in PAI-1 mRNA levels is transcriptic

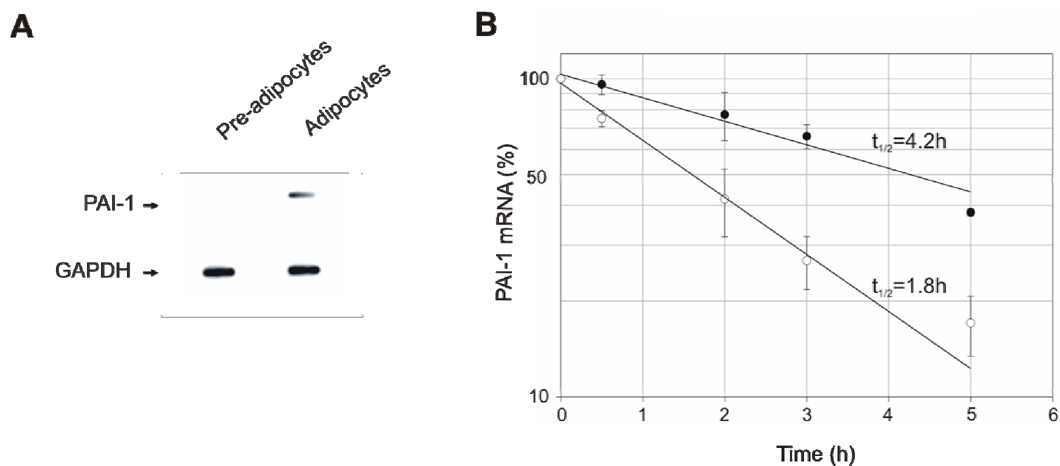
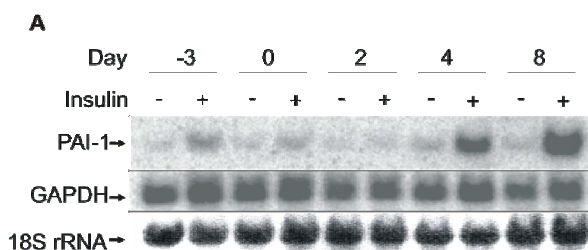


***Fig. 6.2. PAI-1 mRNA stability during adipogenesis.** (A) Transcriptionally active nuclei from both pre-adipocytes and adipocytes were subjected to run-on assays. Purified mRNA from both samples was hybridized to nylon membranes containing linearized and immobilized cDNA from PAI-1 and GAPDH and then exposed to a phosphoimager. (B) Both pre-adipocytes (filled circles) and adipocytes (open circles) were treated with DRB (20*

μg/ml) and cells were collected at various time points. PAI-1 levels normalized to GAPDH levels were plotted on a semi-log scale and linear regression lines drawn using SigmaPlot.

The increase in mRNA levels could be due to an increase in either mRNA synthesis, mRNA stability, or both. To investigate this, we looked at the state of PAI-1 gene transcription in both pre-adipocytes and adipocytes by nuclear run-on assays. PAI-1 transcription appears to be limited in pre-adipocytes, while substantial in adipocytes (Figure 6.2A). Furthermore, we examined the half-lives of PAI-1 mRNA in pre-adipocytes and adipocytes using DRB chase experiments. As shown in Figure 6.2B, the half-lives of PAI-1 mRNA in pre-adipocytes and adipocytes were 4.2 and 1.8 h, respectively. This result indicates that PAI-1 mRNA becomes rather unstable during adipogenesis. Therefore, the elevation of PAI-1 mRNA levels in adipocytes is due to transcriptional activation of the PAI-1 gene and does not involve an increase in mRNA stability.

Increase in PAI-1 gene expression is a result of enhanced biosynthetic potential acquired during adipogenesis



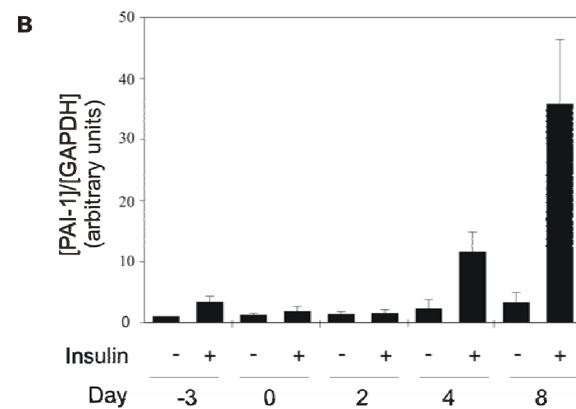


Fig. 6.3. The role of serum and insulin in effecting PAI-1 gene expression during adipogenesis. *(A) Cells at various stages of adipocyte differentiation were subjected to overnight serum-starvation and then induced with 10 μg/ml insulin (1.7 μM) for 2 h. Total RNA was prepared and analyzed for PAI-1 and GAPDH levels by Northern blot analysis. (B) PAI-1 gene expression from three independent experiments were quantified and normalized with GAPDH.*

Cellular gene expression can be influenced by both extrinsic (e.g. extracellular growth factors) and intrinsic factors (e.g. transcription factors). To understand the relative role of these factors in the increase of PAI-1 gene expression associated with adipogenesis, the cells were deprived of extracellular inducers by culturing them overnight in the absence of serum. Interestingly, serum-starved cells, irrespective of their state of differentiation, expressed very low levels of PAI-1 mRNA, which did not change during adipogenesis (Figure 6.3). On the other hand, when supplemented with insulin, these cells could induce PAI-1 gene expression in a manner positively correlating with the extent of differentiation. Even high concentrations of insulin (1.7 µM) could barely induce PAI-1 gene expression in pre-adipocytes, whereas in terminally differentiated adipocytes more than 7-fold induction was observed. These results strongly suggest that adipogenesis as such may not induce PAI-1; rather, it enhances the potential of cells to respond to PAI-1 inducers (e.g. insulin) in serum.

Inducibility of the PAI-1 gene is associated with a decrease in free E2F levels

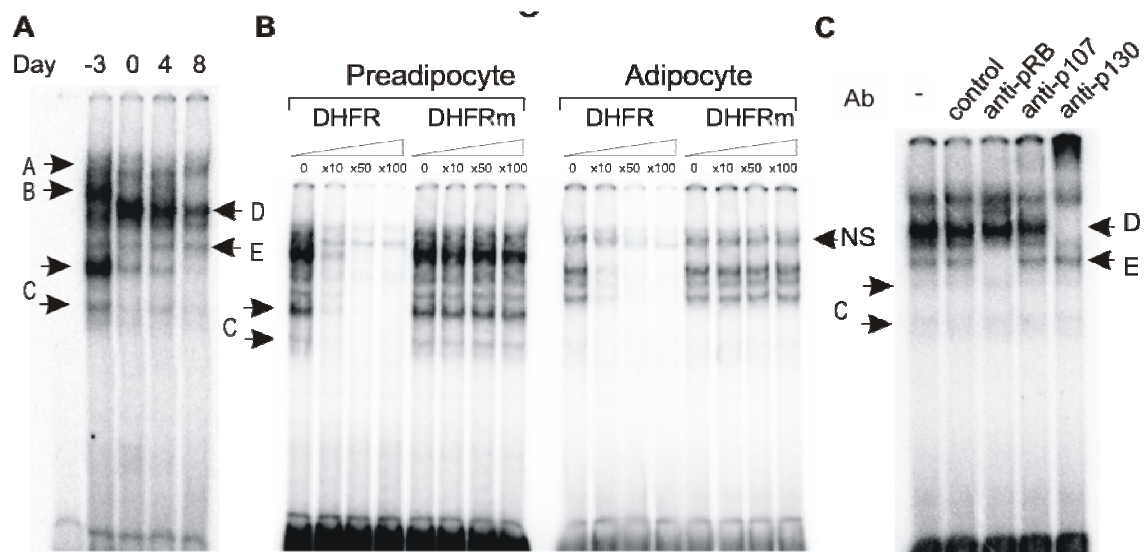


Fig. 6.4. E2F DNA-binding activity during adipogenesis. *(A) E2F DNA-binding activity of nuclear extracts from various days of adipogenesis was assessed by EMSA. (B) In the gel-shift assays, nuclear extracts prepared from adipocytes (day 8) were incubated with the same radioactive*

oligonucleotide used in (A) together with an increasing amount of a cold oligonucleotide DHFR containing an E2F-binding site or mutant DHFRm. (C) Nuclear extracts from adipocytes (day 8) were preincubated with different antibodies for 15 min and then analyzed for E2F DNA-binding activity by EMSA.

We have previously shown that free E2F1-3 that is not bound to pocket-proteins can act as a transcriptional repressor of PAI-1 gene expression. Removal of repressor activity is a plausible mechanism through which inducers can gain biosynthetic potential. To find out if an inverse correlation between E2F activity and PAI-1 gene expression during adipogenesis exists, we employed gel-shift assays to assess the state of E2F activity (free E2F) using an E2pro oligonucleotide probe that contains E2F recognition sites derived from the adenovirus E2 promoter (37). Both free and complex forms of E2F1-6 are able to bind to this E2F recognition element, and can be distinguished from each other by differential migration in DNA gel-shift assays. The pattern of protein-DNA complexes showed differentiation-associated changes in gel-shift assays (Figure 6.4A). In proliferating pre-adipocytes (day −3), several sizes of DNA-protein complexes were detected and, upon reaching confluency (day 0), the intensity of a higher band (B) and lower bands (C) declined and a new band (D) appeared. During differentiation (day 0-8), a further decrease in the intensity of the lower bands (C) was observed. The specificities of these DNA-protein complexes were examined by competition experiments using a competitor oligonucleotide with an E2F recognition sequence derived from the dihydrofolate reductase (DHFR) promoter (38). Except for band A, all complexes found in extracts of pre-adipocytes and adipocytes were out-competed by the DHFR promoter-derived specific oligonucleotide, but not by a mutated oligonucleotide, in a dose-dependent manner (Figure 6.4B), suggesting that these bands represents E2F isoforms that are either free or in complexes with other protein(s). Super-shift assays of adipocyte (day 8) nuclear extracts using specific antibodies revealed that complex D contained E2F bound to p130 and complex E contained E2F bound to pRB (Figure

6.4C). The fast-migrating DNA-protein complexes (C) are present only in proliferating pre-adipocytes, but are progressively reduced during differentiation. These bands, super-shifted with antibodies against E2F (data not shown), may reflect free, active E2F. Other complexes may represent higher molecular weight forms comprising E2F and other proteins such as p107, HDAC, cdk2, cyclin A and DP (39, 40). Taken together, a significant reduction in free E2F levels is found to be associated with adipogenesis.

The decrease in free E2F levels may be due to changes in protein levels of E2F1 and pRB, and the phosphorylation status of pRB

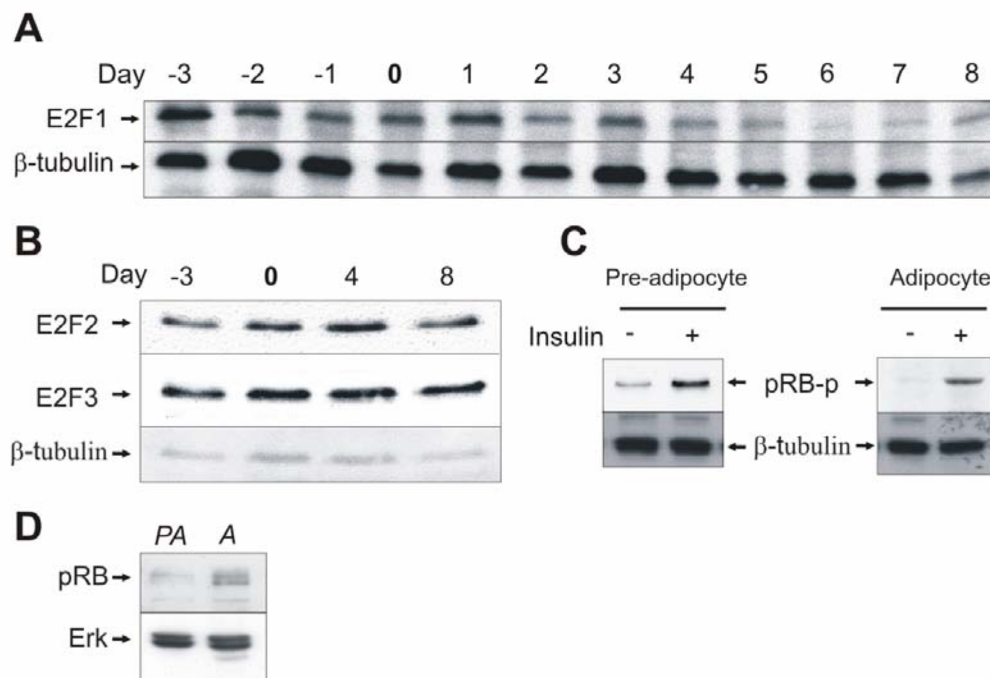


Fig. 6.5. Analysis of the mechanisms that contribute to a decrease in free E2F levels during adipogenesis*. (A) E2F1 and (B) E2F2/3 protein levels were measured at various stages of adipogenesis. (C) Both pre-adipocytes and adipocytes were treated with or without insulin (100 nM) for 10 min and analysed for pRB phosphorylation. (D) 500 µg total cell lysates of pre-adipocytes and adipocytes (day 8) were first immunoprecipitated and then immunoblotted with anti-pRB antibody.*

In principle, the decrease in free E2F levels could be due to a decrease in E2F protein levels, an increase in pRB protein levels or a decrease in pRB phosphorylation. To understand how E2F activity is reduced during adipogenesis, the protein levels of E2F1-3 and pRB, as well as the phosphorylation levels of pRB, were assessed. E2F1 levels were progressively reduced during adipogenesis, while E2F2 and E2F3 levels remained unchanged (Figure 6.5A,B). It appears that adipogenesis results in increasing pRB phosphorylation status (Figure 6.5C) and decreasing pRB protein levels (Figure 6.5D).

Increase in PAI-1 gene expression is a result of a decrease in E2F activity

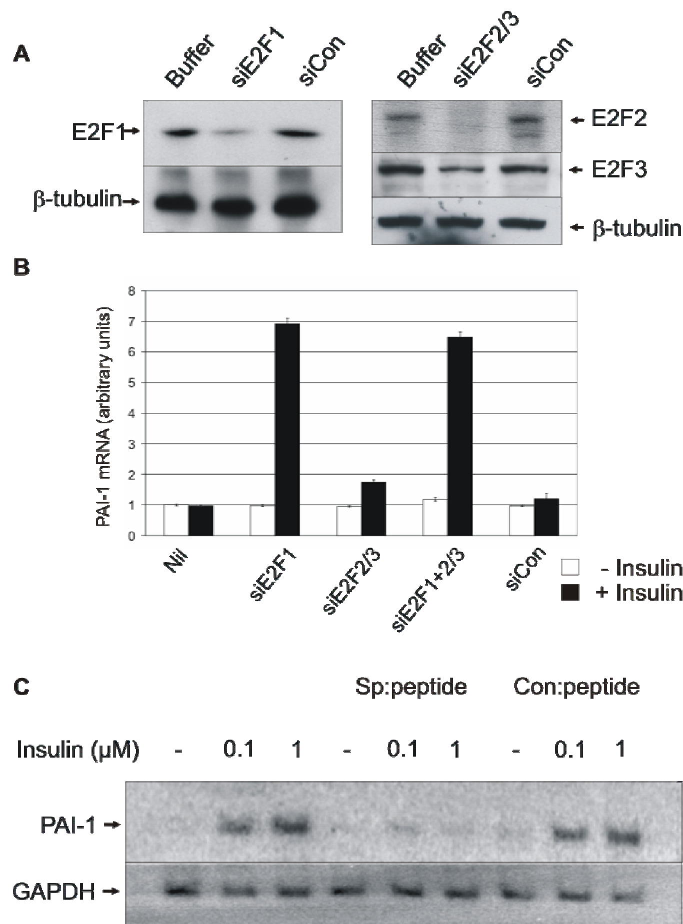


Fig. 6.6. Effect of E2F1-3 on PAI-1 gene expression in pre-adipocytes and adipocytes. *(A) 3T3L1 pre-adipocytes were electroporated with siRNA against respective E2F isoforms. After 48 h, the cells were collected and analyzed for E2F1-3 isoforms using Western blot analysis. (B) Cells were electroporated with siRNA against respective E2F isoforms. After 32 h, the cells were subjected to serum starvation for about 16 h, followed by treatment with or without insulin (1 μM) for 2 h. The total RNA was extracted from the cells and then analyzed for PAI-1 mRNA levels*

using RT-PCR with 18S rRNA as an internal control. (C) Fully differentiated adipocytes were treated with specific or control peptide for 16 h followed by treatment with insulin for 2 h. PAI-1 and GAPDH mRNA levels were analyzed by Northern blot.

Further to our demonstration of an inverse correlation between E2F activity and PAI-1 levels (Figure 6.4), we sought to verify whether this correlation is non-consequential or if the decrease in E2F activity is indeed responsible for the increase in PAI-1 levels during adipogenesis. To address this question, we performed RNAi-mediated gene silencing of E2F1-3 in pre-adipocytes by electroporating siRNAs that target E2F mRNA. Downregulation of E2F isoforms was found to be most effective 48 h after electroporation using protocol T-20 (data not shown). As shown in Figure 6.6A, transfection of cells with specific siRNAs directed against E2F1 and E2F2/3 mRNAs downregulated the respective proteins. Under serum-starved conditions, neither siE2F1, siE2F2/3 nor insulin treatment by themselves had any significant effect on PAI-1 levels, but insulin strongly induced PAI-1 mRNA in cells pre-treated with siE2F (Figure 6.6B). The magnitude of PAI-1 induction was highest upon depletion of E2F1-3 or E2F1 alone followed by E2F2/3. These results indicate that downregulation of E2F1-3 enhances the potential of insulin to induce PAI-1 gene expression in pre-adipocytes.

Conversely, the cell-penetrating peptide that physically disrupts the E2F-pRB binding, and thereby releases E2F isoforms (34), was found to significantly reduce insulin-induced PAI-1 mRNA levels in differentiated adipocytes (Figure 6.6C). This indicates that an increase in free E2F1-3 levels diminishes the potential of insulin to induce PAI-1 gene expression in adipocytes.

Reactivation of E2F by cell-penetrating interfering peptide reduces PAI-1 gene expression without causing cell-cycle re-entry

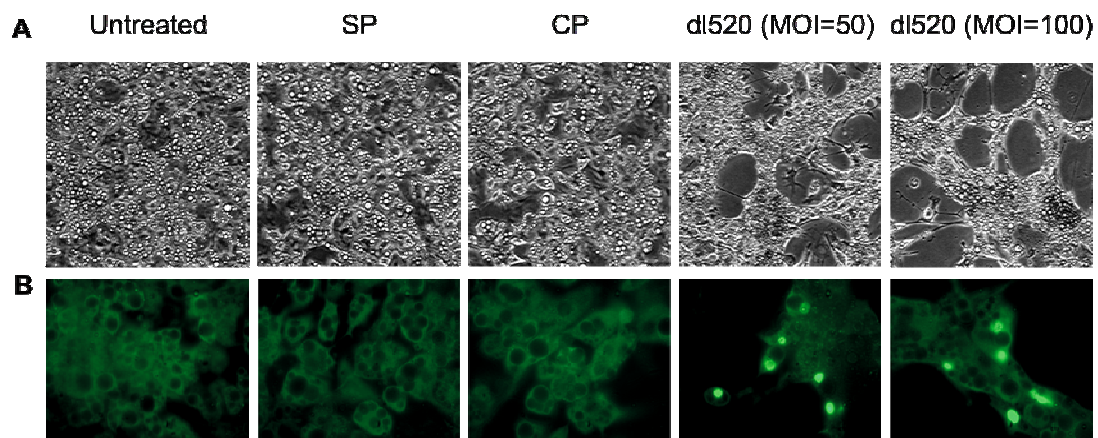


Fig. 6.7. Effect of cell-penetrating interfering peptide on BrdU incorporation. *Differentiated adipocytes were treated either with specific peptide (18 h), control peptide (18 h) or dl520 (48 h). (A) The cells were analyzed using microscopic examination and subjected to (B) BrdU incorporation assay.*

The dramatic reduction of insulin-induced PAI-1 levels by the cell-penetrating interfering peptide is supportive of the novel pharmacological paradigm of disrupting E2F-pRB interaction to suppress PAI-1 levels. The main concern in this strategy is that the forced release of free E2F may induce cell-cycle re-entry and uncontrolled cell growth in terminally differentiated cells. We therefore examined the effect of these peptides on DNA synthesis in adipocytes. Terminally differentiated adipocytes were treated with specific (40 μM) and control peptide (40 μM) for 48 h, analyzed by light microscopy and then subjected to BrdU incorporation assay. A mutant of human adenovirus type 5 (dl520) that is known to induce cell-cycle re-entry in adipocytes was used as a positive control (41). Untreated cells and cells treated with specific peptide or control peptide showed similar morphology and absence of BrdU incorporation (Figure 6.7), while the dl520-treated cells showed altered morphology and BrdU incorporation. This suggests that interfering peptide treatment does not lead to cell-cycle re-entry in terminally differentiated adipocytes.

6.6 Discussion

Adipose tissue, a major source of plasma PAI-1, is thought to be the largest endocrine organ in obese patients (42), thus offering a plausible explanation as to why elevated plasma PAI-1 levels are a biochemical hallmark of obesity. This study was aimed at understanding the molecular mechanisms which enable adipocytes to be a major PAI-1 producing organ and to pharmacologically modulate those mechanisms to inhibit PAI-1 production in adipocytes. In agreement with other studies (21), we have shown that PAI-1 gene expression is significantly upregulated during adipogenesis (Figure 6.1C). The results of nuclear run-on assay suggest that this increase is transcriptional, while DRB chase experiments rule out a role for mRNA stability (Figure 6.2). Interestingly, 3T3L1 cells, irrespective of their state of differentiation, failed to induce PAI-1 under serum-starved conditions (Figure 6.3). However, in the presence of serum (Figure 6.1C) or insulin (Figure 6.3), adipocytes, but not pre-adipocytes, showed significant PAI-1 gene expression, corresponding to the state of differentiation. Taken together, these results indicate that adipogenesis as such does not induce PAI-1; rather, it enhances the potential of cells to respond to PAI-1 inducers (e.g. insulin) in serum.

Previous studies from our laboratory have shown that free E2F1-3 act as transcriptional repressors of PAI-1 (27). This study clearly shows an association between the decrease in free E2F levels (Figure 6.4A) and the increase in PAI-1 gene expression (Figure 6.1C) during adipogenesis. The decrease in free E2F levels is mediated through at least three mechanisms: a decrease in E2F1 protein levels; an increase in pRB levels; and a decrease in pRB phosphorylation (Figure 6.5). In this analysis we did not consider the other pocket-proteins, p107 and p130, as they do not bind to E2F1-3.

Adipogenesis is accompanied by a substantial increase in insulin receptor expression (43). One could argue that the increase in insulin-induced PAI-1 gene expression during adipogenesis could be a direct result of enhanced insulin signaling (e.g. via the mitogen-activated protein kinase pathway) (44)

and the inverse correlation between free E2F1-3 levels and PAI-1 gene expression is non-consequential. In pre-adipocytes under serum-starved conditions, neither siE2F1, siE2F2/3, nor insulin treatment by themselves had any significant effect on PAI-1 mRNA levels (Figure 6.6B). However, insulin treatment could strongly induce PAI-1 gene expression in cells treated with siE2F1-3. This tells us three things. Firstly, removal of repressor activity alone is not sufficient to induce PAI-1. This requires inducers and that might explain why high plasma PAI-1 levels are often associated with high levels of inducers such as insulin and tumor necrosis factor-α (TNF-α). Secondly, in the absence of E2F1-3, insulin signaling is robust enough to induce PAI-1 in pre-adipocytes. In other words, these results challenge the notion that increases in insulin-induced PAI-1 gene expression during adipogenesis is merely a result of an increase in expression of the insulin receptor. Thirdly, the results also suggest that E2F1 is a stronger repressor than E2F2/3 in pre-adipocytes. Conversely, release of free E2F using an interfering peptide was sufficient to significantly reduce insulin-induced PAI-1 gene expression in adipocytes (Figure 6.6C). These results provide strong evidence that, irrespective of the state of differentiation, PAI-1 gene expression during adipogenesis is dependent on free E2F levels. This doubtless confirms that a reduction in free E2F levels, hence the relieving of transcriptional repression, is partly, if not wholly, responsible for the increase in PAI-1 gene expression during adipogenesis. Our findings are further supported by data from E2F1$^{-/-}$ mice that show a 5.7-fold increase in hepatic PAI-1 mRNA levels when compared to their wildtype counterparts (32).

Recently, an orally active small molecule antagonist of PAI-1 (PAI-039) was shown to inhibit plasma PAI-1 activity and accelerate fibrinolysis of coronary artery thrombosis in dogs (45) and provide protection against angiotensin II-induced aortic remodeling in mice (46). These studies offer the much-awaited proof-of-concept for PAI-1 inhibitors in treating cardiovascular complications, thereby endorsing the concept of PAI-1 inhibitors to treat secondary complications of obesity. Since the reactivation of E2F using cell-penetrating interfering peptide could be successfully employed to reduce

PAI-1 levels in terminally differentiated adipocytes (Figure 6.6), small molecule antagonists of E2F-pRB interaction may offer therapeutic value. E2F isoforms that bind to pRB (E2F1-3) are transcriptional factors that drive cell proliferation and apoptosis. Therefore, the pharmacological strategy of disrupting E2F-pRB interaction raises a major concern because it may lead to unwanted effects such as cell-cycle re-entry in terminally differentiated adipocytes. For example, adenoviruses harboring E1A could force terminally differentiated Adipo5-2 adipocytes and C2C12 myoblasts to re-enter the cell cycle (41). Although E1A is known to act on multiple targets, including E2F, p300, pRB, p107, p130, TBP and AP-1, E2F activity was required for E1A-mediated cell-cycle re-entry (47). On the other hand, it has been shown that E2F activation alone, either by overexpressing E2F (47) or by downregulating pRB (48), does not cause cell-cycle re-entry in terminally differentiated skeletal muscles. In line with these observations, we show that the cell-penetrating peptide that releases free E2F and thereby suppresses PAI-1 gene expression does not cause cell cycle re-entry (Figure 7).

Although our data linking the decrease in E2F activity to an increase in PAI-1 gene expression are limited to adipocyte differentiation, the results could have wider implications. It has been shown that E2F1-3 regulate many genes that are involved in cell proliferation and differentiation (33). Furthermore, reduction in the activity or levels of E2F isoforms (mainly E2F1) is critical for cell cycle arrest and subsequent differentiation of many cell types, including cardiomyocytes (49), C2C12 myoblasts (50), hepatocytes (51), platelets (52), keratinocytes (53), quail neuronal retina (54), P19 neurocytes (50) and embryonic granulocytes (55). This might explain why the major sources of plasma PAI-1 happen to be differentiated cell types such as adipocytes, skeletal muscles, hepatocytes, vascular endothelial cells and platelets, and therefore warrant further investigation into the role of E2F in mediating PAI-1 gene expression in these cell types.

Elevated levels of PAI-1 in obesity are considered to be at least in part due to direct stimulation of adipocytes by growth factors, cytokines and

hormones (e.g. insulin, TNF-α and TGF-β) that are by themselves elevated under conditions of obesity (17). This may be achieved by the enhanced sensitivity acquired by adipocytes to respond to PAI-1 inducing agents. By using peptides that release endogenous E2F (bound to pRB), and thereby restoring the E2F-mediated transcriptional repression, we could desensitize the adipocytes to PAI-1-inducing agents without causing unwanted effects such as cell-cycle re-entry.

6.7 Working model

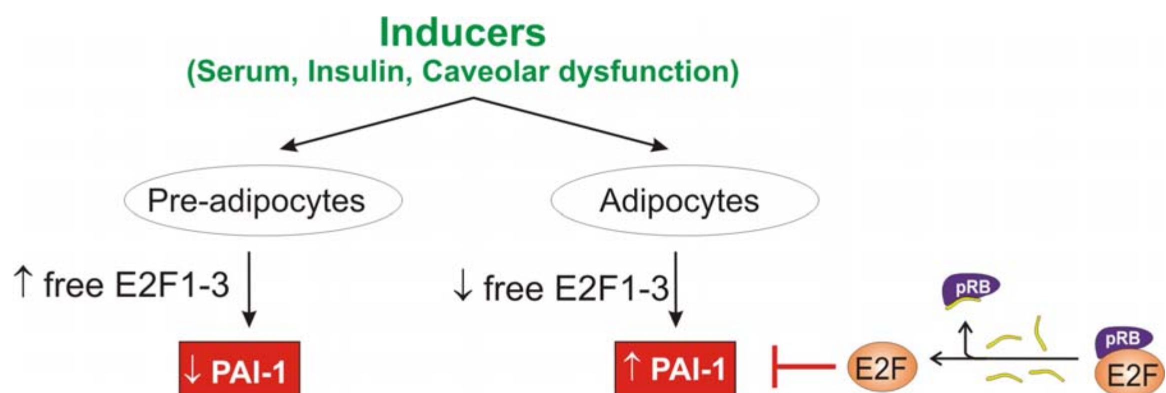


Figure 6.8: *Inducers such as serum, insulin (show in part-3) and caveolar dysfunction (show in part-1) can upregulate PAI-1 gene expression in adipocytes but not so much in pre-adipocytes. Reduction of free E2F-mediated transcriptional repression during adipocyte differentiation is primarily responsible for the increase in PAI-1 biosynthetic capacity of adipocytes, allowing inducers to elicit an increased response. Antagonists of E2F-pRB interaction that can release free E2F in adipocytes can suppress PAI-1 production in adipocytes and may thus offer therapeutic value.*

6.8 Acknowledgements

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Institute, Dublin) and Sandra Kleiner (Friedrich Miescher Institute) for critical reading of the manuscript. This work was partly supported by the Roche Research Foundation (fellowship to K.H.).

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7. General discussion of the thesis

This thesis shows that caveolar microdomains play an important role in maintaining the integrity of cellular signaling. We reason, but do not prove, that obesity is accompanied by caveolar dysfunction. We show that perturbation of caveolar function leads to insulin resistance in 3T3L1

adipocytes. We also present some evidence that caveolar dysfunction activates PKC and glucosamine pathways probably by the inhibition of membrane-associated glycolysis in adipocytes. Insulin resistance is the central feature of T2DM, while inhibition of glycolysis and activation of PKC and glucosamine pathways are the characteristic signaling defects considered to be associated with diabesity®-induced cardiovascular disorders. Using caveolin-1 deficient mice as a prototype for caveolar dysfunction, we will discuss (see next page) if caveolar dysfunction *per se* can lead to diabetes and vascular defects *in vivo*. Notably, all these signaling defects mentioned earlier can independently lead to (but also activate several other pathways) the upregulation of PAI-1 that by its own can induce obesity-driven secondary disorders. Hence, PAI-1 may represent a nodal and downstream drug target. Our studies show that pharmacological antagonism of E2F-pRB interaction can be employed to suppress PAI-1 gene expression.

7.1 *In vivo* evidence for the causative role of caveolar dysfunction in diabetes

Cav-1 null mouse develop an interesting metabolic phenotype. Preliminary analysis of this phenotype showed that Cav-1 null mice are resistant to diet-induced obesity and developed progressive adipose tissue atrophy (1). Metabolically, the abnormalities in Cav-1 (–/–) mice are characterized by elevated free fatty acid and triglyceride levels, decreased leptin and Acrp30 levels, and no changes in plasma insulin or glucose levels (1). However, when these mice were later placed on a high-fat diet for 9 mo, they were found to develop postprandial hyperinsulinemia (2). Additionally, when young Cav-1 null mice were challenged with an insulin tolerance test (ITT), they showed markedly decreased glucose uptake compared with wild-type control animals. These metabolic derangements are similar to those seen in prediabetic individuals in the human population, suggesting that caveolin-1 does indeed play a critical role in insulin signaling in vivo. It was also shown that the metabolic signaling downstream of insulin receptor was adversely affected in this mice, although dramatic downregulation of insulin receptor

was found to be the cause. It has to be noted that this does not correspond to the patient or animal models of diabetes. However the insulin signaling defects in our studies correspond well with the clinical and animal data. The discrepancy between the insulin signaling defects observed in the caveolin null mice and caveolar perturbation (in vitro) could be due to the duration (acute/chronic) and dosage (reduction/absence).

In light of current data, caveolin-1 can be thought of as a major player in insulin signaling in tissues where it is expressed; however, loss of caveolin-1 is not sufficient to produce fulminant diabetes. This is consistent with other mouse models, specifically the adipose tissue selective insulin receptor knockout mouse (FIRKO) (3). These mice phenotypically have relatively mild full-body insulin resistance despite a significant reduction in the insulin-responsiveness of isolated adipocytes. Also of note are findings that a subset of patients with severe insulin resistance were found to have mutations in the caveolin-binding motif of the insulin receptor (4). Taken together, these findings further support the idea that caveolin-1 may be critical for insulin signaling in the human population and that perhaps some diabetic patients exist with caveolin-1 mutations.

7.2 In vivo evidence for the causative role of caveolar dysfunction in cardiovascular complications

Cardiovascular complications represent one of the most striking phenotypes of caveolin knock out mice. Analysis of the phenotype of mice with genetic deletions or overexpression of specific caveolin isoforms has provided key evidence for the importance of caveolins and caveolae in several aspects of the cardiovascular biology, including vascular contractility, lipid metabolism, angiogenesis, or the control of cardiac hypertrophy. The cardiovascular defects observed in these mice are summarized in the following table:

Genes	Expression	Cardiovascular Phenotypes/Abnormalities in Cav-Knockout (KO) Mice

Cav-1	Endothelia, smooth muscle cells, macrophages, and cardiac fibroblasts	Cav-1 KO: lung hypercellularity; pulmonary hypertension/fibrosis; increased microvascular permeability; decreased angiogenesis capacity; athero-protection; increased neointimal hyperplasia (smooth muscle cell proliferation); arterial hyper-relaxation/decreased vascular tone; hypertrophic cardiomyopathy due to the hyperproliferation of cardiac fibroblasts; reductions in life span; hyperactivation of eNOS and Ras-p42/44 MAP kinase signaling cascades in endothelial cells and cardiac fibroblasts, respectively.
Cav-2	Same as Cav-1	Cav-2 KO: lung hypercellularity; pulmonary hypertension/fibrosis without hypertrophic cardiomyopathy.
Cav-3	Smooth muscle cells and cardiac myocytes	Cav-3 KO: hypertrophic cardiomyopathy and hyperactivation of the Ras-p42/44 MAP kinase signaling cascade in cardiac myocytes. Also, a human Cav-3 (T63S) mutation has been discovered in patients with familial hypertrophic cardiomyopathy (FHCM) (inherited).
Cav-1+3		Cav-1/3 dKO: severe hypertrophic cardiomyopathy in double-KO mice due to loss of caveolins in both cardiac fibroblasts (Cav-1) and cardiac myocytes (Cav-3).

Table. 7.1: Summary of the cardiovascular pheotype of the caveolin knock out mice

In an attempt to find the underlying pathological mechanism, some of these studies have identified certain signal transduction defects that are know to result in cardiovascular complications. It has been shown that the caveolar platform is required for muscarinic and β-adrenergic receptor mediated activation of eNOS signaling (5). Based on this and other evidence, it has

recently been proposed that the cardiovascular defects in caveolin deficient mice may partly be due to defective eNOS signaling. Our study proposes a strong link between caveolar dysfunction and inhibition of glycolysis and subsequent activation of four pathways that are implicated in the hyperglycemia-induced vascular damage. This is in agreement with other studies, which propose that glycolysis may be localized to the caveolar microdomain. Our study is the first report that links caveolar dysfunction to the unifying theory of diabetes-induced cardiovascular complications (Brownlee's hypothesis) (6) and may further explain why caveolin null mice develop severe cardiovascular abnormalities.

7.3 Can caveolar dysfunction lead to cancer as well?

Our studies primarily address the potential link between caveolar dysfunction and two major complications of obesity, namely diabetes and cardiovascular complications. However there is increasing evidence linking caveolar dysfunction to cancer, whose association with obesity is currently being proposed (7). It has been suggested that caveolin-1 acts as a tumor suppressor protein, inhibiting the signaling of several proto-oncogenes and consequently disrupting the process of cellular transformation (8) (9) (10, 11). Numerous follow-up studies designed to test this hypothesis have contributed a myriad of evidence suggesting that caveolin-1 may indeed possess tumor suppressor capabilities. For instance, caveolin-1 mRNA and protein expression are downregulated in NIH-3T3 cells transformed with several activated oncogenes, such as v-Abl, Bcr-Abl, and H-Ras (G12V) (8, 12). The ability of these transformed cells to grow in soft agar, a hallmark of cellular transformation, was found to inversely correlate with caveolin-1 protein levels. The reintroduction of caveolin-1 under the control of an inducible promoter was sufficient to inhibit the anchorage-independent growth of these cells, thus reverting them to their transformed phenotype (8). It has been shown that targeted downregulation of caveolin-1 using a vector-based anti-sense approach resulted in the transformation of NIH-3T3 cells, enhanced their anchorage independent growth, and hyperactivated the Ras-

p42/44 mitogen-activated protein (MAP) kinase cascade (10). When injected into nude mice, these NIH-3T3 cells expressing the caveolin-1 anti-sense construct were capable of forming large tumors, compared with matched NIH-3T3 cells lacking the caveolin-1 anti-sense vector.

Genetic evidence supporting the role of caveolin-1 as a tumor suppressor has emerged from gene mapping studies, which revealed that the human CAV-1 gene maps to the long arm of human chromosome 7 (7q31.1). This region, the D7S522 locus, encompasses a known fragile site (FRA7G) and is often associated with loss of heterozygosity (LOH) in various cancers, including breast, prostate, ovarian, and renal carcinomas (13, 14). While no genes have been directly mapped to the D7S522 locus, the closest genes to this region encode caveolin-2 and caveolin-1 (15) (16). Furthermore, the CAV-1 promoter has been reported to be hypermethylated in several cancer cell lines (16).

Thus the loss of caveolin-1 alone appears insufficient to induce cell transformation in vivo, but loss of caveolin-1 potentiates this process when combined with a transforming agent (a carcinogen or tumor-prone genetic background) (17, 18). This is not surprising, as the process of cell transformation and the development of cancer in vivo is a multistep process that involves the selective and progressive loss of several tumor suppressors (such as p53, INK4a, and Rb), as well as the mutational activation or upregulation of certain key protooncogenes [Ras(G12V), c-Myc, and c-Neu/ErbB2]. Indeed, the etiology of most cancers does not reflect alterations in a single gene, but rather the functional loss or induction of a series of key regulatory proteins that, in combination, disrupt the normal regulation of the cell cycle and subsequently lead to uncontrolled cell growth.

7.4 PAI-1: is it a viable drug target?

We have now discussed the effects of caveolar dysfunction in-vitro and in-vivo. Based on these observations it is perfectly plausible to assume that

caveolar dysfunction can lead to insulin resistance, activation of PKC and glucosamine pathways. We also show that these signaling defects independently converge to activate PAI-1 gene expression, an important mediator of obesity-driven disorders (19). These observations taken together, PAI-1 may represent a nodal and downstream drug target.

There has been considerable interest in the pharmaceutical industry to develop inhibitors of PAI-1. Inhibitory monoclonal antibodies to PAI-1 have been used in preclinical models of acute thrombosis and shown to enhance endogenous fibrinolysis (20). The modulation of PAI-1 by orally-active drugs has however remained a significant challenge. The rational design of inhibitors has proved a challenge due to the lack of a pharmacophore. Wyeth undertook the HTS path, screening 35000 compounds for PAI-1 inhibitory activity. From the 300 hits that were investigated in follow up studies the small molecule inhibitor; tiplaxtinin (PAI-039) was selected for development. This inhibitor has been reported to produce pro-fibrinolytic activity in a rodent model of acute arterial thrombosis (21). Recently, in the canine model of coronary thrombosis, pre-treatment with PAI-039 produced beneficial effects (22). In this paper the authors report that under control conditions, electrolytic injury to the coronary artery caused progressive occlusion and blood flow was eventually permanently interrupted. Pretreatment with PAI-039 prolonged the onset of coronary occlusion and even when permanent occlusion was observed spontaneous coronary reperfusion occurred in 60% of the dogs studied. This was accompanied by a decrease in thrombus weight. However, there was no observable effect on platelet aggregation or bleeding time.

This study offers the pre-clinical proof-of-concept for the efficacy of a small molecule inhibitor of PAI-1 in a model of thrombosis. Therapeutic activity is related to the removal of ongoing inhibition of endogenous t-PA. Thus PAI-039 could be developed as a monotherapy with a wide therapeutic window. Inhibiting PAI-1 rather than administering exogenous t-PA represents a rational approach since PAI-1 levels are elevated in type 2 diabetes and

atherosclerosis, both risk factors for myocardial infarction, peripheral arterial disease and stroke. Hence increased PAI-1 activity could conceivably shift the homeostatic balance in favor of thrombosis in these disease states. A combinatorial approach based on the administration of PAI-039 along side t-PA also offer significant opportunities since elevated PAI-1 has been suggested to contribute to the therapeutic failure of t-PA in the treatment of peripheral arterial thrombolysis. Incorporating PAI-039 into the treatment of these patients could therefore improve the therapeutic activity of t-PA in these patients. More broadly the use of PAI-039 could be employed as a general strategy to reduce the dosage of t-PA administered to patients in a variety of acute cardiovascular settings. Reducing the dosage in this fashion is anticipated to widen the therapeutic window for t-PA by improving the thrombolytic activity of t-PA while at the same time reducing the risk of intracerebral hemorrhage and bleeding.

Early research on PAI-1 was primarily focused on its role in cardiovascular system and it was only recently that its potential role in T2DM was discovered (23-25) (26). Very recently, it was shown that PAI-039 can increase insulin sensitivity in wild-type mice (27).

Wyeth is now developing an orally administered PAI-1 inhibitor (PAI-749) for the treatment of peripheral vascular disease and other thrombolytic disorders. The compound had entered Phase I clinical studies in the US by June 2004. Other pharmaceutical firms such as Xenova also have pre-clinical research programs to develop PAI-1 inhibitors.

Our studies show that certain tissues such as adipose tissue are responsive to PAI-1 inducing signals because of the loss of free E2F-mediated transcriptional repression of the PAI-1 promoter during cellular differentiation. By disrupting the E2F-pRB interaction and thereby releasing free E2F we could desensitize the differentiated adipocytes to both physiological (insulin) and pathological (caveolar dysfunction) inducers of PAI-1 gene expression without causing unwanted effects such as cell cycle re-entry. This may

highlight a new drug target that is highly amenable to pharmacological modulation, especially since the crystal structure of E2F-1 bound to pRB has recently been solved, and high throughput screening for small molecular antagonists of E2F-pRB interaction is currently being carried out.

7.5 References for discussion

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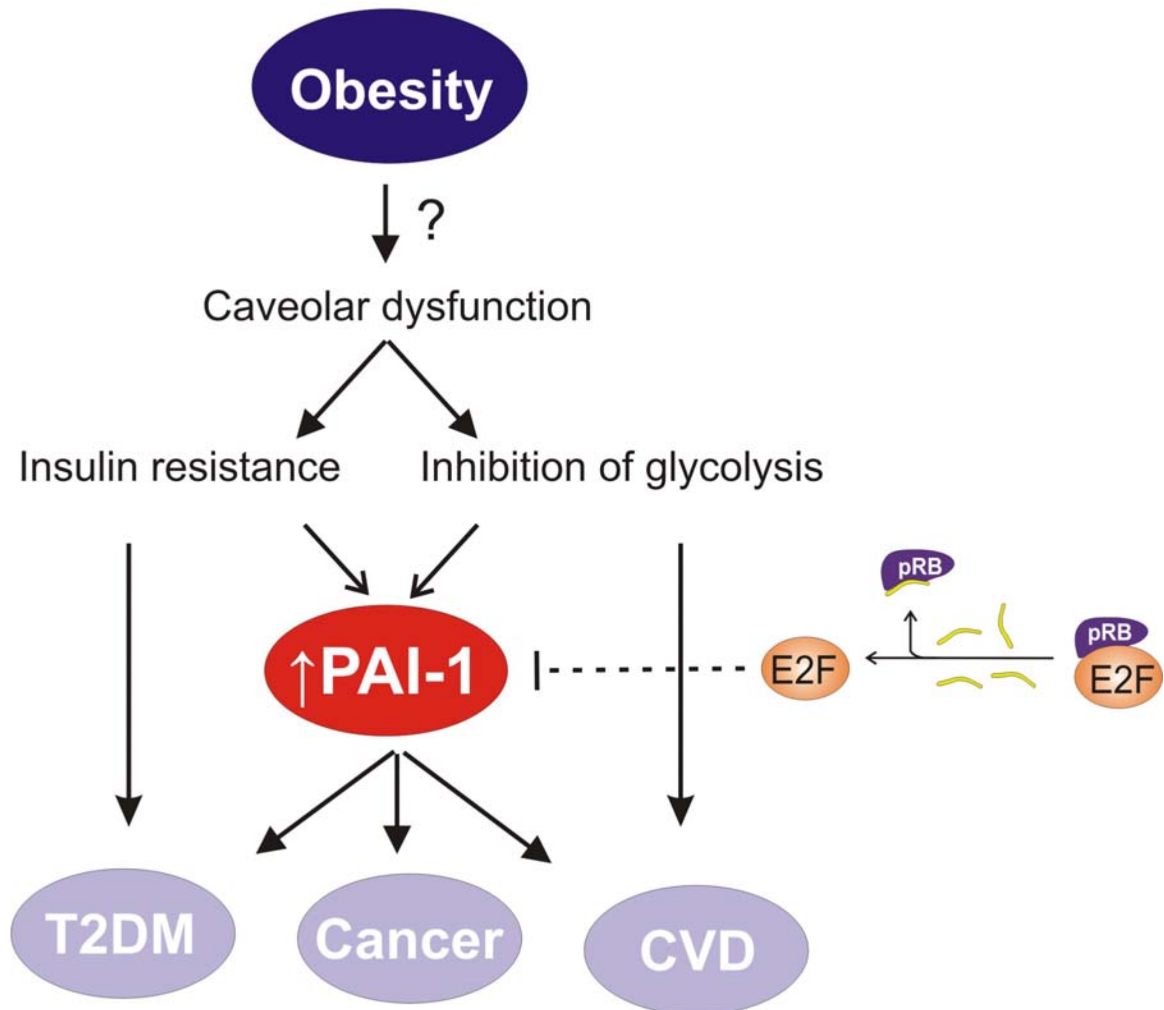
8. Conclusion



In effect, we show that caveolar microdomains are a key signal transduction platform, whose perturbation can lead to a plethora of signaling defects that are known to elicit secondary-disorders of obesity. Elevated levels of PAI-1 represent a 'nodal' and 'downstream' mediator of these signaling defects. By using peptide antagonists of E2F-pRB interaction, we could inhibit PAI-1 gene expression in adipocytes without causing unwanted effects such as cell-cycle reentry.

9. Outlook

Caveolar dysfunction: natural cause of obesity-driven disorders?

The in-vitro data presented in this thesis and the in-vivo data discussed earlier, offer strong evidence for the ability of caveolar dysfunction to induce secondary disorders of obesity such as T2DM and cardiovascular disorders. The reduction in the levels of two essential components of caveolae in obesity raises the possibility of caveolar dysfunction to occur in obese patients. However, it remains to be investigated if caveolar dysfunction can indeed be a natural cause of obesity-induced secondary disorders. This can be experimentally verified in in ob/ob mice and obese patients by checking the structural and functional integrity of caveolae using microscopic analysis and functional studies respectively. Conversely, one could also look at the susceptibility of transgenic mice overexpressing caveolin-1/3 to high-fat diet induced obesity, insulin resistance and cardiovascular disorders. Additionally this mouse can be crossed with ob/ob mice and the same experiments can be performed.

Role of PAI-1 in mediating caveolar dysfunction induced disorders

We have shown that caveolin-1 knock-down leads to PAI-1 upregulation in-vitro. To verify this in-vivo, plasma PAI-1 levels in caveolin-1 knock out mice needs to be measured. If the PAI-1 levels are elevated as expected, then it would be of considerable interest to study the role of PAI-1 in mediating the secondary disorders. This can be achieved by studying the effect of PAI-1 inhibitors in retarding the insulin resistance and vascular disorders observed Cav-1 KO mice. Another approach would be to cross Cav-1 KO mice with PAI-1 KO mice and study the insulin sensitivity and vascular function in the offspring. It would also be interesting to test how effective inhibitors of E2F-pRB inhibitors are in inhibiting PAI-1 production.

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11. Appendix-1: Chief components of the plasminogen System

Plasminogen consists of 791 amino acids as determined by cDNA sequencing, although originally 790 amino acids were identified by protein sequencing. It is organized in seven structural domains, comprising a "preactivation peptide" (amino acid residues 1-77), 5 sequential homologous kringle domains (disulfide bonded triple loop structures of about 80 residues each), and the proteinase domain (residues 562-791). The kringle domains contain lysine-binding sites that play a crucial role in the specific binding to fibrin, cell surfaces and α2-antiplasmin. Plasminogen is converted to plasmin by cleavage of a single Arg561-Val562 peptide bond.

Tissue-type plasminogen activator (t-PA) consists of 530 amino acids, although originally 527 were identified. It is composed of several domains with homologies to other proteins: a finger domain (residues 4-50), a growth factor domain (residues 50-87), two kringles of about 80 residues, and the protease domain (residues 276-527), comprising the catalytic triad. Binding of t-PA to fibrin is most likely mediated via the finger and the second kringle domains.

Urokinase-type plasminogen activator (u-PA) is composed of an epidermal growth factor domain, one kringle domain and a protease domain containing the catalytic triad. The epidermal growth factor domain is responsible for the binding of u-PA to its receptor, which is present on the surface of a variety of cells. Single chain u-PA is converted to two chain u-PA by cleavage of the Lys158-Ile159 peptide bond.

α2-Antiplasmin (α2-AP, α2-plasmin inhibitor) was originally isolated as a glycoprotein containing 452 amino acids but it was later shown that native α 2-AP contains 464 amino acids. It is unique among serpins by having a COOH-terminal extension of 51 amino acid residues, which contains a binding site that reacts with the lysine-binding sites of both plasminogen and plasmin. The NH2-terminal Gln14 residue of α2-AP (Gln2 in the original numbering system) can cross-link to Aα-chains of fibrin, in a process which requires Ca2+ and is catalyzed by activated coagulation factor XIII.

The two most important **plasminogen activator inhibitors (PAIs) are PAI-1 and PAI-2**. PAI-1 is stabilized by a tight binding to the cell adhesion protein vitronectin. PAI-2 exists in two different forms with comparable kinetic properties, a 47 kDa intracellular non-glycosylated form with pI 5.0 and a 60 kDa secreted glycosylated form.

The specific cell surface **u-PA receptor (u-PAR)** is synthesized as a 313 amino acid polypeptide, which is post-translationally processed at the COOH-terminus into a protein of 283 amino acids anchored to the plasma membrane by a glycosyl phosphatidylinositol (GPI) moiety. It binds all forms

of u-PA containing an intact growth factor domain, with high affinity. It is composed of three distantly related homologous structural domains, of which the NH2-terminal one binds u-PA.

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Nov 2000 - Sep 2001	**Temporary Lectureship,** **National University of Ireland**, Galway, Republic of Ireland	Introduced new technologies; wrote a successful research grant; supervised two post-graduate projects; framed new curriculum.
July 1999 - Sep 1999	**Consultant,** **Spidersoft Services**, Trivandrum, India	Developed retail pharmacy management software that commands the largest local market share. Key role in strategic planning and database development.

Key Research Publications

- **Venugopal J**, Hanashiro K, and Nagamine Y **(2006)** Acquired PAI-1 biosynthetic capacity during adipogenesis: role of E2F-mediated transcriptional repression, *(under editorial review).*
- **Venugopal J**, Hanashiro K, Yang ZZ and Nagamine Y **(2004)** Identification and pharmacological modulation of a caveolae-dependent signal pathway that upregulates PAI-1 in insulin-resistant adipocytes, **Proceedings of the National Academy of Sciences (USA)** 101(49), 17120-171205.
- **Venugopal J (2003)** Pharmacological modulation of natriuretic peptide system, **Expert Opinion in Therapeutic Patents**, 13(9), 1389-1409.
- **Venugopal J (2001)** Cardiac natriuretic peptides: Hope or hype? **Journal of Clinical Pharmacology & Therapeutics**, 26(1), 15 – 31.

Awards and Honours

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- Listed as an **Expert in public communications of sciences** by **Europa** (EU portal).
- **Editorial Board Advisor** of the journal '**Recent Patents on CNS Drug Discovery'**.
- **Special Achievement Award** from the **XVIIth ISFP Congress** (2004), Melbourne.
- **International PhD Program Scholarship** from **Friedrich Miescher Institute**.
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Strategic Thinking (Public communication)

- **Venugopal J (2005)** Virtual laboratories: globalizing research, **Nature** (invited; under preparation)
- **Venugopal J (2005)** The power of collective wisdom, **The Financial Express**, Dec 11, 4.
- **Venugopal J (2005)** Drug imports: the free-rider paradox, **Express Pharma Pulse**, 11(9), 8
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- **Venugopal J (2001)** The question of ethics in biomedical research, **Express Pharma Pulse,** 8(5), S23-S25
- **Venugopal J (2000)** R&D: What are we upto?, **Express Pharma Pulse**, 7(1), 4 -5

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- **Workshop on intellectual property protection** organised by the Swiss Academy of Sciences, Nov 4-6, 2004, **Thun**, Switzerland *(invited to attend).*
- **FMI Annual meeting,** 16-19 Sep 2004, **Crans Montana**, Switzerland *(Oral presentation).*
- XVIIth **ISFP Congress**, 21-25 March, 2004, **Melbourne,** Australia *(Oral presentation).*
- **Novartis-FMI joint symposium,** March, 2004, **Furingen,** Switzerland *(Oral presentation).*
- **IXth International Workshop** on Molecular & Cellular Biology of PA, 19-23 Oct, 2003, **Isle of Capri,** Italy (Poster presentation).
- **Summer session of Royal Academy of Medicine, Limerick,** June 2001, Ireland (Oral presentation).

Other interests

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