Fatty Acids and their Metabolism Critically Regulate Podocyte Survival

Inauguraldissertation

zur
Erlangung der Würde eines Doktors der Philosophie
vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel

von

Kapil Dev Kampe

aus Hyderabad, Indien

Basel, 2014

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät auf Antrag von

Prof. Dr. Ed Palmer

Prof. Dr. Marc Donath

PD Dr. Andreas Jehle

Basel, den 10. Dezember 2013

Prof. Dr. Jörg Schibler

Dekan der Philosophisch-Naturwissenschaftlichen Fakultät

ABSTRACT

Diabetic nephropathy (DN) is the most common cause of end-stage renal disease in industrialized countries, and most affected patients have type 2 diabetes. Podocyte injury and loss are considered critical in the development, and progression of DN. Several factors of the diabetic milieu are well known to impair function and survival of podocytes. However, the role of free fatty acids (FFAs), which are elevated in type 2 diabetes, and the role of their metabolism are just emerging in the pathogenesis of DN. FFAs were reported to regulate podocyte survival. Saturated FFAs, i.e. palmitic acid, were found to induce endoplasmic reticulum (ER) stress and podocyte death, whereas monounsaturated FFAs, i.e. palmitoleic acid or oleic acid, were protective.

The aims of the present study were to investigate whether FFA metabolism is regulated in glomeruli of type 2 diabetic patients with DN and whether regulation of FFA metabolism affects the susceptibility of podocytes towards palmitic acid. Particularly, I aimed to investigate whether regulation of fatty acid oxidation (FAO) modifies palmitic acid-induced podocyte death. As genome wide association studies suggest that acetyl CoA carboxylase (ACC) 2, an important enzyme in the regulation of FAO, is involved in the pathogenesis of DN, I performed detailed studies investigating the role of ACCs in podocytes. Furthermore, I explored the effect of palmitic acid on podocytes in combination with well-known proapoptotic stimuli of the diabetic milieu.

The present study uncovered that palmitic acid can aggravate the toxicity of other factors which are known to be important in the pathogenesis of DN and which are considered to cause podocyte loss. In particular the toxicity of high glucose concentrations and transforming growth factor (TGF)-β are substantially increased by palmitic acid, whereas the effect of palmitic acid on tumor necrosis factor (TNF)-α induced podocyte death is discret.

In the main part of this study FFA metabolism and its effect on palmitic acid induced podocyte death was investigated. The study finds that in glomeruli of type 2 diabetic patients mRNA expression levels of several key enzymes involved in fatty acid metabolism are altered. Of particular relevance for my detailed studies on FAO, a significant upregulation of all three isoforms of carnitine palmitoyltransferase (CPT)-1, the rate-limiting enzyme for

FAO, and a downregulation of ACC-2, which catalyzes the formation of the CPT-1 inhibitor malonyl-CoA, are found which suggest a disposition for increased FAO. In vitro, stimulation of FAO by aminoimidazole-4-carboxamide-1β-D-ribofuranoside (Aicar) or by adiponectin, activators of the low-energy sensor AMP-activated protein kinase (AMPK), protect from palmitic acid induced podocyte death. Conversely, inhibition of CPT-1, a downstream target of AMPK, by etomoxir augments palmitic acid toxicity and impedes the protective Aicar effect. Etomoxir blocked the Aicar induced FAO measured with tritium labeled palmitic acid. Of note, only double knockdown of ACC1 and ACC2 has a protective effect on palmitic acid induced cell death, which indicates that both isoforms contribute to the regulation of FAO in podocytes. Furthermore, the effect of Aicar is associated with a reduction of ER-stress as indicated by a significant attenuation of the palmitic acid induced upregulation of immunoglobulin heavy chain binding protein (BiP), an ER chaperone, and of the proapoptotic transcription factor C/EBP homologous protein (CHOP).

In conclusion, palmitic acid increases the toxicity of other factors known to contribute to podocyte loss, which underlines the potentially important contribution of elevated saturated FFAs in the pathogenesis of DN. An important role of FFAs and of their metabolism in the pathogenesis of DN is further suggested by profound changes in gene expression levels of key enzymes of FFA metabolism in glomerular extracts of type 2 diabetic patients. The changed expression profile indicates a compensatory, protective response. Moreover, the results of this study uncover that stimulation of FAO by modulating the AMPK-ACC-CPT-1 pathway protects from palmitic acid induced podocyte death. The results of this study should encourage further investigations to evaluate the therapeutic potential of interfering with FFA metabolism specifically with stimulating FAO for the prevention and therapy of DN.

TABLE OF CONTENTS

ABSTRACT ..... **I**

LIST OF FIGURES AND TABLES ..... **VI**

List of Figures ..... **VI**

List of Tables ..... **VII**

List of ABBREVIATIONS ..... **VIII**

1. Introduction **1**

1.1. Diabetic Nephropathy (DN) **1**

1.1.1. Incidence and prevalence of DN 1

1.1.2. Pathophysiology of DN 2

1.1.3. The role of podocytes in the pathogenesis of DN 2

1.1.4. Factors contributing to apoptosis of podocytes in DN 3

1.2. Lipotoxicity **4**

1.2.1. Lipotoxicity in Diabetic Nephropathy 4

1.2.2. Lipotoxicity: The role of free fatty acids 5

1.2.2.1. The toxicity of saturated free fatty acids 5

1.2.2.2. Modulating pathways and the role of monounsaturated free fatty acids 8

1.2.3. Free fatty acids and their metabolism in podocytes 8

1.2.3.1. Regulation of podocyte survival by free fatty acids 8

1.2.3.2. Susceptibility of Podocytes to Palmitic Acid Is Regulated by Stearoyl-CoA Desaturases 1 and 2 …..... 10

1.2.3.3. Lipotoxicity: Modulation by fatty acid oxidation and the role of ACCs 11

1.3. Aim of the study **13**

2. MATERIALS and METHODS **14**

2.1. Cell culture **14**

2.2. Agonists, inhibitors and cytokines 15

2.3. Free fatty acids preparation 15

2.4. Apoptosis assay 15

2.5. ACC1 and ACC2 knock down 16

2.5.1. shRNA sequences and lentiviral expression vector 16

2.5.2. Lentiviral production 16

2.6. Western Blot 17

2.7. β-oxidation measurement 18

2.8. Statistical analysis 19

3. Results 20

3.1. Palmitic acid induced podocyte death: Modification by high glucose, TGF-β, and TNF α 20

3.1.1. Palmitic acid uncovers the toxicity of high glucose concentrations 20

3.1.2. TGF-β aggravates palmitic acid induced podocyte death 21

3.1.3. TNF alpha aggravates palmitic acid induced podocyte death 22

3.2. Regulation of fatty acid oxidation in palmitic acid induced podocyte cell death: Critical role of Acetyl CoA carboxylase 1 and 2 23

3.2.1. Differential regulation of genes involved in fatty acid metabolism in glomeruli of patients with established DN 23

3.2.2. Modulation of fatty acid oxidation and its effect on palmitic acid induced podocyte death 23

3.2.2.1. AMPK activation protects from palmitic acid induced cytotoxicity 23

3.2.2.2. Inhibition of AMPK exacerbates palmitic acid induced cell death and reversed the protection caused by Aicar. 26

3.2.2.3. Etomoxir aggravates palmitic acid induced podocyte death and reverses the protective Aicar effect 27

3.2.2.4. Aicar stimulates and etomoxir inhibits fatty acid oxidation 28

3.2.2.5. Combined silencing of ACC1 and ACC2 protects from palmitic acid induced podocyte death 29

3.2.2.6. Aicar reduces ER-stress and the upregulation of CHOP 32

4. Discussion 34

4.1. Aggravation of palmitic acid induced podocyte death by high glucose, TGF-β, and TNF-α 34

4.2. Regulation of fatty acid oxidation in palmitic acid induced podocyte death: critical role of acetyl CoA carboxylase 1 and 2 35

5. Conclusion 38

6. References 40

7. Acknowledgements 49

7.1 Acknowledgements for micro-array data 50

APPENDIX ..... a

American Journal of Physiology, Renal Physiology Article ..... a

LIST OF FIGURES AND TABLES

List of Figures

Figure 1: Prevalence of ESRD by primary diagnosis 1

Figure 2: Structure of the glomerular filtration barrier 2

Figure 3: ACC2 inhibits CPT-1 by the production of malonyl-CoA from acetyl-CoA..... 5

Figure 4: ER-stress and the unfolded protein response (UPR) 7

Figure 5: Palmitic acid induces apoptosis and necrosis of podocytes in a dose-dependent manner 9

Figure 6: Dose-and time-dependent induction of CHOP in podocytes by palmitic acid 9

Figure 7: Overexpressing SCD-1 partially protects from palmitic acid-induced apoptosis ... 10

Figure 8: Aicar stimulates fatty acid oxidation 11

Figure 9: High glucose accentuates the to toxicity caused by palmitic acid in podocytes 21

Figure 10: TGFβ increases the toxicity caused by palmitic acid in podocytes 21

Figure 11: TNFα induces podocyte death and aggravates palmitic acid induced toxicity..... 22

Figure 12: Differential expression of genes related to fatty acid metabolism in glomeruli of DN patients 23

Figure 13: Scheme representing the metabolic path activated by Aicar and Adiponectin 24

Figure 14: Aicar phosphorylates AMPK and ACC..... 24

Figure 15: Aicar attenuates palmitic acid induced podocyte death..... 25

Figure 16: Adiponectin decreases apoptosis induced by palmitic acid..... 25

Figure 18: Compound C accentuates palmitic acid induced podocyte death and partially reverses the protection by Aicar 27

Figure 19: Etomoxir exacerbates palmitic acid induced podocyte death..... 28

Figure 20: Etomoxir reverses the protection by Aicar for palmitc acid induced podocyte death 28

Figure 22: Etomoxir prevents the Aicar induced β-oxidation 29

Figure 23: Immunoblot for ACC1, ACC2 and ACC1/2 knock down 30

Figure 24: ACC1 or ACC2 single knockdown is not protective for palmtitic acid induced podocyte death 30

Figure 25: Combined knock down of ACC1 and ACC2 protects podocytes from palmitic acid induced cell death 31

Figure 26: Combined knock down of ACC1 and ACC2 with a second set of shRNAs against ACC1 and ACC2 protects podocytes from palmitic acid induced cell death 31

Figure 27: Modest effect of Aicar in ACC1/ACC2 double silenced podocytes 32

Figure 28: Aicar mitigates palmitic acid induced ER-stress 33

Figure 29: A working model for the Aicar activated AMPK-ACC-CPT-1 pathway and the prosurvival effects of oleic acid and Scd-1/-2 on palmitic acid-induced podocyte death. 39

List of Tables

Table 1: Agonists, inhibitors and cytokines 15

Table 2: shRNA sequences and respective plasmids. 16

Table 3: Summary of transfection conditions for the production of lentiviral particles. 17

Table 4: List of primary and secondary antibodies for western blot. 18

LIST OF ABBREVIATIONS

ACC	Acetyl-CoA carboxylase
Aicar	5-aminoimidazole-4-carboxyamide ribonucleoside
AMPK	AMP-activated protein kinase
ATF	Activating transcription factor
ATP	Adenosine triphosphate
BiP	Immunoglobulin heavy chain binding protein
BSA	Bovine serum albumin
CHOP	C/EBP homologous protein
CPT-1	Carnitine palmitoyl transferase 1
DAG	Diglyceride
DGAT1	DAG acyltransferase 1
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DN	Diabetic nephropathy
DNA	Desoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
DPM	Disintegrations per minute
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid
ER	Endoplasmic reticulum
ERAD	ER associated protein degradation
ESRD	End-stage renal disease
FBS	Fetal bovine serum
FFA	Free fatty acid
GBM	Glomerular basement membrane
HEK	Human embryonic kidney
IRE-1	Inositol-requiring enzyme 1
IRS	Insulin receptor substrate
JNK	c-Jun NH_2-terminal kinase
LXR	Liver X receptor
MUFA	Monounsaturated fatty acid

PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PERK	Double-stranded DNA-dependent protein kinase (PKR)-like ER kinase
PI	Propidium iodide
PPARα	Peroxisome proliferator-activated receptor α
RIPA	Radioimmunoprecipitation assay
ROS	Reactive oxygen species
RPM	Revolutions per minutes
RPMI	Roswell Park Memorial Institute
SCD	Stearoyl-CoA desaturase
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamid gel electrophoresis
SFA	Saturated fatty acid
SNP	Single nucleotide polymorphism
TBS	Tris-buffered saline
TG	Triglyceride
TGF-ß	Transforming growth factor ß
TO	TO901317
UPR	Unfolded protein response
VSV	Vesicular stomatitis virus

1. INTRODUCTION

In the following I will give an introduction into diabetic nephropathy (DN). Thereby, the main focus will lie on podocytes which are highly specialized epithelial cells of the glomerular filtration barrier and which are thought to be critically involved in the pathogenesis of DN. In the second part I will introduce the term "lipotoxicity" and its potential relevance in the pathogenesis of DN. As saturated and monounsaturated free fatty acids critically determine lipotoxicity, I will review the literature with a special focus on their cellular effects and the mechanisms involved herein. Lastly, I will summarize two recent studies related to free fatty acids (FFAs) and FFAs metabolism in podocytes in which I have been involved as a coauthor and which are directly linked to my main PhD thesis project on fatty acid oxidation and acetyl CoA carboxylases (ACCs) in podocytes.

1.1. Diabetic Nephropathy

1.1.1. Incidence and prevalence of DN

Diabetic nephropathy (DN) is the most common cause of end-stage renal disease (ESRD) in industrialized countries, e.g. over 40% in the US (Figure 1) (USRDS Annual report, (2013)). The majority of diabetic patients starting renal replacement therapy today have type II diabetes as the prevalence of type II diabetes is much higher. Of the patients with type II diabetes 20-40% develop ESRD (Foley et al., 1998). The five-year survival rate of patients with DN and renal replacement therapy is significantly worse than in patients with other renal diseases mainly as a result of an increased cardiovascular mortality (Locatelli et al., 2004; USRDS, 2011). Therefore, it is important to better understand the pathogenesis of DN, to identify new strategies and additional therapeutic targets for the prevention and treatment of DN.

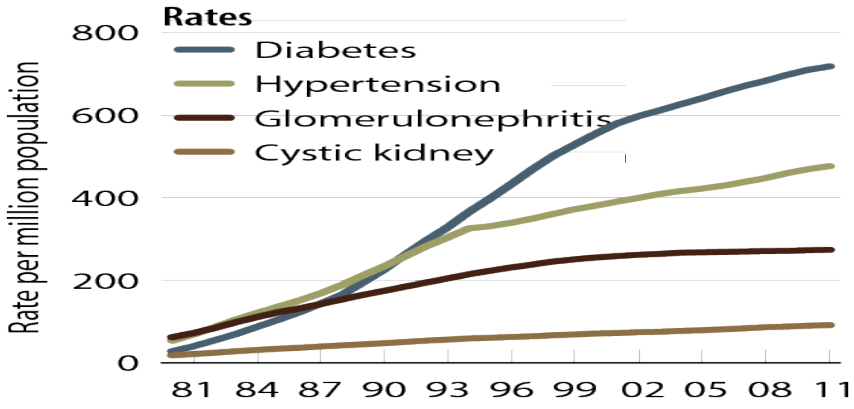


Figure 1: Prevalence of ESRD by primary diagnosis (U.S. Renal Data System, USRDS 2013 Annual Data Report, Chapter One, Figure 1-15, Volume Two)

1.1.2. Pathophysiology of DN

Dating back to the first description by Kimmelstiel and Wilson (Kimmelstiel P, 1936) the typical lesion of DN is mesangial matrix expansion accompanied by hypertrophy of mesangial cells, and thickening of the glomerular basement membrane (GBM). In addition, already in these seminal reports intraglomerular lipid deposits were described.

The classical, earliest clinical sign of DN is microalbuminuria, i. e. loss of small amount of albumin in the urine, resulting from damage to the glomerular filtration barrier. Consequently, attention to mechanisms focusing on alterations of the glomerular filtration barrier seems most warranted.

The glomerular filtration barrier is made of three interdependent layers (Figure 2), which are fenestrated endothelial cells, the GBM, and very specialized epithelial cells, the so called podocytes (Figure 2). All these layers form a size- and charge selective renal filtration sieve to prevent albumin and other molecules to be lost in the urine.

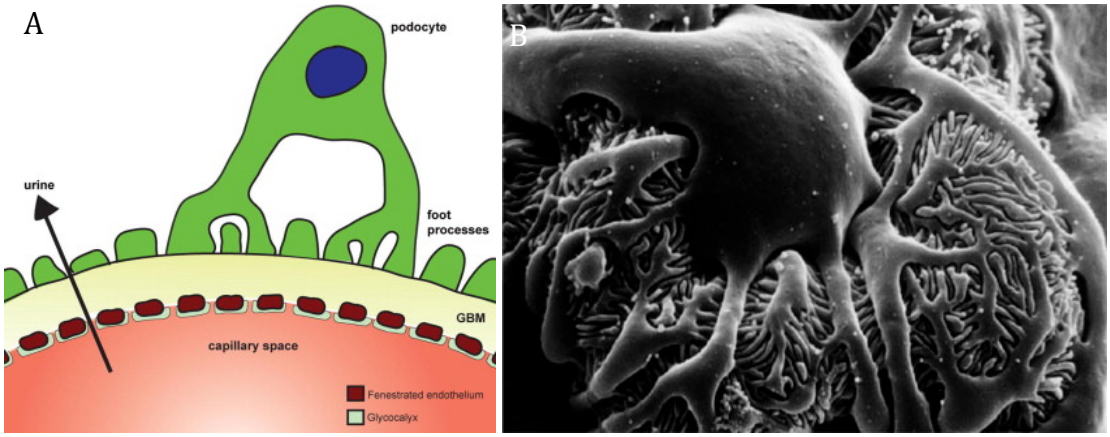


Figure 2: Structure of the glomerular filtration barrier. A) Schematic picture of the glomerular filtration barrier consisting of fenestrated endothelium cells, the glomerular basement membrane (GBM), and podocytes with their interdigitating foot process (Image from: J Patrakkaa and K Tryggvasona. Biochem Biophys Res Comm., 2010 (Patrakka and Tryggvason, 2010)); B) Image taken using a scanning electron microscope of a podocyte wrapped around a glomerular capillary (Image from: Smoyer WE & Mundel P, J Mol Med, 1998 (Smoyer and Mundel, 1998)).

1.1.3. The role of podocytes in the pathogenesis of DN

Over the past two decades by elucidating the genetic origin of a number of single human gene defects that result in congenital or early onset focal segmental glomerulosclerosis with massive proteinuria, it has become apparent that podocytes are the primary affected cell and critically determine the proper function of the glomerular filtration barrier (Kriz, 2003; Reidy and Kaskel, 2007). Also, in many other renal diseases with proteinuria including DN

increasing evidence suggests that podocyte dysfunction and loss mainly contribute to the development of proteinuria (Jefferson et al., 2008; Shankland, 2006). Podocytes are pericyte-like cells. They have a complex cellular architecture that includes a cell body, primary processes that further ramify into fine secondary foot processes. Individual foot processes interdigitate with foot processes of neighboring cells, and the filtration slits between the processes are bridged by slit diaphragms, which critically contribute to the selective permeability of the glomerular filtration barrier (Mundel and Kriz, 1995; Reiser et al., 2000). Moreover, podocytes critically determine the biophysical characteristics of the GBM and they are important to counteract the hydrostatic pressure from the glomerular capillaries (Endlich and Endlich, 2006).

Podocytopathy in DN is characterized by foot process widening. Importanly, this morphological change correlates in type I diabetic subjects with the urinary albumin excretion rate (Berg et al., 1998). Both, in patients with type I or type II diabetes the number and density of podocytes have been reported to be decreased (Dalla Vestra et al., 2003; Pagtalunan et al., 1997; Steffes et al., 2001; White and Bilous, 2000; White et al., 2002), and this podocyte loss relates to proteinuria (White and Bilous, 2004). A study performed in Pima Indians with type II diabetes suggests that a reduced number of podocytes per glomerulus predicts progressive kidney disease (Meyer et al., 1999).

Podocytes have no or very limited ability to replicate (Marshall and Shankland, 2006), therefore podocyte death and/or podocyte detachment from the GBM are thought to account for podocyte loss. Indeed, podocyturia has been documented in patients with type II diabetes and seems to correlate with disease progression (Nakamura et al., 2000).

1.1.4. Factors contributing to apoptosis of podocytes in DN

It is likely that multiple hits are necessary for the occurrence of injury and ultimately apoptosis in podocytes during the development of diabetic nephropathy.

In vitro, high glucose levels induce apoptosis in podocytes, and increased ROS have been shown to be important mediators of glucotoxicity (Susztak et al., 2006). In db/db mice chronic inhibition of NADPH oxidase was able to reduce podocyte apoptosis and ameliorated podocyte depletion, urinary albumin excretion, and mesangial expansion (Susztak et al., 2006). Of note, increased ROS levels in diabetes do not result from hyperglycemia alone, but

angiotensin II (Haugen et al., 2000) as well as elevated free fatty acids (Piro et al., 2002)) may also be important contributors.

TGF-β1 mRNA and protein levels are increased in various models of diabetes in rodents, and TGF-β signaling can be activated by a large number of mediators in diabetes including ROS, angiotensin II, and advanced glycation products (Ziyadeh, 2004). In vitro, TGF-β has been reported to induce apoptosis in murine podocyte (Schiffer et al., 2001). In podocytes derived from glomeruli of rats angiotensin II was shown to have a pro-apoptotic effect also (Ding et al., 2002). Interestingly, this effect was shown to depend on angiotensin II induced TGF-β production and could be attenuated by anti- TGF-β antibodies. Most importantly, in db/db mice administration of a neutralizing anti-TGF-β antibody was found to prevent the mesangial matrix expansion and to protect from a decline in kidney function (Ziyadeh et al., 2000). Also, tumor necrosis factor (TNF)-α was reported to induce podocyte death (Ryu et al., 2012; Tejada et al., 2008), and a variety of direct and indirect evidence suggests that TNF-α plays an important role in the pathogenesis of DN (Navarro-Gonzalez et al., 2009).

1.2. Lipotoxicity

Obesity, the metabolic syndrome, and type 2 diabetes are associated with elevated serum triglycerides and free fatty acids (FFAs). This leads to lipid accumulation in nonadipose tissues, including pancreas, heart, liver, and kidneys. Accumulation of excess lipids in these organs causes cell dysfunction and cell death. This process is termed lipotoxicity (Brookheart et al., 2009).

1.2.1. Lipotoxicity in Diabetic Nephropathy

In the kidneys of diabetic humans, intraglomerular lipid deposits were described first in 1936 by Kimmelstiel and Wilson and subsequently observed by other researchers (Kimmelstiel and Wilson, 1936; Lee et al., 1991). Upregulated lipogenic genes and development of glomerular and tubular lipid deposits have been observed in different animal models of obesity and diabetes mellitus (mice fed high-fat diets, leptin impaired db/db and ob/ob mice, streptozotocin-treated rats) (Jiang et al., 2007; Kume et al., 2007; Sun et al., 2002; Wang et al., 2005).

Importantly, recent data from genome wide association studies (GWAS) suggest a potentially important role of FFA metabolism in the pathogenesis of DN. In detail, two GWAS in type 2 diabetic patients found a polymorphism in a noncoding region of acetyl-CoA carboxylase (ACC) 2 with a strong association with proteinuria (Maeda et al., 2010; Tang et al., 2010). ACCs catalyze the carboxylation of acetyl-CoA to produce malonyl-CoA, which inhibits CPT-1, the rate limiting enzyme of FAO (Figure 3). As the DN-risk SNP of ACC2 results in a higher ACC2 expression (Maeda et al., 2010), it can be postulated that this leads to increase in malonyl-CoA levels and increased inhibition of CPT-1 with subsequent impairment of FAO.

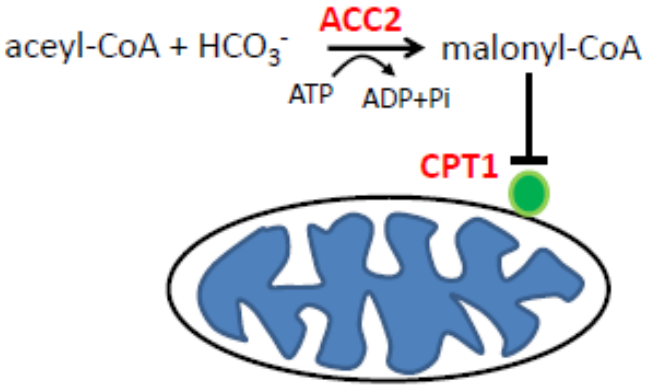


Figure 3: ACC2 inhibits CPT-1 by the production of malonyl-CoA from acetyl-CoA

1.2.2. Lipotoxicity: The role of free fatty acids

Elevated FFAs and disturbed FFA metabolism are critical determinants of lipotoxicity (Brookheart et al., 2009). Toxicity has been attributed mainly to saturated fatty acids (SFAs) whereas monounsaturated fatty acids (MUFAs) exert beneficial and cytoprotective effects (Brookheart et al., 2009; Nolan and Larter, 2009). Up to 80% of the plasma FFAs consist of the saturated palmitic (C16:0) and stearic acid (C18:0) as well as the monounsaturated oleic acid (C18:1) (Hagenfeldt et al., 1972).

1.2.2.1. The toxicity of saturated free fatty acids

The toxicity of SFAs has been attributed to multiple cellular mechanisms. One mechanism is related to decreased triglyceride (TG) synthesis and accumulation of cytotoxic metabolites such as diacylglycerides (DAGs). Of note, MUFAs can induce fatty acid oxidation and increase lipid storage in form of TGs, thereby reducing cytotoxic metabolites such as DAGs (Nolan and Larter, 2009), and DAG-mediated lipotoxicity may depend on the saturation of

fatty acids incorporated in DAGs (Bergman et al.), i. e. DAG with a higher content of MUFAs are less toxic. A second mechanism is related to ceramide synthesis. Palmitic acid is a substrate for the production of ceramide (Listenberger and Schaffer, 2002). Ceramide is a lipid secondary messenger involved in initiation of apoptosis. Mechanistically, ceramide induces insulin resistance and thereby may affect the prosurvival effect of insulin signaling; also, ceramide induces apoptotic pathways via increased membrane permeability of mitochondria (Bikman and Summers, 2011; Siskind, 2005). Inhibition of ceramide synthesis prevents lipotoxicity in pancreatic β-cells but not fibroblasts, suggesting that cell type-specific metabolic channeling of FFAs may be important (Brookheart et al., 2009). In cardiomyocytes, palmitic acid leads to depletion of cardiolipin, a phospholipid localized to the inner mitochondrial membrane and important for optimal mitochondrial function (Chicco and Sparagna, 2007), and reduced cardiolipin levels are thought to contribute to disruption of the mitochondrial inner membrane with release of cytochrome c. (Ostrander et al., 2001). SFAs as palmitic acid also effect other mitochondrial membrane phospholipids and thereby disturb mitochondrial function and lead to increase production of ROS (Brookheart et al., 2009). Importantly, oxidative stress can impair membrane integrity, organelle function, and gene expression, and thereby contributing to cell death. A third mechanism linked to the toxicity of SFA is related to the endoplasmic reticulum (ER). Palmitic acid rapidly increases the saturated lipid content of the ER leading to compromised ER morphology and integrity (Borradaile et al., 2006). In pancreatic β-cells palmitic acid depletes ER Ca^{2+} and slows ER Ca^{2+} uptake (Cunha et al., 2008). Disturbed ER homeostasis with the accumulation of mis- or un-folded proteins referred to as ER-stress (Rasheva and Domingos, 2009). ER-stress results in several signaling pathways, collectively known as unfolded protein response (UPR). The UPR cascade involves three signaling branches that are mediated by ER transmembrane receptors: double-stranded DNA-dependent protein kinase (PKR)-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE-1), and activating transcription factor 6 (ATF6) (Figure 4). These receptors are bound by the ER chaperone immunoglobulin heavy chain binding protein (BiP, also termed GRP78 or HSPA5) that keeps them silenced. Accumulation of unfolded and/or misfolded proteins is leading to dissociation of BiP and therefore to the activation of PERK, IRE-1 and ATF6. The UPR is primarily an adaptive response to maintain and restore proper ER function (Kaufman, 2002; Ma and Hendershot, 2001). However, the UPR is also linked to inflammatory signals (Figure 4), which themselves can trigger or maintain ER-stress. In addition, ER-stress leads to insulin resistance, at least in part through serine phosphorylation of insulin receptor substrate 1 (IRS1) by IRE1-activated JNK1 (Figure. 4)

(Ozcan et al., 2004). Unresolved and severe ER-stress may lead to apoptosis through up-regulation of the proapoptotic transcription factor CHOP (also known as DDIT3) (Zinszner et al., 1998), and loss of CHOP protects β cells from apoptosis in the db/db mouse model (Song et al., 2008). Conversely, overexpression of BiP in pancreatic beta-cells can reduce palmitic acid induced apoptosis (Laybutt et al., 2007) which may be explained by the ability of BiP to bind to and thereby repress the activity of the ER-stress transducers (Bertolotti et al., 2000) (Figure 4).

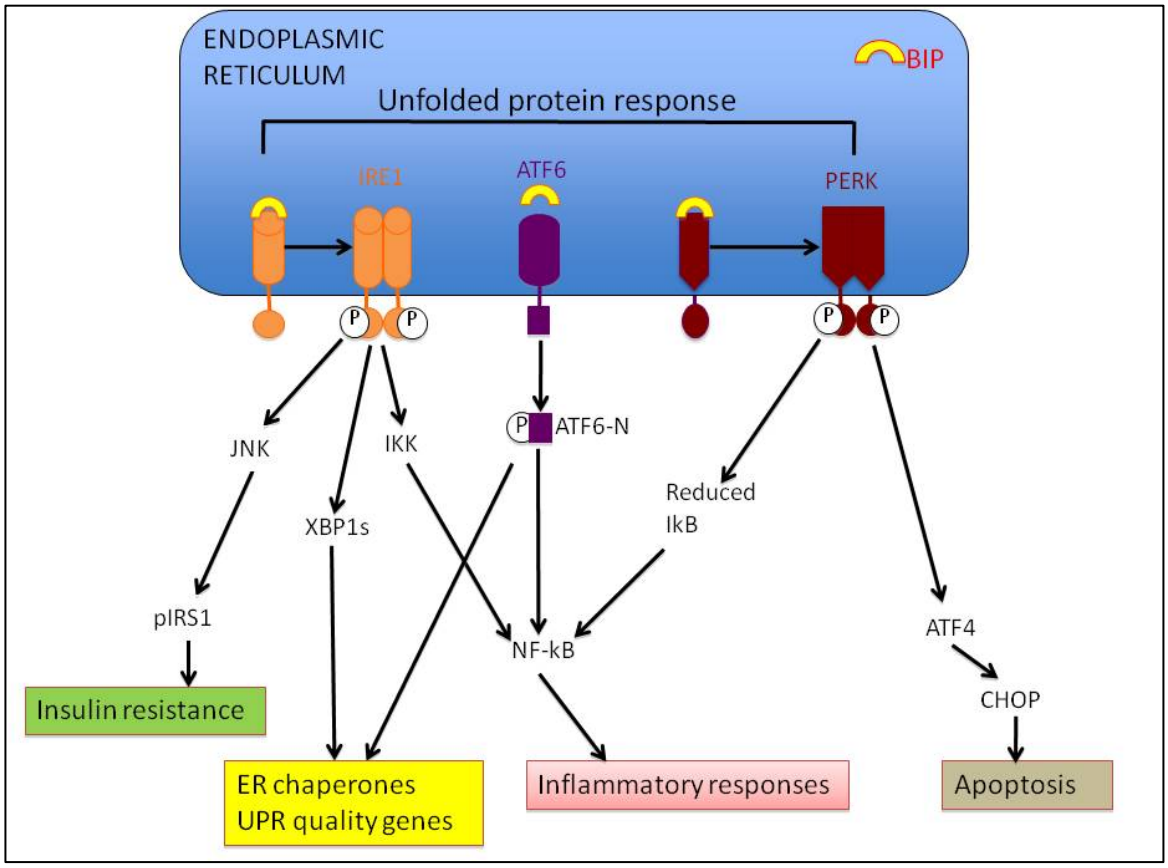


Figure 4: ER-stress and the unfolded protein response (UPR). In eukaryotic cells monitoring of the ER lumen is mediated by three ER membrane-associated proteins , PERK (PKR-like eukaryotic initiation factor 2α kinase), IRE1 (inositol requiring enzyme 1), and ATF6 (activating transcription factor-6). In a well-functioning and "stress-free" ER, these three transmembrane proteins are bound by a chaperone, BiP/GRP78, in their intralumenal domains and rendered inactive. Accumulation of improperly folded proteins and increased protein cargo in the ER results in the recruitment of BiP away from these UPR sensors. This results in oligomerization and activation of the two kinases, PERK and IRE1. Activation of the third branch of the UPR requires translocation of ATF6 to the Golgi apparatus where it is processed to an active transcription factor. The endoribonuclease activity of IRE1α cleaves the mRNA of the X-box binding protein-1 (XBP1), creating an active (spliced) form of the transcription factor (XBP1s). XBP1s, alone or in conjunction with ATF6α, launches a transcriptional program to produce chaperones (e.g. BiP) and proteins involved in ER-associated protein degradation (ERAD). ATF6 regulates XBP1 mRNA expression, and in addition ATF6 regulates together with XBP1s the expression of ER chaperones and UPR quality control genes. PERK activation results in phosphorylation of eIF2α (eukaryotic translational initiation factor 2α), which converts eIF2α to a competitor of eIF2B resulting in reduced global protein synthesis and a subsequent reduction in the workload of the ER. All three branches of the UPR are involved in the regulation/activation of the NF-κB-IKK pathway leading to an inflammatory response. If the ER-stress is prolonged, the UPR can induce apoptosis involving PERK mediated

activation of ATF4, which induces genes involved in apoptosis, e.g. C/EBP homologous protein (CHOP). (Adapted from (Hotamisligil, 2010))

1.2.2.2. Modulating pathways and the role of monounsaturated free fatty acids

In contrast to SFAs, monounsaturated fatty acids (MUFAs) exert mainly cytoprotective effects (Brookheart et al., 2009; Nolan and Larter, 2009). MUFAs are more potent ligands of the peroxisome proliferator-activated receptor α (PPARα), a transcription factor regulating lipid metabolism (Keller et al., 1993). PPARα is inducing transcription of genes involved in mitochondrial ß-oxidation (Hihi et al., 2002), and increasing ß-oxidation is thought to be a mechanism to detoxify cells from SFAs. In addition, MUFAs favor the incorporation of SFAs in TG, and this is thought to be cytoprotective as palmitic acid and its metabolites incorporated in TG are thought to be "biologicaly inert", i. e. they are stored away in "safe lipid pools" (Nolan and Larter, 2009). Mechanistically, incorporation of SFA-derived acyl-CoAs has been suggested to be more efficient in the presence of MUFAs as MUFAs are the preferred substrates for acyl-CoA:diacylglycerolacyltransferases (DGATs), that transfer acyl-CoAs to DAGs to form TGs (Cases et al., 1998; Cases et al., 2001; Hardy et al., 2003; Ricchi et al., 2009). Similarly, overexpression of stearoyl-CoA desaturases (SCDs) that desaturate saturated FFAs to form MUFAs has been shown to result in resistance to palmitic acid induced cell death (Listenberger et al., 2003). Furthermore and as previously discussed, ER-stress plays an important role in lipotoxicity. Importantly, MUFAs are directly linked to attenuation of ER-stress (Diakogiannaki et al., 2008; Holzer et al., 2011).

1.2.3. Free fatty acids and their metabolism in podocytes

In the following I will summarize recent studies, which are closely linked, to my own PhD project and in which I was able to contribute as a coauthor. Also, I will introduce in more detail the role of fatty acid oxidation and its regulation by ACCs.

1.2.3.1. Regulation of podocyte survival by free fatty acids

Only recently the effect FFAs has been investigated in podocytes. For these studies our laboratory used conditionally immortalized mouse podocytes, a well-established model to study podocyte biology *in vitro* (Mundel et al., 1997). As shown in figure 5 palmitic acid dose dependently increased both apoptosis and necrosis in podocytes. Podocyte cell death was assessed by flow cytometry after staining with Annexin V and propidium iodide (PI).

Annexin V-positive/PI-negative podocytes were considered apoptotic, whereas annexin V-positive/PI-positive podocytes were considered (late apoptotic) necrotic cells .

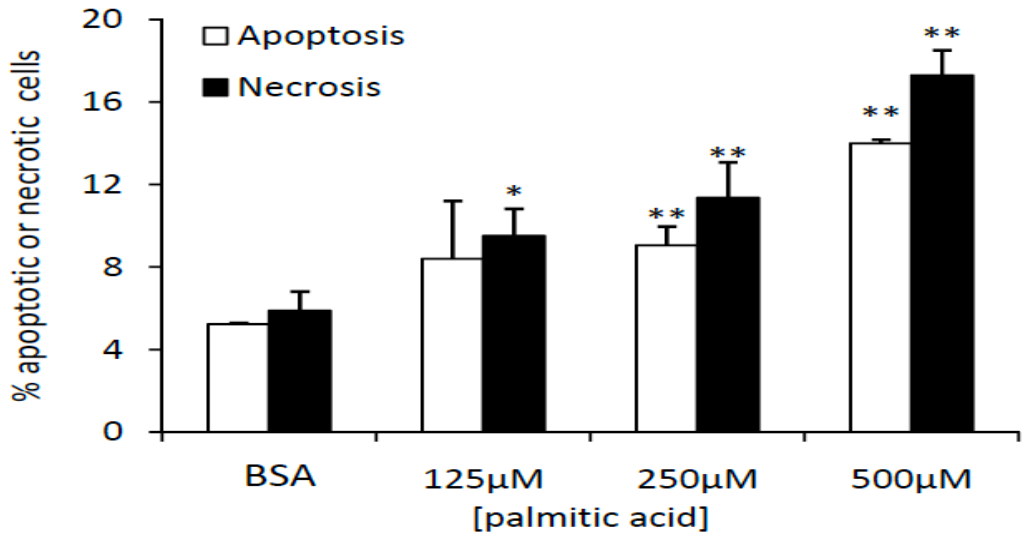


Figure 5: Palmitic acid induces apoptosis and necrosis of podocytes in a dose-dependent manner. Podocytes were exposed to palmitic acid (125 – 500 µM) or BSA (at a concentration quivalent to cells treated with 500 µM palmitic acid complexed to BSA) for 38 h. Quantitative analysis of palmitic acid induced podocyte cell death. Bar graph represents the mean percentages +/- SD of annexin V-positive/PI-negative (early apoptotic) or annexin V-positive/PI-positive (late apoptotic/necrotic) podocytes (n=3; * $p<0.05$, ** $p < 0.01$).(Sieber et al., 2010)

Similar to other cell type (Guo et al., 2007; Kharroubi et al., 2004; Martinez et al., 2008; Wei et al., 2009; Wei et al., 2006) we observed that palmitic acid dose- and time-dependently results in ER-stress as indicated by the induction of the proapoptotic transcription factor CHOP, which is typically upregulated during severe ER-stress (Zinszner et al., 1998) (figure 6), (Sieber et al., 2010).

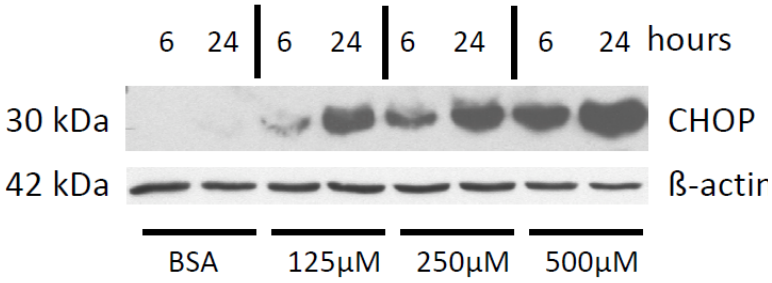


Figure 6: Dose-and time-dependent induction of CHOP in podocytes by palmitic acid (

Importantly, further experiments demonstrated that palmitic acid induced podocyte death as well as ER-stress can be prevented by coincubation with palmitoleic or oleic acid (Sieber et al., 2010). Finally, we were able to demonstrate that palmitic acid induced cell death at least in part is mediated by CHOP as knockdown of CHOP led to a significantly reduction of podocyte death (Sieber et al., 2010). In addition, we and other observed that palmitic acid

leads to insulin resistance in podocytes (Sieber and Kampe, unpublished observation, and (Lennon et al., 2009)). As insulin signaling has a strong prosurvival effect in many cell types (Huber et al., 2003) this may further increase the susceptibility of podocyte to proapoptotic stimuli such as palmitic acid.

1.2.3.2. Susceptibility of Podocytes to Palmitic Acid Is Regulated by Stearoyl-CoA Desaturases 1 and 2

To study the impact of type 2 diabetes and diabetic nephropathy (DN) on glomerular fatty acid metabolism microarray analysis of key enzymes involved in fatty acid metabolism was performed. Specifically, our laboratory investigated the gene expression in glomeruli of patients with type 2 diabetes mellitus compared to pretransplantation living donors (Sieber et al., 2013). Interestingly, the most prominent change was the upregulation of stearoyl-CoA desaturases (SCD)-1, and by immunohistochemistry the increased signal for SCD-1 could be predominantly observed in podocytes (Sieber et al., 2013). To address the potential contributory role of FFAs to the altered gene expression profile in glomeruli and podocytes of patients with DN, cultured podocytes were treated with 200µM palmitic acid complexed to BSA, compared to uncomplexed BSA, and significantly increased expression of Scd-1 (1.7 ± 0.7-fold) and Scd-2 (1.9 ± 0.6-fold), the most abundant SCD isoforms in murine kidneys (Ntambi and Miyazaki, 2003) and murine podocytes was observed. Further experiments with pharmacological and genetic overexpression of SCDs as well as gene silencing of SCD-1, and -2 elegantly demonstrated that both isoforms are protective for palmitic acid induced podocyte death Figure 7, (Sieber et al., 2013).

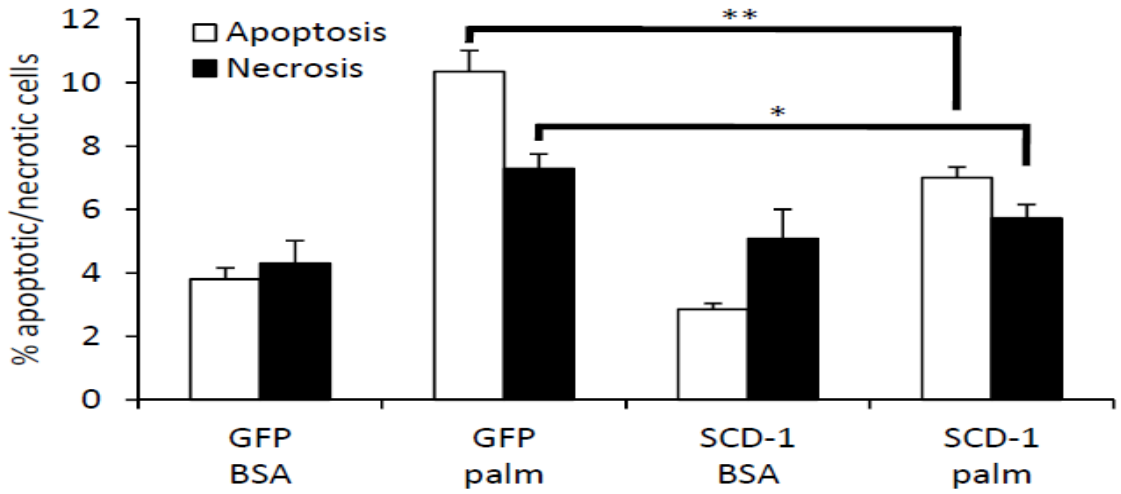


Figure 7: Overexpressing SCD-1 partially protects from palmitic acid-induced apoptosis. Podocytes with SCD-1 overexpressing were compared to podocytes with overexpression of GFP. SCD-1 reduced palmitic acid-induced apoptosis and necrosis in podocytes compared to GFP controls. Bar graph shows mean percentages ± SD of apoptotic and necrotic cells after exposure to 200µM palmitic acid for 48 h (n = 3, * $p < 0.05$, ** $p < 0.01$, (Sieber et al., 2010)

Mechanistically, MUFAs or stimulation of SCDs, which convert saturated FFAs to MUFAs, promote the incorporation of palmitic acid into TG, suggesting that the protective effect at least in part results from compartmentalization of palmitic acid in "safe lipid pools" (Sieber et al., 2013).

1.2.3.3. Lipotoxicity: Modulation by fatty acid oxidation and the role of ACCs

Studies in endothelial cells suggest that stimulation of fatty acid oxidation (FAO) protects from palmitic acid induced cell death (Borradaile et al., 2006). In these studies FAO was stimulated by the by the AMPK agonist aminoimidazole-4-carboxamide-1β-D-ribofuranoside (Aicar) which leads to phosphorylation of ACCs resulting in lower malonyl-CoA levels and disinhibtion of CPT-1 (Fig. 9), the rate-limiting enzyme of FAO (Muoio and Newgard, 2008).

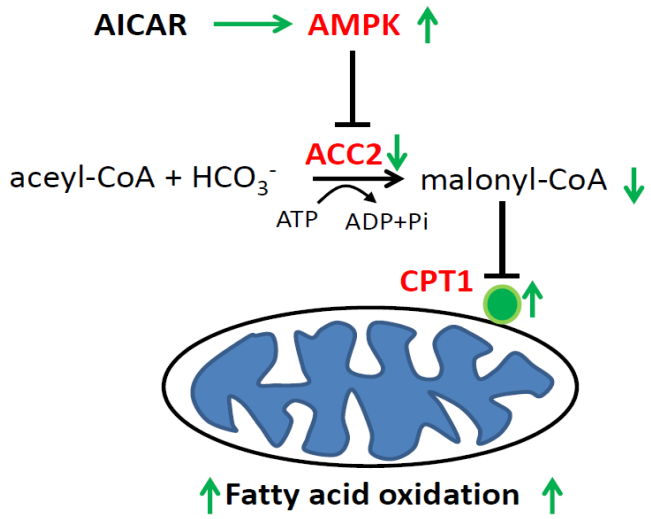


Figure 8: Aicar stimulates fatty acid oxidation. Figure depicts the metabolic pathway activated by Aicar, which results in stimulation of fatty acid oxidation.

As mentioned above (section 1.2.1.), two recent GWAS in type 2 diabetic patients found a polymorphism in a noncoding region of ACC2 with a strong association with proteinuria (Maeda et al., 2010; Tang et al., 2010). The DN-risk single nucleotide polymorphism of ACC2 results in a higher ACC2 expression (Maeda et al., 2010) potentially leading to increased malonyl-CoA levels and decreased FAO.

In humans and rodents there are two ACC isoforms, ACC1 (ACC alpha) and ACC2 (ACC beta) (Savage et al., 2006), which share considerable sequence identity and the same domain structure responsible for enzyme activity (Savage et al., 2006). In contrast to ACC1, ACC2 has an extra N-terminal hydrophobic domain, which facilitates its localization to the

mitochondrial membrane (Abu-Elheiga et al., 2005), where it preferentially regulates local malonyl-CoA levels and CPT-1 activity. In contrast cytosolic ACC1 is classically thought to regulate malonyl-CoA synthesis for incorporation into fatty acids in lipogenic tissues. However, more recently this classical view has been challenged, and at least in some cell types, e.g. hepatocytes, both isoforms have been shown to regulate CPT-1 activity synergistically (Savage et al., 2006).

1.3. Aim of the study

Increasing evidence suggests that damage and loss of podocytes are early events in DN and critically determine disease progression. Several factors of the diabetic milieu are known to impair function and survival of podocytes. Although lipid accumulation is a well known feature of DN, only recently the potentially important role of FFAs and FFA metabolism in this process were acknowledged.

Over the last five years our laboratory systematically analyzed the effects of FFAs as well as FFA metabolism in podocytes. A key finding was that FFAs can regulate podocyte survival. Specifically, SFAs, i.e. palmitic acid, were found to induce endoplasmic reticulum (ER) stress and podocyte death, whereas monounsaturated FFAs, i.e. palmitoleic acid or oleic acid, were protective.

The aims of the present study were to investigate whether FFA metabolism is regulated in glomeruli of type 2 diabetic patients with DN and whether regulation of their metabolism affects the susceptibility of podocyte towards palmitic acid. The main focus was to understand whether regulation of FAO modifies palmitic acid-induced podocyte death. As genome wide association studies suggest that acetyl CoA carboxylase (ACC) 2, an important enzyme in the regulation of FAO, is involved in the pathogenesis of DN, detailed studies investigated the role of ACCs in podocytes. Furthermore, I investigated whether palmitic acid modifies podocyte death induced by other factors of the diabetic milieu, which have been shown to be involved in the pathogenesis of DN and which are thought to contribute to damage and loss of podocytes.

2. MATERIALS AND METHODS

2.1. Cell culture

Podocytes were cultured following the protocol described by Mundel et al (Shankland et al., 2007). Conditionally immortalized mouse podocyte cell lines were established from the immortomouse, which carries a thermosensitive (ts58A) variant of the SV 40 T antigen as a transgene (Shankland et al., 2007). Podocytes having the passage number from 4 to 14 are utilized for performing the experiments. First, podocytes were cultured or proliferated in permissive conditions which include growing in 33°C with 50U/ml interferon gamma (IFN-γ, # CTK-358-2PS, MoBiTec GmbH, Germany) for first two passages, later IFN-γ concentration can be brought down to 10U/ml. Differentiation of podocytes is done in non-permissive conditions which includes thermoshift to 37°C without IFN-γ. Podocytes were allowed to undergo differentiation atleast 11 days prior to the start of experiments.

Podocytes are cultured in RPMI-1640 (#21875, Invitrogen) supplemented with 10% FBS (#10270, Invitrogen), 100 U/ml penicillin and 100 μg/ml streptomycin (#15140, Invitrogen). For apoptosis experiments, β-oxidation experiments 6-well plates were employed and for isolating mRNA and protein 10-cm dishes were employed from BD biosciences. All the plates and dishes were coated with 0.1 mg/ml type I collagen (BD biosciences) prior to seeding the cells. Freezing of the cells was performed in complete culture medium supplemented with 8% (v/v) dimethylsulfoxide (Sigma). For the production of lentiviral particles, HEK293 cells were employed as packaging cells. HEK293 cells were cultured in DMEM (#41965, Invitrogen) supplemented with 10% FBS and penicillin/streptomycin.

2.2. Agonists, inhibitors and cytokines

Substance	Supplier (catalog number)	Physiological role	Concentration applied
Aicar	Cell signaling (#9944)	AMPK agonist	0.5mM
Adiponectin	BioVision (#4902-100)	AMPK agonist	0.5µg/ml
Compound C	Sigma (#5499)	AMPK inhibitor	4µM
Etomoxir	Sigma (#E1905)	CPT-1 inhibitor	10, 30 and 200µM
TGFβ	Roche (#10874800)		5ng/ml
TNFα	Sigma (#T7539)		5ng/ml

Table 1: Agonists, inhibitors and cytokines

2.3. Free fatty acids preparation

Sodium palmitate and oleic acid (both from Sigma) were dissolved overnight at 10 mM in glucose-free RPMI-1640 medium (#11879) containing 11% essential fatty-acid free BSA (Sigma) under N_2-atmosphere at 55°C, sonicated for 10 min and sterile filtered (stock solution). The molar ratio of fatty acid to BSA was 6:1.The effective free fatty acid concentrations were measured with a commercially available kit (Wako). Endotoxin concentration was equal or less than 0.5ng/ml, as determined by a kit (#L00350) from Genscript (Piscataway, NJ, USA).

2.4. Apoptosis assay

The cells were trypsinized, washed once with PBS, and resuspended in 120 µl annexin V binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl2, pH 7.4). 100 µl of the cell suspension was used for the staining procedure. Alexa-647 annexin V (#A23204, Invitrogen) staining was applied for 15 min at room temperature at a dilution of 1:100 (see producer protocol) and before analyzing an additional 400 µl of annexin V binding buffer was added along with 0.5µg propidium iodide (#P3566, Invitrogen) were added. 20'000 – 25'000 cells were analyzed by flow cytometery with CyAn™ ADP Analyzer (Beckman Coulter). Data from flow cytometry was analyzed by FLOWJO (Tree Star, Inc. Ashland, OR, USA) software program. Annexin V-positive/PI-negative podocytes were considered apoptotic, whereas annexin V-positive/PI-positive podocytes were considered (late apoptotic) necrotic cells.

2.5. ACC1 and ACC2 knock down

2.5.1. shRNA sequences and lentiviral expression vector

For the knock down, ACC1, ACC2 and scrambled shRNA sequences were cloned into pSIH-H1-puro lentiviral expression plasmid, which is a kind gift of Dr. Markus Heim, (University Hospital Basel, Switzerland). Respective details of shRNA sequences are furnished in table 1 along with information of plasmids and references. pSIH-H1-puro was first linearized with EcoR1/BamH1 restriction enzymes and at the same time shRNA oligos of respective genes were designed and ordered with restriction sites for EcoR1/BamH1 flanking them to facilitate cloning into the pSIH-H1-puro vector. Next, shRNA oligos were ligated with digested pSIH-H1-puro and ligation product was transformed into competent E.coli strain, DH5-α. Following day of bacterial transformation, colonies were randomly picked and miniprep was done to isolate plasmid and which further sent to sequencing for confirmation.

Gene	shRNA sequence (5' – 3')	Vector	GeneBank number	Reference
ACC1	GCAGATTGCCAACATCCTAGA	pSIH-H1-puro	NM_133360	(Jeon et al., 2012)
ACC2	GTGGTGACGGGACGAGCAA	pSIH-H1-puro	NM_133904	(Jeon et al., 2012)
ACC2 (2)	GAGGTTCCAGATGCTAATG	pSIH-H1-puro	NM_133904	Optimized
Scrambled	GACCGCGACTCGCCGTCTGCG	pSIH-H1-puro		(Sieber et al., 2010)

Table 2: shRNA sequences and respective plasmids.

2.5.2. Lentiviral production

A 4-plasmid based lentiviral system (kindly provided by Dr. Markus Heim, University Hospital Basel, Switzerland) was employed with following helper plasmids: pRSV-REV (Rev expression vector), pMDLg/pRRE (Gag-Pol expression vector), and pMD2.G (VSV-G expression vector). All helper plasmids along with pSIH-H1-puro were mixed with 45µl of FuGene HD (Promega, Madison, WI, USA) transfection agent in total 3ml of Opti-MEM (#31985, Gibco) and incubated at room temperature for 20min. Next HEK293 were transfected in a 10-cm dish with 5ml of DMEM (#41965) without antibiotics and medium was changed after 8-12h, followed by addition of fresh complete 10ml of DMEM (conditions for transfection is detailed in table 2). HEK293 cells were grown upto 60 to 70% confluence

before transfection. 48h post transfection, the supernatant medium enriched with lentiviral particles was harvested, spun at 780g for 5min and filtered through 0.45µm filter.

Transduction of podocytes was done by adding virus containing media with pre-treating podocytes with 5µg/ml polybrene (Sigma) for 5min. 8-24 hours after transduction, medium was exchanged. Experiments were performed three to five days after viral transduction. Amount of viral particles to be used for transducing cells was standardized separately by employing a GFP-based lentiviral expression plasmid FUGW, which facilitates the visual inspection of the efficiency of viral transduction. For all the experiments a viral titer to achieve efficiency of 70 – 80% was employed.

Plasmid/Reagent	Amount
pSIH-H1-puro (with respective shRNA sequence)	9 µg
Rev expression vector (pRSV-REV)	1.8 µg
Gag-Pol expression vector (pMDLg/pRRE)	4.5 µg
VSV-G expression vector (pMD2.G)	2.7 µg
Total plasmid DNA	18 µg
FuGene HD	45 µl
Total Opti-MEM	3 ml

Table 3: Summary of transfection conditions for the production of lentiviral particles.

2.6. Western Blot

For protein isolation cells were always cultured in 10-cm dishes. For isolating protein, medium was sucked off and cells were washed with ice cold PBS and scraped in 180 µl RIPA lysis buffer (50mM Tris-HCl, pH 7.5, 200 mM NaCl, 1% Triton, 0.25% deoxycholic acid, 1 mM EDTA, 1mM EGTA) containing EDTA-free protease inhibitors (#11873580001, Roche) and phosphatase inhibitors (#78420, Pierce). Then collected cells were lysed mechanically and rotated for 1 h at 4°C. To remove nuclei, the samples were spun down (10'000 rpm, 10 min) and the protein concentration of the supernatant was determined by D_C Protein Assay (Bio-Rad). 20 - 80 µg of protein was complemented with 6x sample buffer (200 mM Tris-HCl pH 6.8, 26% glycerol, 10% SDS, 0.01% bromphenol blue) and DTT (final concentration of 100 mM) and heated for 10 min at 95°C. Protein samples were loaded on 7-12% gels and SDS-PAGE was performed at 150 V. Transfer to nitrocellulose membranes (Protran BA83, Whatman Schleicher und Schuell) was applied at 100 V in the cold room for 1 hour and the blots were blocked for 2 hours with 5% milk powder in TBS-Tween (50 mM Tris HCl pH

7.4, 150 mM NaCl, 0.02% Tween). Primary antibodies were applied overnight and the secondary antibodies for 1 hour in 5% milk in TBS-Tween. The immunoblots were detected by enhanced chemiluminescence (#34094, Pierce) on Kodak BioMax light films (#Z370398-50EA, Sigma). The list of primary and secondary antibodies employed with respective dilutions is detailed in table 3.

Antigen	Species	Conjugate	Supplier (catalog number)	Dilution
ACC1/2	Rabbit	Purified	Cell signaling (#3676)	1:500
AMPK	Rabbit	Purified	Cell signaling (#2532)	1:1000
BiP	Rabbit	Purified	Cell signaling (#3177)	1:500
CHOP	Mouse	Purified	Santa Cruz (#sc-7351)	1:200
Mouse IgG	Rabbit antiserum	HRP	Dako (#P0260)	1:4000
pAMPK	Rabbit	Purified	Cell signaling (#2531)	1:200
pACC	Rabbit	Purified	Cell signaling (#3661)	1:500
Rabbit IgG	Goat antiserum	HRP	Dako (#P0448)	1:1600
ß-actin	Mouse	Purified	Sigma (#A5441)	1:50,000

Table 4: List of primary and secondary antibodies for western blot.

2.7. β-oxidation measurement

For measuring the β-oxidation of palmitic acid, tritium labeled palmitic acid (^{3}H-palmitic acid, #NET043001MC Perkin Elmer, Schwerzenbach, Switzerland) was employed. For these experiments, Aicar was pre-incubated for 1h if necessary and serum starvation medium was employed having 0.2% FBS, 5 mM glucose which is supplemented with 0.5% FFA-free BSA. For all the experiments 200µM of palmitic acid and 0.5µCi/ml ^{3}H-palmitic acid was applied. After the incubation times of experiment, 1ml of the supernatant medium was taken and added to 5ml of chloroform/methanol/5N HCl (2:1:0.05, v/v) and rotated for 5min. Later, the mixture was spun down at 350xg for 5 min, which apparently separates upper aqueous and lower organic phase. Now 500µl of upper aqueous phase was taken and added to 2ml of scintillation liquid (Insta-gel Plus, Packard, Groningen, The Netherlands) in a special scintillation reading tubes (Perkin elmer). Tubes were thoroughly mixed before they were put in scintillation reader (Packard, Canberra, CT, USA) for measuring radioactivity. β-oxidation values were obtained as disintegrations per minute (DPMI) and were normalized to total protein.

2.8. Statistical analysis

All the experiments were performed at least 4 times and representative result was shown. Data are expressed as means ± SD unless otherwise mentioned. One way ANOVA was performed and for calculating significance of differences, Bonferroni post hoc test was employed. The prism 6 program was used for the analysis and differences were considered significant when P value was < 0.05.

3. RESULTS

3.1. Palmitic acid induced podocyte death: Modification by high glucose, TGF-β, and TNF-α

As reported by us and confirmed in independent studies, podocytes are highly susceptible to palmitic acid induced cell death (Sieber et al., 2010) (Sieber et al., 2013; Tao et al., 2012). Previously other factors of the diabetic milieu have been reported to induce podocyte death, including high glucose concentrations (Susztak et al., 2006), TGF-β (Schiffer et al., 2001), and TNF-α (Ryu et al., 2012; Tejada et al., 2008). I explored the effect of these factors in combination with palmitic acid in podocytes.

3.1.1. Palmitic acid uncovers the toxicity of high glucose concentrations

High glucose concentrations (20 – 30 mmolar) have been reported to induce apoptosis in podocytes (Susztak et al., 2006). In these studies apoptosis was quantified by assessment of nuclear condensation following DAPI staining and by a caspase 3 activity assay (Susztak et al., 2006). To see whether high glucose in combination with palmitic acid affects podocyte survival, I treated podocytes with normal glucose (NG, 11mM) or high glucose (HG, 22mM) in the presence or absence of 200µM palmitic acid for 48h (Figure 9). Podocyte cell death was assessed by flow cytometry after staining with Annexin V and propidium iodide (PI). Annexin V-positive/PI-negative podocytes were considered apoptotic, whereas annexin V-positive/PI-positive podocytes were considered (late apoptotic) necrotic cells (Sieber et al., 2010). As shown in figure 9, high glucose alone had no significant effect on podocyte death in the presence of BSA which was used as the appropriate control for podocytes treated with palmitic acid complexed to BSA. However, high glucose significantly increased podocyte apoptosis in the presence of palmitic acid (155.3 ± 16.8%, $p < 0.01$). As the toxicity of high glucose was only seen in the presence of palmitic acid in these experiments we can also say that toxicity of glucose was “uncovered” by palmitic acid.

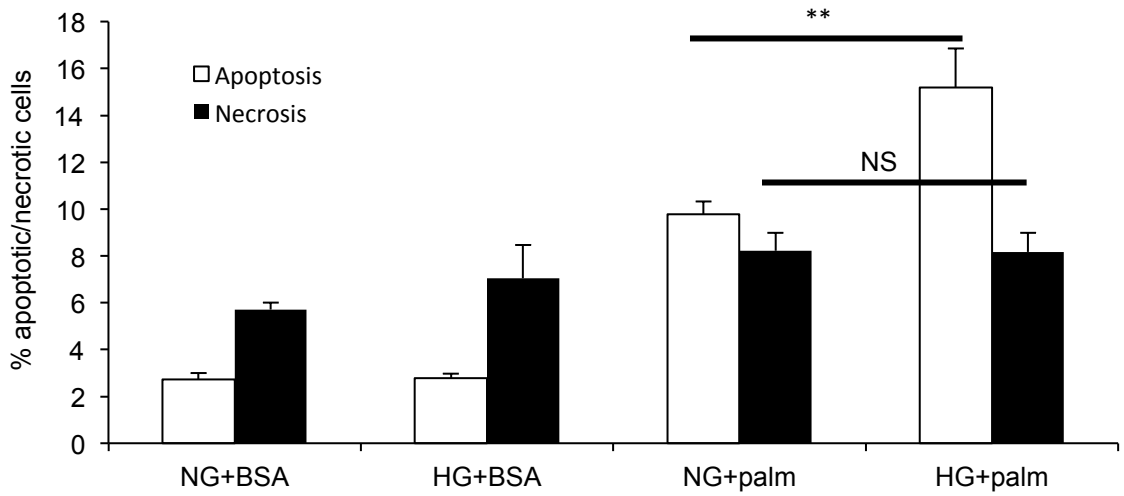


Figure 9: High glucose accentuates the to toxicity caused by palmitic acid in podocytes. Podocytes were incubated with either 11mM glucose (NG) or 22mM glucose (HG) in presence of 200µM palmitic acid or BSA (control) for 48h. 11mM of mannitol was employed to NG conditions to correct for osmolality. Bar graph represents mean percentages ± SD of apoptotic and necrotic cells (n = 3, * *p < 0.01).

3.1.2. TGF-β aggravates palmitic acid induced podocyte death

Considerable evidence suggests that TGF-β plays an important role in the pathogenesis of DN (Chen et al., 2003), and TGF- β has been reported to induce apoptosis in murine podocyte (Schiffer et al., 2001). Therefore podocytes were treated with either 200µM palmitic acid or BSA (control) in the presence or absence of 5ng/ml TGF-β for 48h (figure 10). TGF-β could accentuate the toxicity caused by palmitic acid, as both apoptosis and necrosis levels were increased by 157.4 ± 10.2% ($p<0.01$) and 145.5 ± 10.6% ($p<0.01$) respectively. The effect of TGF-β alone on podocyte death was minimal and did not reach statistical significance, however the toxicity of TGF-β was again uncovered by the co-treatment with palmitic acid.

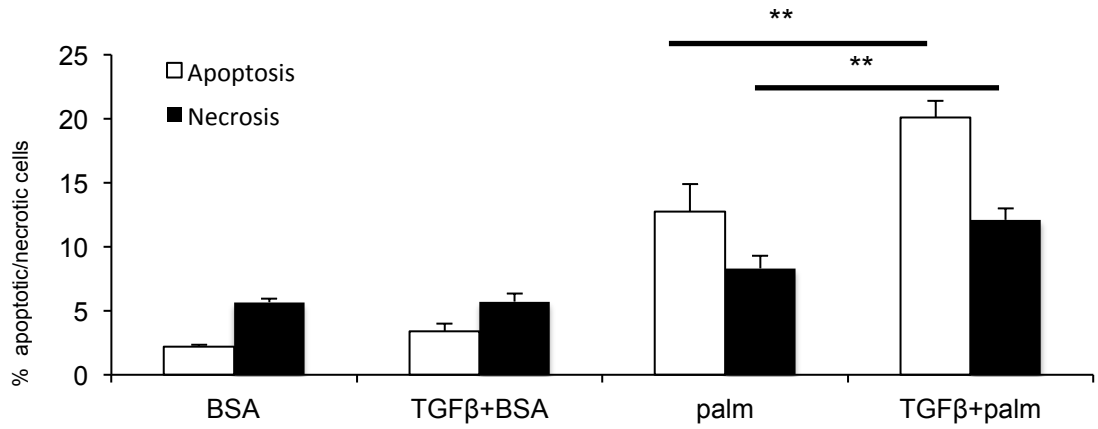


Figure 10: TGF TGFβ increases the toxicity caused by palmitic acid in podocytes: Podocytes were treated either with 200µM palmitic acid or BSA (control) with or without 5ng/ml TGFβ for 48h. Bar graph represents mean percentages ± SD of apoptotic and necrotic cells (n = 3, * *p < 0.01).

3.1.3. TNF alpha aggravates palmitic acid induced podocyte death

A variety of direct and indirect evidence suggests that tumor necrosis factor alpha (TNF-α) plays a role in the pathogenesis of DN (Navarro-Gonzalez et al., 2009). Also, TNF-α was reported to induce podocyte death (Ryu et al., 2012; Tejada et al., 2008). To examine whether TNF-α affects palmitic acid induced podocyte death, podocytes were treated with 5ng/ml of TNFα alone or in combination with 200µM palmitic acid for 48h (Figure 11). TNF-α significantly increased podocyte death, specifically apoptosis was increased by 168.1 ± 20.9% ($p < 0.01$), and necrosis by 120.6 ± 10.2% ($p < 0.05$). However, the increase of palmitic acid induce podocyte death by TNF-α was modest, and only apoptosis was significantly increased by 116.0 ± 7.9 ($p < 0.05$).

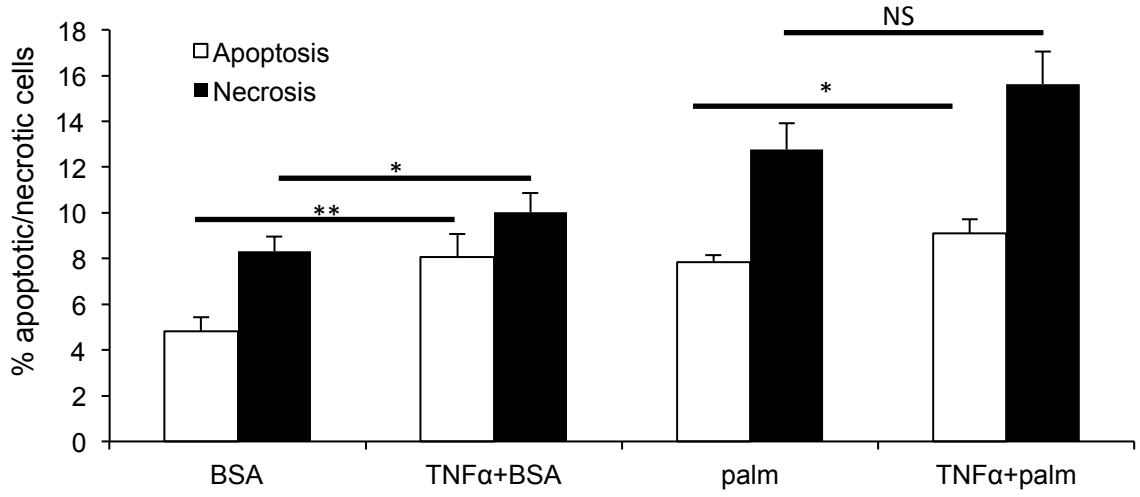


Figure 11: TNFα induces podocyte death and aggravates palmitic acid induced toxicity. Podocytes were treated with either 200 µM BSA or palmitic acid in presence or absence of 5 ng/ml TNFα for 48 h. Bar graph represents mean percentages +/- SD of apoptosic necrotic cells (n=3, **$p < 0.01$, *$p < 0.05$).

3.2. Regulation of fatty acid oxidation in palmitic acid induced podocyte cell death: Critical role of Acetyl CoA carboxylase 1 and 2

3.2.1. Differential regulation of genes involved in fatty acid metabolism in glomeruli of patients with established DN

To see whether enzymes of fatty acid metabolism might be regulated in DN, we performed microarray analysis of different enzymes involved in fatty acid metabolism in glomeruli from type 2 diabetic patients with DN and compared them to glomeruli from pretransplantation living donors (Sieber et al., 2013). Significantly altered expression levels of several enzymes involved in FAO and TG synthesis was observed (Figure 12). The most prominent change was the induction of SCD-1, which provides DGATs with their preferential substrates, MUFAs. Together with the positive regulation of DGAT1, which catalyzes the incorporation of exogenous FFAs into TG, this implies a disposition towards increased TG synthesis. Furthermore, we saw a significant upregulation of all three isoforms of CPT-1, the rate-limiting enzyme for fatty acid oxidation, and a downregulation of ACC-2, which catalyzes the formation of the CPT-1 inhibitor malonyl-CoA, which suggests a disposition for increased fatty acid oxidation.

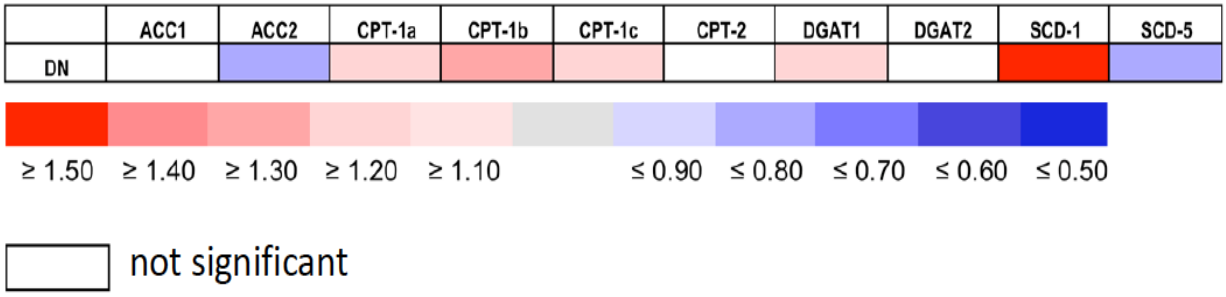


Figure 12: Differential expression of genes related to fatty acid metabolism in glomeruli of DN patients. Microarray data were obtained from isolated glomeruli of type 2 diabetic patients with DN and controls (pretransplant allograft biopsies). Expression of fatty acid oxidation related genes such as ACC2, CPT-1a, CPT-1b and CPT-1c, were significantly regulated in DN compared to controls. Up regulated enzymes are indicated in red, down regulated enzymes in blue colors.

3.2.2. Modulation of fatty acid oxidation and its effect on palmitic acid induced podocyte death

3.2.2.1. AMPK activation protects from palmitic acid induced cytotoxicity

To investigate whether stimulation of fatty acid oxidation (FAO) plays a protective role in palmitic acid treated podocytes, we took advantage of the AMP-activated protein kinase (AMPK) activator 5-aminoimidazole-4-carboxamide-1β-D-ribofuranoside (Aicar). Aicar (as

well as adiponectin (Sharma et al., 2008)) acts by phosphorylating AMPK, which in turn phosphorylates and inhibits ACC resulting in disinhibition of CPT-1 (Figure 13).

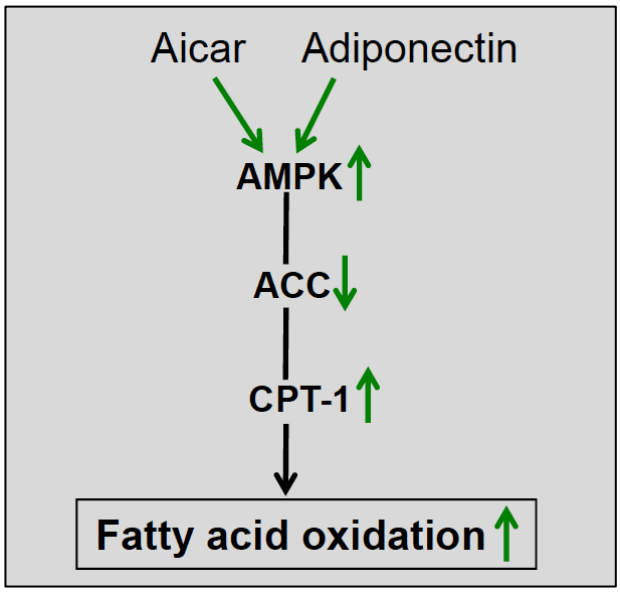


Figure 13: Scheme representing the metabolic path activated by Aicar and Adiponectin. Aicar and Adiponectin activate AMPK by phosphorylation. AMPK phosphorylates and inhibits ACC which results in decreased synthesis of malonyl CoA and disinhibition of CPT-1 resulting in upregulation of fatty acid oxidation.

In a first step, phosphorylation of AMPK and ACC by Aicar in podocytes was examined by Western immunoblotting (Figure 14).

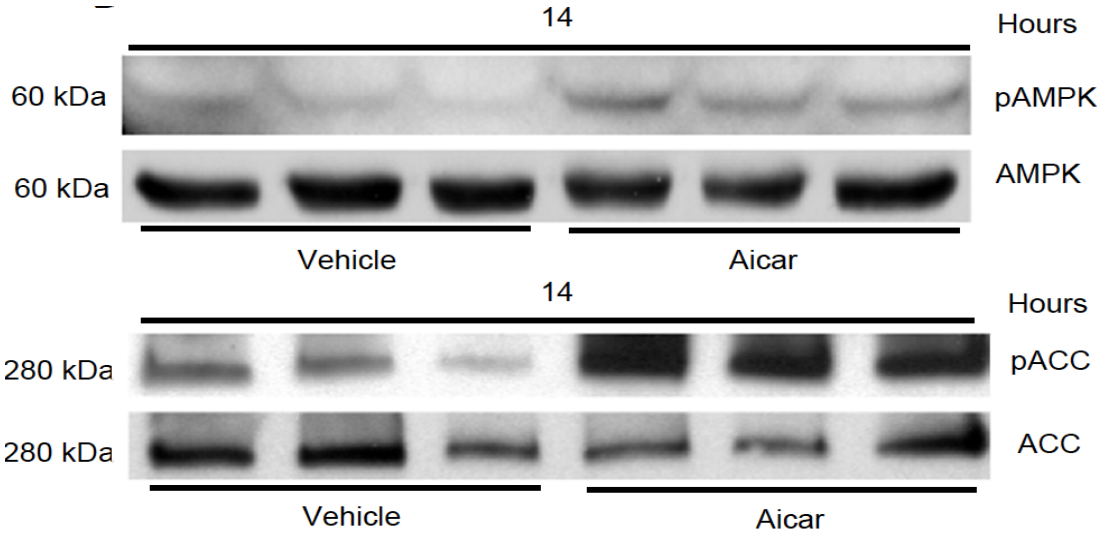


Figure 14: Aicar phosphorylates AMPK and ACC. Immunoblot shows phosphorylation of AMPK and ACC after incubation of podocytes with either (PBS) vehicle or 0.5 mM Aicar for 14 hours. Total AMPK and total ACC served as loading controls.

Functionally, as shown in Figure 15, Aicar significantly prevented palmitic acid induced podocyte death assessed by flow cytometry after staining for Annexin V and propidium

iodide (PI). Specifically, Aicar reduced palmitic acid induced apoptosis and necrosis by 50.5 ± 1.5% ($p < 0.01$) and 42.5 ± 6.1% ($p < 0.05$) respectively.

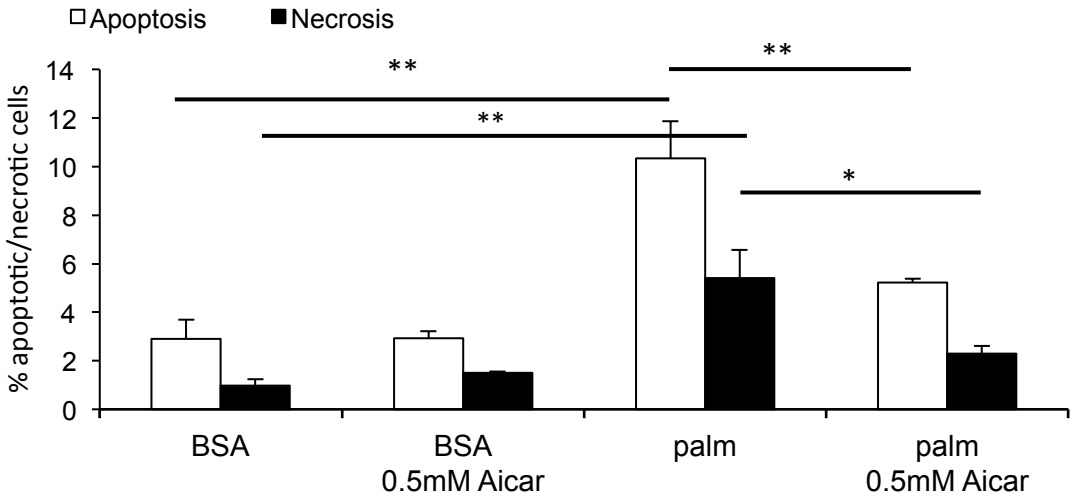


Figure 15: Aicar attenuates palmitic acid induced podocyte death. Podocytes were treated with either 200µM palmitic acid or BSA (control) with or without 0.5mM Aicar for 48h. Podocytes were preincubated with Aicar for 1h. Representative bar graph shows mean percentages ± SD apoptotic and necrotic podocytes. (n=3, *p<0.05, **p<0.01)

Similarly to Aicar, the physiological AMPK agonist adiponectin (Sharma et al., 2008) also reduced palmitic acid induced podocyte death, although to a lesser extent than Aicar (figure 16). Specifically, adiponectin significantly decreased apoptosis by 14.1 ± 4.7% ($p < 0.05$), but the reduction of necrosis by 9.9 ± 6.3% did not reach statistical significance. To see the protective effect of adiponectin podocytes were kept at a high glucose concentration of 22 mmol/L, which is known to reduce phosphorylation of AMPK (Sharma et al., 2008) and increases the susceptibility of podocytes to AMPK activation (Sharma et al., 2008).

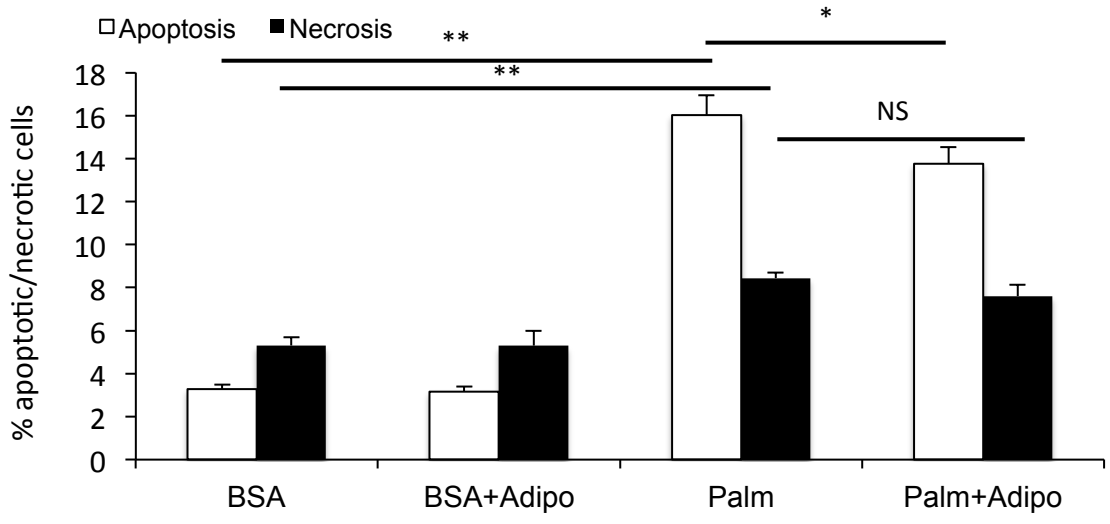


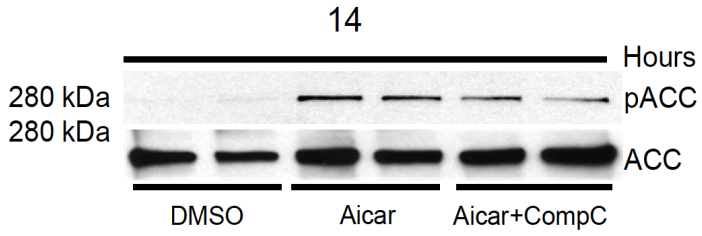
Figure 16: Adiponectin decreases apoptosis induced by palmitic acid. Podocytes were treated with either 200µM palmitic acid or BSA (control) with or without 0.5µg/ml adiponectin for 48h. 22mM of glucose was employed.

Podocytes were preincubated with adiponectin for 1h. Bar graph represents mean percentages ± SD of apoptotic and necrotic cells (n = 3, ** $p < 0.01$, * $p < 0.05$).

3.2.2.2. Inhibition of AMPK exacerbates palmitic acid induced cell death and reversed the protection caused by Aicar.

To further to explore the role of AMPK we used the AMPK inhibitor compound C. Compound C was used at a low concentration of 4 μM, as higher concentrations were toxic, i.e. podocyte death was markedly increased for BSA control (data not shown). The Aicar induced ACC phosphorylation was significantly reduced by compound C (Figures 17A, and 17B, $p < 0.05$).

A)



B)

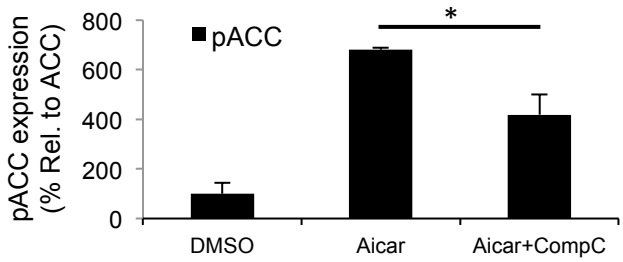


Figure 17: Compound C inhibits Aicar induced phosphorylation of ACC. **A.** Podocytes were incubated with DMSO, 0.5mM Aicar and Aicar in combination of 4μM of compound C. pACC was immunoblotted and total ACC served as loading control. **B.** Quantification of pACC by densitometry. Bar graph represents the relative expression ± SD (*$p < 0.05$). DMSO treated controls were set to 100%.

In line with the inhibitory effect of compound C on the AMPK-ACC-CPT-1 pathway, compound C treatement increased palmitic acid induced apoptosis and necrosis in podocytes by 140.1 ± 20.1 % ($p < 0.01$) and 130.9 ± 14.0% ($p < 0.01$), respectively. In agreement with the partial reduction of the Aicar induced ACC phosphorylation, (Figure 17) cotreatment with compound C compared to Aicar alone only moderately increased palmitic acid induced podocyte death, i.e. apoptosis was increase by 128.2 ± 9.3 % (NS) and necrosis by 176.7 ± 9.7% ($p < 0.01$) (Figure 18).

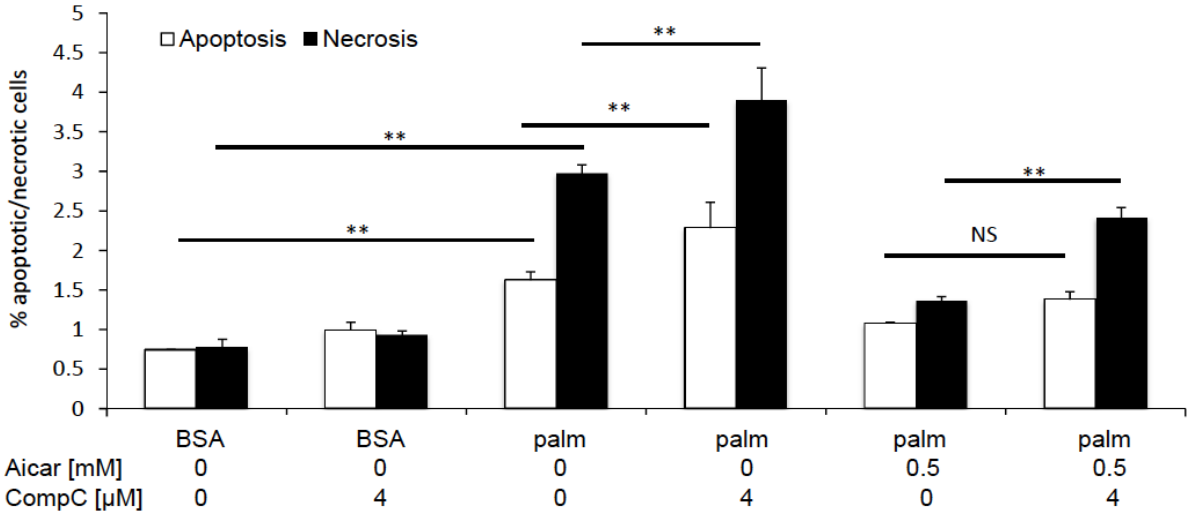


Figure 18: Compound C accentuates palmitic acid induced podocyte death and partially reverses the protection by Aicar. Podocytes were incubated with 200µM palmitic acid or BSA (control) either with or without 4 µM of Compound C, or either with 0.5mM Aicar alone or in combination with Compound C for 48h. Bar graph represents mean percentages ± SD of apoptotic and necrotic cells (n = 3, $^{**}p < 0.01$).

3.2.2.3. Etomoxir aggravates palmitic acid induced podocyte death and reverses the protective Aicar effect

To further investigate the impact of FAO on palmitic acid induced podocyte death I made use of the CPT-1 inhibitor etomoxir (Figure 19A). Etomoxir exacerbated palmitic acid induced podocyte death (Figure 19B). Specifically, apoptosis was increased by 184.3 ± 6.0% ($p < 0.01$) and necrosis by 185.1 ± 16.3% ($p < 0.01$). Moreover, etomoxir reversed the protective effect of Aicar (Figure 20). Of note, this effect could already be seen at a very low etomoxir concentration (10 µM), which by itself had no significant effect on palmitic acid induced podocyte death (data not shown). Compared to podocytes treated with Aicar alone, the presence of 10µM etomoxir increased palmitic acid mediated apoptosis by 131.1 ± 5.0% ($p < 0.05$) and necrosis by 127.3 ± 10.7% ($p < 0.05$). At 200 µM, etomoxir completely reversed the protective effect of Aicar (Figure 20).

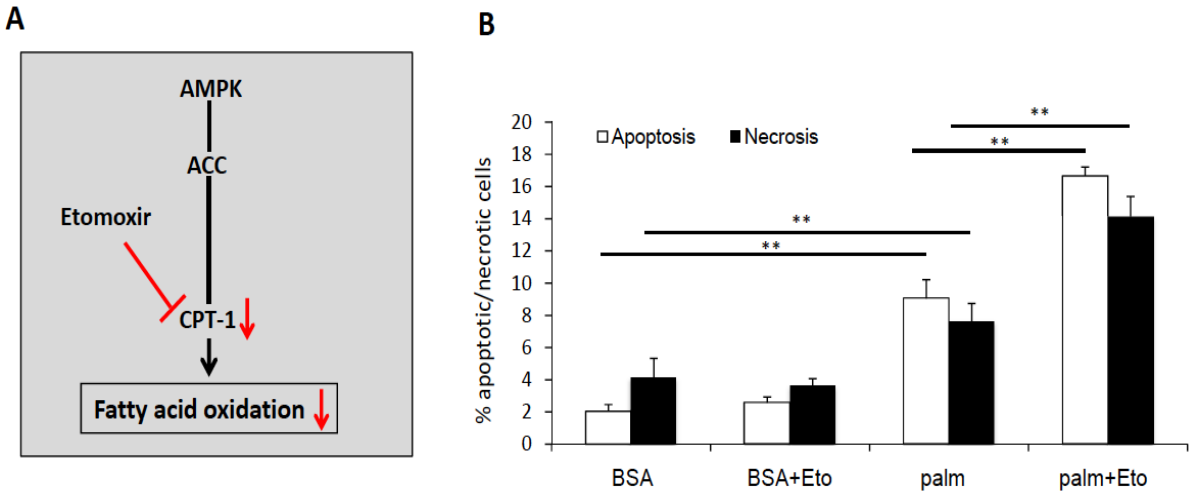


Figure 19: Etomoxir exacerbates palmitic acid induced podocyte death. A: Scheme shows mechanism of action of etomoxir, a CPT-1 inhibitor, in inhibiting fatty acid oxidation. B: Etomoxir aggravated palmitic acid induced cell death after 48h. Podocytes were treated with 200µM palmitic acid or BSA (control) in the presence or absence of etomoxir. Bar graph represents mean percentages ± SD of apoptotic and necrotic cells (n = 3, **p<0.01).

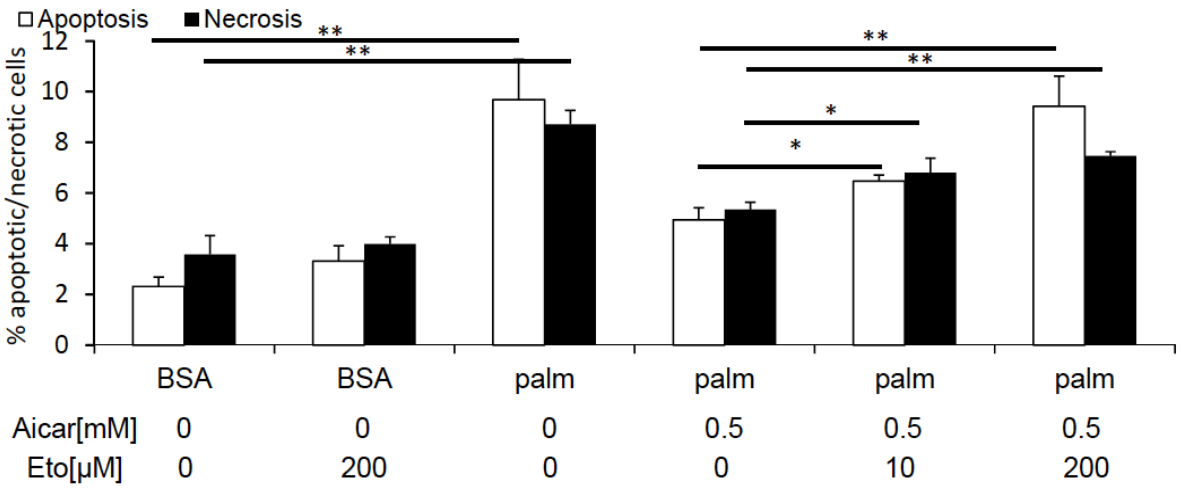


Figure 20: Etomoxir reverses the protection by Aicar for palmitc acid induced podocyte death. Podocytes were treated with 200 µM palmitic acid or BSA (control) in the presence or absence of etomoxir (10 µM or 200 µM) and/or 0.5 mM Aicar. Bar graph represents mean percentages ± SD of apoptotic and necrotic cells (n = 3, * p < 0.05, **p < 0.01).

3.2.2.4. Aicar stimulates and etomoxir inhibits fatty acid oxidation

To directly measure the effect of Aicar on palmitic acid oxidation, I treated podocytes with 200 µM palmitic acid along with 0.5 µCi/ml tritiated palmitic acid in the absence or presence of Aicar. As a direct read out of palmitic acid β-oxidation, tritiated water released in the supernatants of podocytes was measured. As expected the release of tritiated water gradually increased from 1 to 3 hours (Figure 21). The stimulation of podocytes with Aicar significantly

increased the formation of tritiated water (146.6 ± 22.0%, $p < 0.05$, Figure 21) reflecting the stimulatory effect of Aicar on palmitic acid oxidation.

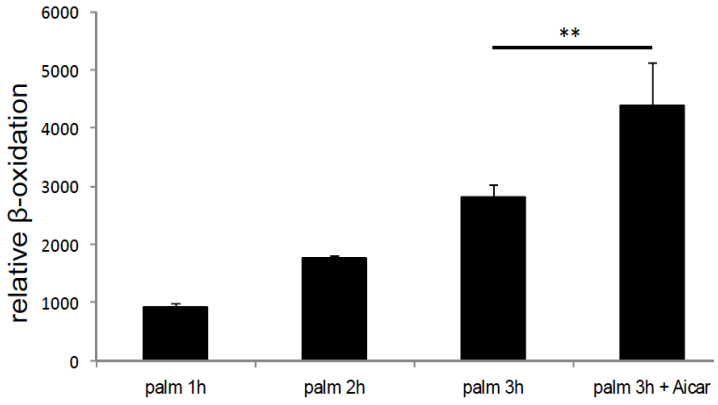


Figure 21: Aicar increased β-oxidation of palmitic acid. Podocytes were treated with 0.5% FFA free-BSA with 200µM palmitic acid in presence of 0.5 µCi/ml [^{3}H]-palmitic acid for indicated time points in presence or absence of 0.5mM Aicar. Podocytes were preincubated for 1h with 0.5mM Aicar. Bar graph represents relative ß-oxidation ± SD (n = 3, ** $p < 0.01$).

Importantly, and as shown in Figure 22, the effect of Aicar could be completely prevented by etomoxir.

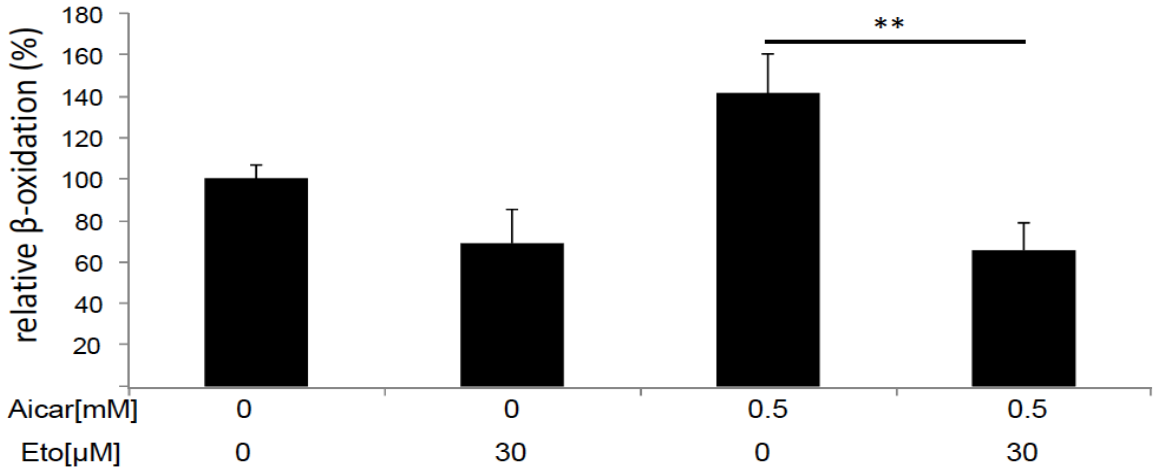


Figure 22: Etomoxir prevents the Aicar induced β-oxidation. Podocytes were treated with 0.5% FFA free-BSA with 200 µM palmitic acid supplemented with 0.5 µCi/ml [^{3}H]-palmitic acid either in the presence of 0.5mM Aicar alone or in combination with 30 µM etomoxir for 3h. Bar graph represents relative ß-oxidation (%) ± SD (n = 3, * $p < 0.05$)

3.2.2.5. Combined silencing of ACC1 and ACC2 protects from palmitic acid induced podocyte death

Two recent genome wide association studies (Maeda et al., 2010; Tang et al., 2010) found a single nucleotide polymorphism in ACC2, leading to increased ACC2 expression (Maeda et al., 2010) to be associated with proteinuria in type 2 diabetic patients. To investigate further the role of both ACC isoforms in podocytes, I generated cells deficient of ACC1, ACC2, or both by lentiviral infection using specific short-hairpin (sh) RNAs. Knock down of ACC1 but not ACC2 strongly reduced the band corresponding to both isoforms. The residual band seen

in ACC1 single knock down podocytes was almost completely gone in ACC1/ACC2 double knock down cells (Figure 23). These data suggest that the expression level of ACC1 is much higher than ACC2 in podocytes.

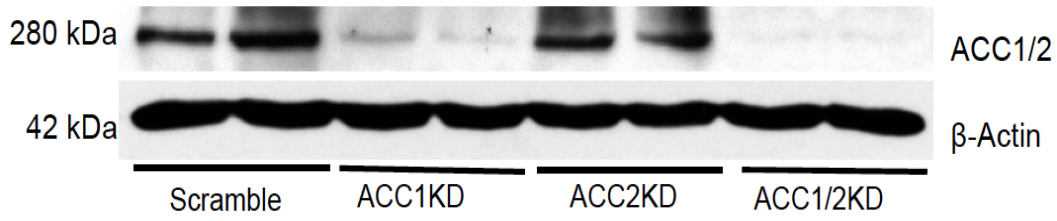


Figure 23: Immunoblot for ACC1, ACC2 and ACC1/2 knock down. ACC1, ACC2 or both were knocked down and an immunoblot was done with an antibody recognizing both isoforms. ß-actin served as a loading control.

As shown in Figure 24, single knock down of ACC1 or ACC2 was not able to protect from palmitic acid induced podocyte death.

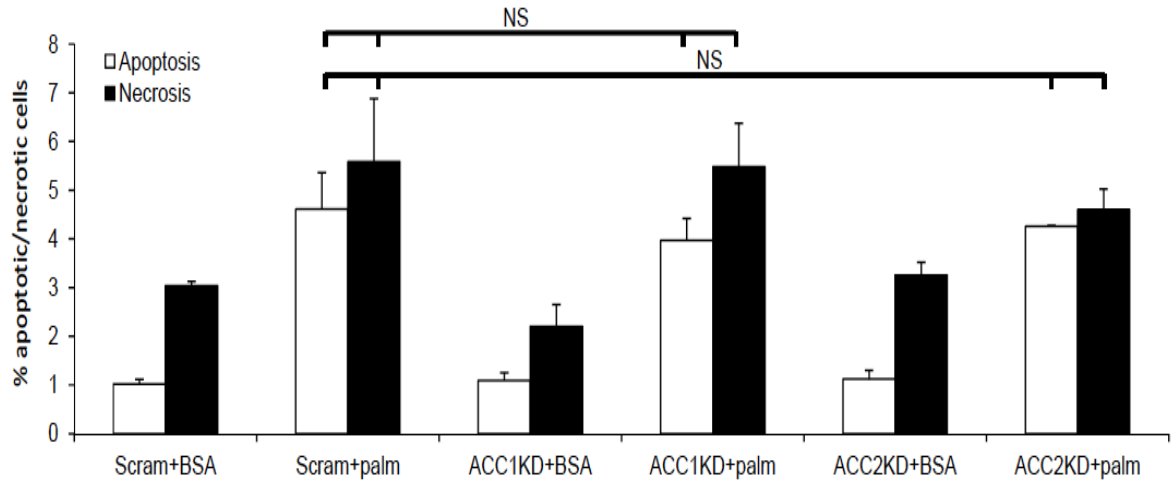


Figure 24: ACC1 or ACC2 single knockdown is not protective for palmtitic acid induced podocyte death. Podocytes were silenced with either ACC-1 or ACC-2, and were treated with either 200 μM palmitic acid or BSA (control) for 48h.The bar graph shows mean percentage apoptotic or necrotic cells ± SD (n=3).

Contrariwise, double knockdown of both isoforms significantly reduced palmitic acid induced podocyte death. Specifically, in ACC1/ACC2 double knockdown podocytes palmitic acid induced apoptosis and necrosis was reduced by 59.6 ± 4.5% ($p < 0.01$) and 64.4 ± 6.4% ($p < 0.01$) compared to podocytes transfected with scrambled shRNA (Figure 25).

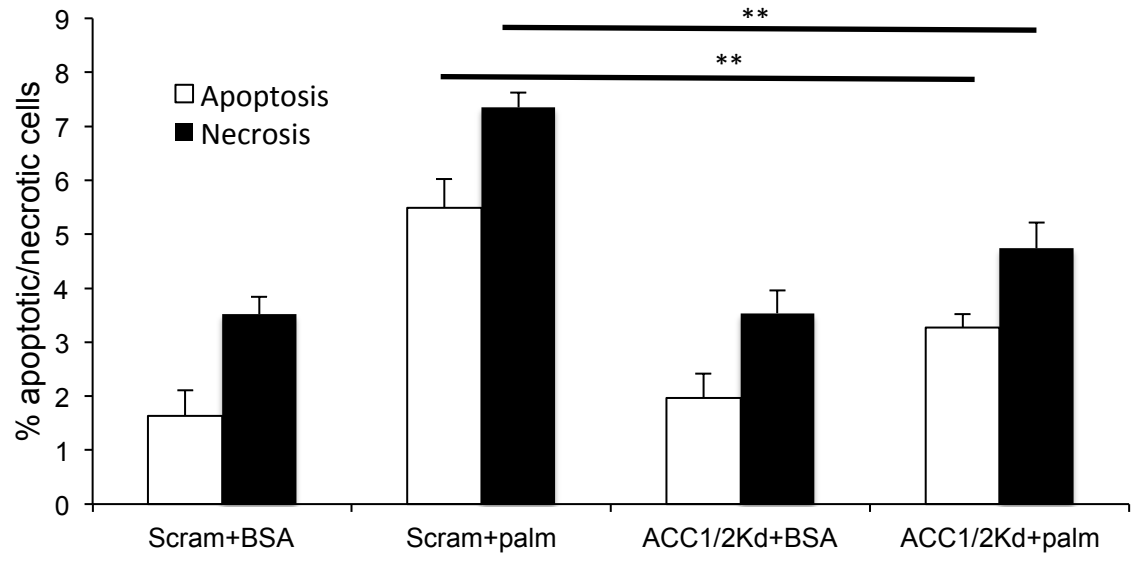


Figure 25: Combined knock down of ACC1 and ACC2 protects podocytes from palmitic acid induced cell death. Podocytes were treated either with 200μM palmitic acid or BSA (control) for 48h. The bar graph represents % mean apoptotic or necrotic cells ± SD. (n=3, **p<0.01).

To further corroborate these results I used a different set of shRNAs directed against ACC1 and ACC2. As shown in Figure 26, the protective effect with this second set was similar to the first set.

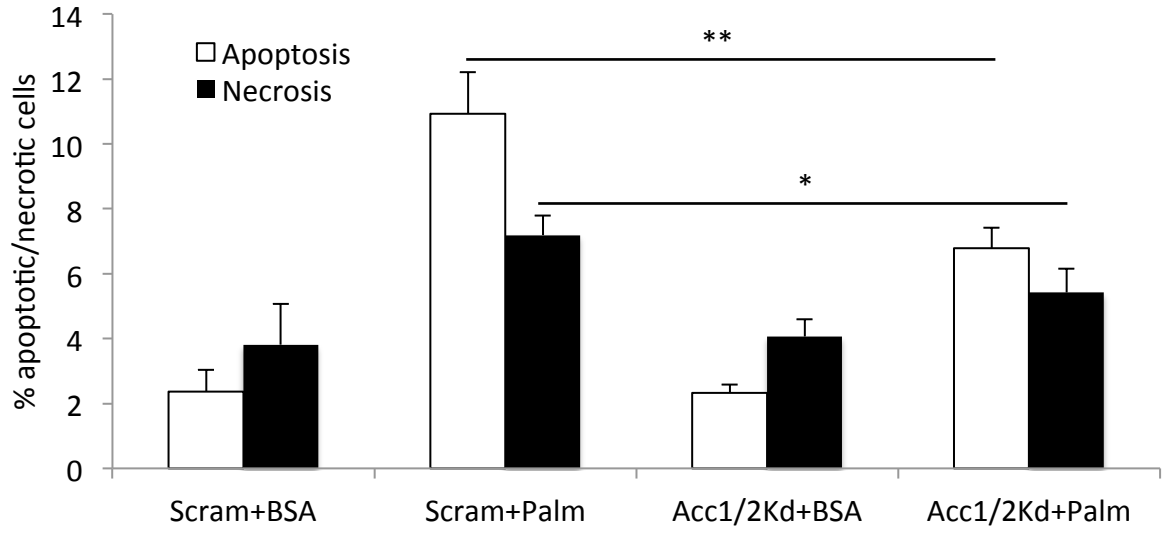


Figure 26: Combined knock down of ACC1 and ACC2 with a second set of shRNAs against ACC1 and ACC2 protects podocytes from palmitic acid induced cell death. Podocytes were treated either with 200μM palmitic acid or BSA (control) for 48h. The bar graphs represent percentages of mean apoptotic or necrotic cells ± SD. (n = 3, * p < 0.05, ** p < 0.01).

However, single knock down of ACC1 or ACC2 was again not protective (data not shown). In a next step the effect of Aicar was tested in ACC1/ACC2 double silenced podocytes. The residual protective effect shown in figure 27 was weak and not consistently seen in all experiments performed.

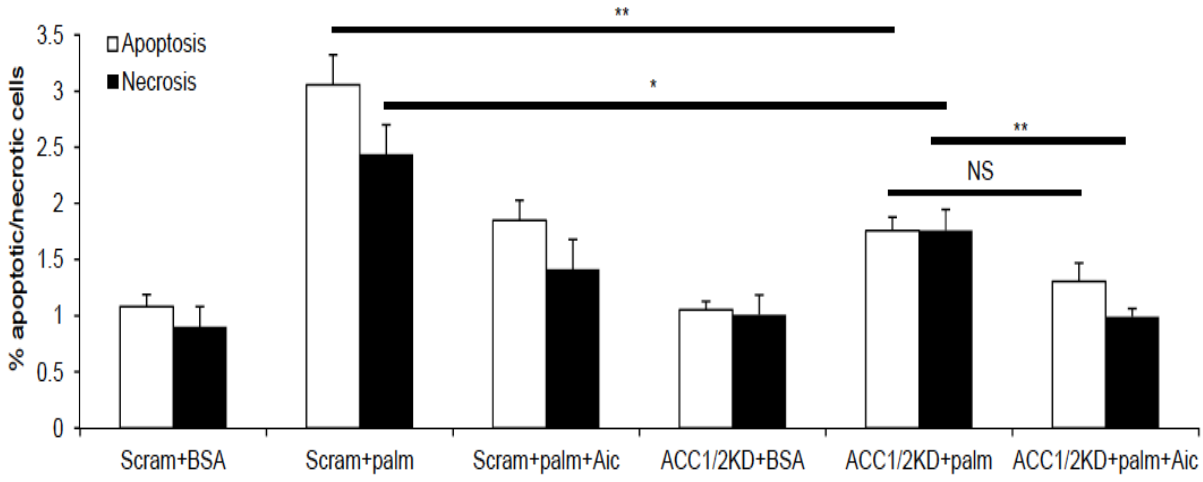


Figure 27: Modest effect of Aicar in ACC1/ACC2 double silenced podocytes. ACC1/ACC2 double silenced podocytes and scramble controls were treated either with 200 µM palmitic acid or BSA (control) for 48h in presence or absence of 0.5 mM Aicar. Bar graph represents % mean apoptotic or necrotic cells ± SD. (n=3, *p<0.05, **p<0.01)

3.2.2.6. Aicar reduces ER-stress and the upregulation of CHOP

As palmitic acid induced podocyte death involves ER-stress and as CHOP gene silencing attenuates palmitic acid induced podocyte death (Sieber et al., 2010), I investigated the effect of Aicar on the ER chaperone BiP and the proapoptotic transcription factor CHOP. Aicar strongly suppressed the upregulation of BiP and CHOP (Figure 28A, and B). (Sieber et al., 2010)

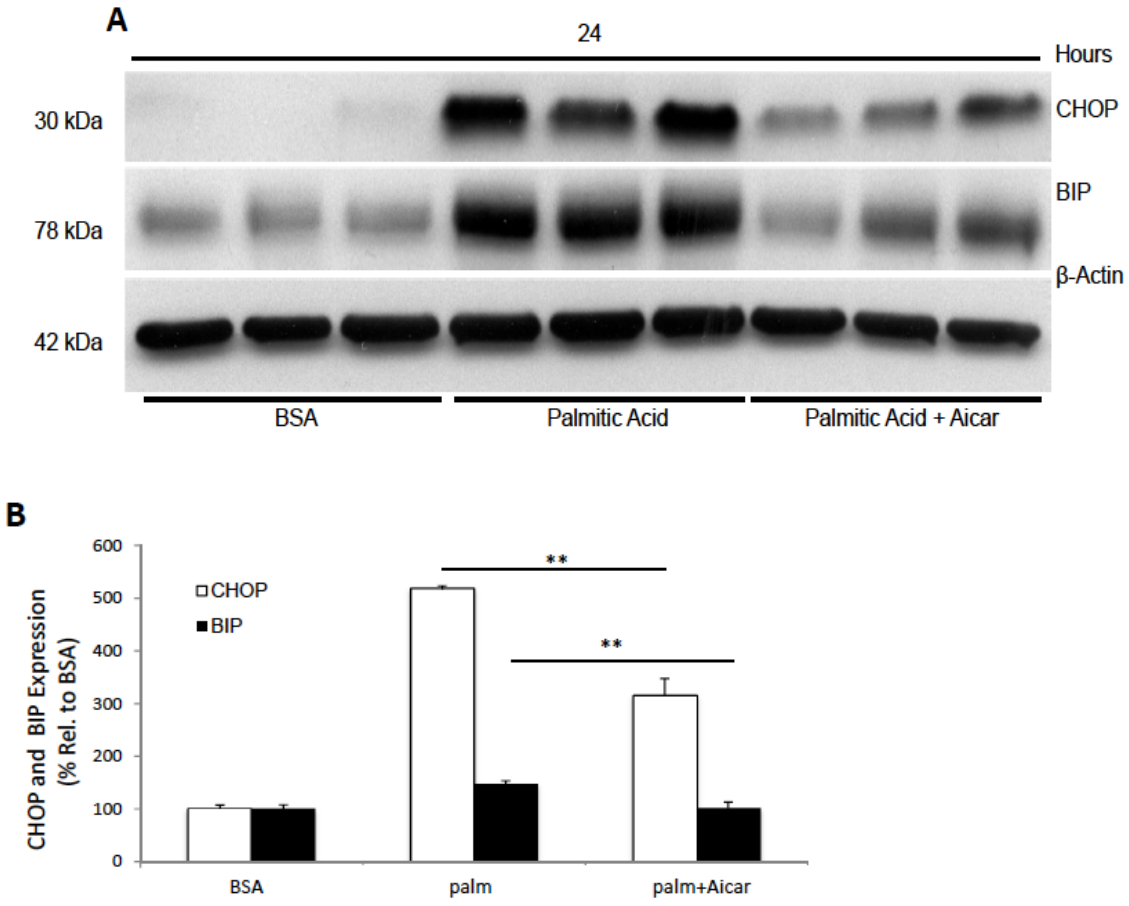


Figure 28: Aicar mitigates palmitic acid induced ER-stress. A: Aicar attenuated palmitic acid-induced induction of CHOP and BiP after 24 hours. CHOP and BIP levels were analyzed by Western immunoblotting. ß-actin served as a loading control. B: Quantification of CHOP and BIP levels. Bar graph represents the relative mean expression levels ± SD (n = 3, ** $p < 0.01$). BSA treated controls were set to 100%.

4. DISCUSSION

4.1. Aggravation of palmitic acid induced podocyte death by high glucose, TGF-β, and TNF-α

Podocytopathy and loss occur at the onset of DN, predict progressive kidney disease (Meyer et al., 1999). Multiple factors of the diabetic milieu have been reported to induce podocyte death, including high glucose concentrations (Susztak et al., 2006), TGF-β (Schiffer et al., 2001), and TNF-α (Ryu et al., 2012; Tejada et al., 2008) . Our group previously reported that podocytes are highly susceptible to palmitic acid induced cell death (Sieber et al., 2010). Importantly, palmitic acid induces podocyte death at relatively low concentration starting from 125μM, a concentration which is well within the reported physiological range of 120 to 340 μM (Fraser et al., 1999; Groop et al., 1989; Hagenfeldt et al., 1972). *In vivo,* likely multiple factors contribute to podocyte death, and this was the reason why I explored to which extent palmitic acid modifies the response of podocyte to previously reported proapoptotic factors such as high glucose levels, TGF-β, and TNF-α.

First, I observed that the effect of palmitic acid *per se* on podocyte death was strong, very robust, and independent of glucose concentrations, a result which further confirms the strong and very robust effect of palmitic acid on podocyte death (Sieber et al., 2010). Interestingly, I observed that a high glucose concentration of 22mM compared to a normal glucose concentration defined as 11mM, which is the concentration in most cell culture media had no or only a weak effect on podocyte death (figure 9). Contrariwise, the toxicity of 22mM glucose was very well visible in the presence of palmitic acid with a significant increase of apoptotic podocytes (figure 9). Previously we reported that the increase of necrotic podocytes can be underestimated, in particular if the necrosis level is already high (Sieber et al., 2010), as necrotic cells break apart and cannot be recovered for the cell death assay. This may explain why the increase could only be seen for apoptotic, and not necrotic podocytes. It is not clear why the effect of high glucose compared to normal glucose was difficult to see in the absence of palmitic acid, and this was in contrast to previous studies (Susztak et al., 2006). Possibly BSA as a control for palmitic acid complexed to BSA did account for this difference although in preliminary experiments without BSA the effect of high glucose alone was also not clearly visible. Certainly methodological differences may also explain these discrepancies. Further studies will be needed clarify this issue.

Next, I studied the effect of TGF-β in the presence and absence of palmitic acid. The effect of TGF-β alone on podocyte death was minimal and did not reach statistical significance (figure 10). In contrast, the toxicity of TGF-β was highly significant in the presence of palmitic acid (figure 10). Why the effect of TGF-β alone was weak is not clear, and contrast previous reports (Schiffer et al., 2001). More studies are needed, and in particular the mechanisms for the increased toxicity of TGF-β in podocyte exposed to palmitic acid will be interesting to study in the future.

The effect of TNF-α on podocyte was robust (figure 11) as reported previously (Ryu et al., 2012; Tejada et al., 2008). In the presence of palmitic acid the additional increase was of modest and only visible for apoptotic podocytes. As mentioned previously, the reason that the effect was not seen for necrotic podocytes may be related to incomplete recovery of necrotic cells (Sieber et al., 2010).

In summary, the results reported here are interesting and suggest that FFAs as palmitic acid can substantially aggravate the toxicity of other factors which are thought to be important in the pathogenesis of DN and which contribute to podocyte loss.

4.2. Regulation of fatty acid oxidation in palmitic acid induced podocyte death: critical role of acetyl CoA carboxylase 1 and 2

The study uncovers that in the glomeruli of type 2 diabetic patients with DN mRNA expression levels of several key enzymes involved in fatty acid metabolism are altered. Of particular relevance for the current work, a significant upregulation of all three isoforms of CPT-1, the rate-limiting enzyme for fatty acid oxidation, and a downregulation of ACC-2, which catalyzes the formation of the CPT-1 inhibitor malonyl-CoA, is found which suggests a disposition for increased fatty acid oxidation. Together with the detailed in vitro studies discussed below, this changed expression profile most likely suggests a compensatory, protective response.

Furthermore, several lines of evidence indicate that regulation of FAO and interference with the AMPK-ACC-CPT-1 pathway affects podocytes exposed to palmitic acid. Specifically, the AMPK agonist Aicar, which significantly stimulates FAO in podocytes, reduces palmitic acid-induced podocyte death (figure 15). Conversely, the AMPK inhibitor compound C increased palmitic acid-induced cell death (figure 18). Furthermore, the CPT-1 inhibitor etomoxir, which completely prevents the Aicar induced increase of FAO in podocytes,

potentiates the toxicity of palmitic acid and dose-dependently reverses the protective effect of Aicar (figure 20). Moreover, gene silencing of ACC1/ACC2 markedly reduced palmitic acid-induced cell death (figure 25).

Adiponectin, a physiological activator of AMPK in podocytes (Sharma et al., 2008), also reduced palmitic acid-induced podocyte death (figure 16). Although its protective effect was relatively small compared to pharmacological activation by Aicar, the sustained action of adiponectin *in vivo* may still be relevant for the protection of podocytes from lipotoxicity. Activation of AMPK by adiponectin or Aicar is also reported to suppress oxidative stress and the NADPH oxidase Nox4 (Sharma et al., 2008). As neither tempol, a membrane-permeable radical scavenger, nor the antioxidant N-acetylcysteine reduce palmitic acid-induced podocyte death (unpublished observation), the modulation of oxidative stress through the AMPK pathway related to lipotoxicity needs further investigation.

To further address the role of AMPK for palmitic acid-induced podocyte death we additionally used the AMPK inhibitor compound C which increased the toxicity of palmitic acid (figure 18). Compound C reduced the Aicar induced phosphorylation of ACC (figure 17) and partially prevented the protective Aicar effect (figure 18). Together, these findings suggest that the susceptibility of podocytes exposed to palmitic acid can be greatly modulated by AMPK.

The present results indicating an important role of FAO and the AMPK-ACC-CPT-1 pathway for the susceptibility of podocytes exposed to toxic FFAs extend and potentially explain the results of recent GWAS which found a SNP in ACC2 with a significant enhancer activity resulting in an increased ACC2 expression associated with proteinuria in type 2 diabetic patients (Maeda et al., 2010; Tang et al., 2010). Moreover the results of this study suggest that the recently published observation of a decreased expression of ACC2 and an increased expression of all CPT-1 isoforms in glomerular extracts of type 2 diabetic patients (Sieber et al., 2013) reflects an adaptive, protective mechanism against toxic FFAs in DN.

The differential role of ACC1 and ACC2 for the regulation of FAO is under debate (Olson et al., 2010). I found that only double knockdown of ACC1 and ACC2 has a protective effect on palmitic acid-induced cell death (figure 25). This indicates that both isoforms contribute to the inhibition of CPT-1 in podocytes as previously suggested for hepatocytes and skeletal muscle cells (Olson et al., 2010; Savage et al., 2006).

Interestingly, Aicar showed a small residual protective effect in ACC1/ACC2 double knockdown podocytes (figure 27). This may be due to residual expression of ACC-isoforms or an additional ACC independent effect. Activation of AMPK stimulates e. g. peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1α (Iwabu et al., 2010) which has been shown to be important for mitochondrial function in podocytes (Yuan et al., 2012). Finally, AMPK-independent off-target effects of Aicar cannot be excluded. Future studies are needed to confirm or refute this hypothesis.

The biguanide metformin is widely used to treat type 2 diabetes (Qaseem et al., 2012). Its mechanism of action is not fully established but is reported to involve indirect activation of the AMPK-ACC-CPT-1 pathway via inhibition of complex I of the respiratory chain and a consequent increase in the AMP:ATP ratio which results in AMPK activation (Zang et al., 2004). Despite this potential mode of action, preliminary experiments showed that metformin from 0.5-2 mM displays no protection from palmitic acid-induced lipotoxicity in podocytes (data not shown). Previously, undesired effects of metformin leading to cell death have been reported for pancreatic β-cells (Kefas et al., 2004). Of interest, a potential beneficial effect of metformin was shown in podocytes exposed to high glucose concentration of 30 mM by decreasing ROS production through reduction of NAD(P)H oxidase activity (Piwkowska et al., 2010). Clearly, more studies are required to reassess the short and long time effects of metformin on podocytes.

Interestingly, Aicar significantly reduces the induction of CHOP in podocytes exposed to palmitic acid (figure 28), which is likely contributes to the protective effect of Aicar as gene silencing of CHOP attenuates palmitic acid-induced cell death (Sieber et al., 2010). The action of Aicar on the AMPK-ACC-CPT-1 pathway may indicate that increased FAO reduces palmitic acid derived toxic metabolites and therefore suppresses the induction of ER-stress. However, the basic unanswered question is how palmitic acid and its metabolites trigger ER-stress. Some reports indicated that palmitic acid rapidly increases the saturated lipid content of the ER leading to compromised ER morphology and integrity (Borradaile et al., 2006). In pancreatic β-cells palmitic acid depletes ER Ca^{2+} and slows ER Ca^{2+} uptake (Cunha et al., 2008) which leads to accumulation of unfolded proteins. However, the detailed molecular mechanisms are not well known (Back et al., 2012). Clearly, more experiments are needed to understand these principle mechanisms and to study whether FAO and ER-stress are causatively related.

5. CONCLUSION

The present study emphasizes the critical role of FFAs and FFA metabolism for podocyte survival. As loss of podocytes critically determines the pathogenesis and progression of DN, the findings contribute to a better understanding of the most common cause leading to ESRD. The results reported here suggest that palmitic acid significantly aggravates the toxicity of other factors such as high glucose concentrations and TGF-β, as their effect on podocyte death was largely increased in the presence of palmitic acid.

My results suggest that modulation of FFA metabolism and stimulating FAO by activation of the AMPK-ACC-CPT-1 pathway critically influences the susceptibility of podocytes exposed to toxic FFAs. Together with our previous studies the following working model is suggested (Figure 29). Palmitic acid increases the generation of toxic metabolites, which leads to ER-stress and podocyte death (Sieber et al., 2010). Oleic acid or induction of Scd-1/-2 expression by the LXR-agonists TO and GW shift palmitic acid and its metabolites into a "safe lipid pool" containing triglycerides (TG), which reduces injurious metabolites and prevents podocyte death (Sieber et al., 2013). In addition, activation of the AMPK-ACC-CPT-1 pathway by Aicar reduces palmitic acid-induced podocyte death by increasing FAO and reducing palmitic acid and its metabolites. Our findings may explain the results of recent genome wide association studies indicating that modulation of FFA metabolism and specifically modulation of FAO directly affects the susceptibility to DN. In addition, our results have potentially important therapeutic implications to prevent and treat DN in type 2 diabetic patients.

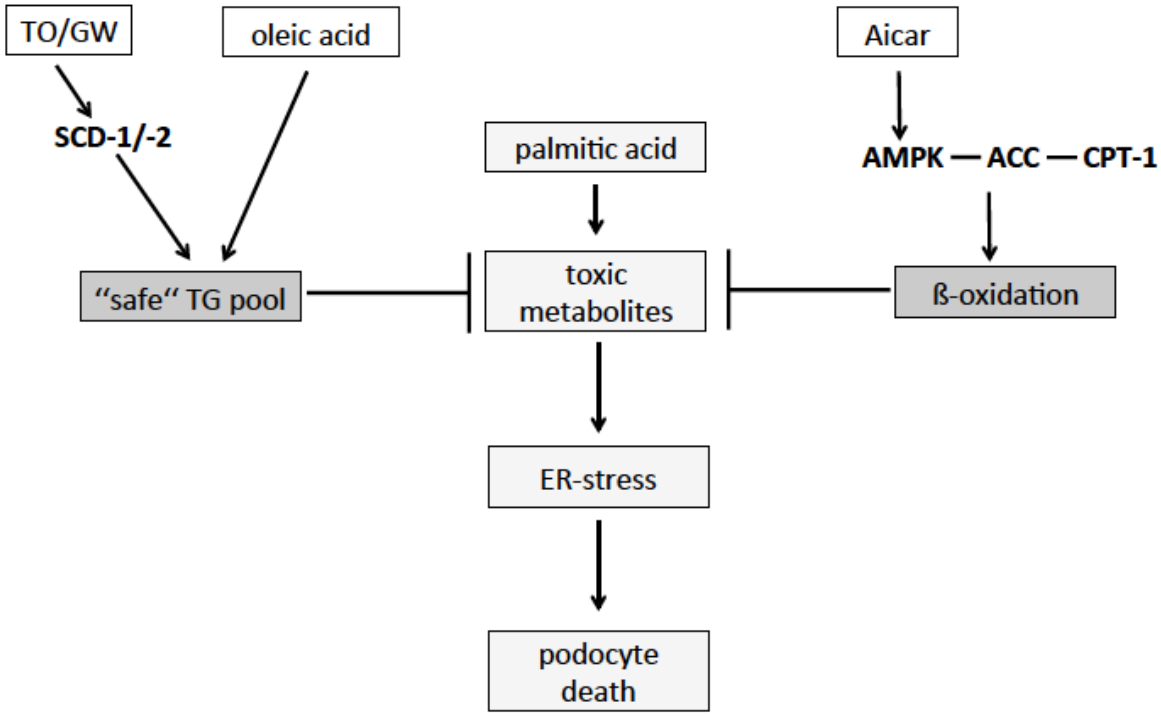


Figure 29: A working model for the Aicar activated AMPK-ACC-CPT-1 pathway and the prosurvival effects of oleic acid and Scd-1/-2 on palmitic acid-induced podocyte death. Palmitic acid increases the generation of toxic metabolites, which leads to ER-stress and podocyte death. The LXR-agonists TO and GW increase Scd-1/2 expression, which in turn elevates the TG safe pool and reduces injurious metabolites and subsequent podocyte death. Similarly, oleic acid ameliorates palmitic acid-induced podocyte death by shifting palmitic acid and toxic metabolites to the TG safe pool. Additionally, Aicar reduces palmitic acid-induced podocyte death by stimulation of the AMPK-ACC-CPT-1 pathway and subsequently β-oxidation, thus decreasing toxic metabolites and eventually podocyte

6. REFERENCES

2013. USRDS 2013 Annual Data Report.

Abu-Elheiga, L., M.M. Matzuk, P. Kordari, W. Oh, T. Shaikenov, Z. Gu, and S.J. Wakil. 2005. Mutant mice lacking acetyl-CoA carboxylase 1 are embryonically lethal. *Proceedings of the National Academy of Sciences of the United States of America*. 102:12011-12016.

Back, S.H., S.W. Kang, J. Han, and H.T. Chung. 2012. Endoplasmic reticulum stress in the beta-cell pathogenesis of type 2 diabetes. *Exp Diabetes Res*. 2012:618396.

Berg, U.B., T.B. Torbjornsdotter, G. Jaremko, and B. Thalme. 1998. Kidney morphological changes in relation to long-term renal function and metabolic control in adolescents with IDDM. *Diabetologia*. 41:1047-1056.

Bergman, B.C., L. Perreault, D.M. Hunerdosse, M.C. Koehler, A.M. Samek, and R.H. Eckel. Increased intramuscular lipid synthesis and low saturation relate to insulin sensitivity in endurance-trained athletes. *J Appl Physiol*. 108:1134-1141.

Bertolotti, A., Y. Zhang, L.M. Hendershot, H.P. Harding, and D. Ron. 2000. Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. *Nature cell biology*. 2:326-332.

Bikman, B.T., and S.A. Summers. 2011. Ceramides as modulators of cellular and whole-body metabolism. *The Journal of clinical investigation*. 121:4222-4230.

Borradaile, N.M., X. Han, J.D. Harp, S.E. Gale, D.S. Ory, and J.E. Schaffer. 2006. Disruption of endoplasmic reticulum structure and integrity in lipotoxic cell death. *J Lipid Res*. 47:2726-2737.

Brookheart, R.T., C.I. Michel, and J.E. Schaffer. 2009. As a matter of fat. *Cell Metab*. 10:9-12.

Cases, S., S.J. Smith, Y.W. Zheng, H.M. Myers, S.R. Lear, E. Sande, S. Novak, C. Collins, C.B. Welch, A.J. Lusis, S.K. Erickson, and R.V. Farese, Jr. 1998. Identification of a gene encoding an acyl CoA:diacylglycerol acyltransferase, a key enzyme in triacylglycerol synthesis. *Proc Natl Acad Sci U S A*. 95:13018-13023.

Cases, S., S.J. Stone, P. Zhou, E. Yen, B. Tow, K.D. Lardizabal, T. Voelker, and R.V. Farese, Jr. 2001. Cloning of DGAT2, a second mammalian diacylglycerol acyltransferase, and related family members. *J Biol Chem*. 276:38870-38876.

Chen, S., B. Jim, and F.N. Ziyadeh. 2003. Diabetic nephropathy and transforming growth factor-beta: transforming our view of glomerulosclerosis and fibrosis build-up. *Semin Nephrol*. 23:532-543.

Chicco, A.J., and G.C. Sparagna. 2007. Role of cardiolipin alterations in mitochondrial dysfunction and disease. *Am J Physiol Cell Physiol*. 292:C33-44.

Cunha, D.A., P. Hekerman, L. Ladriere, A. Bazarra-Castro, F. Ortis, M.C. Wakeham, F. Moore, J. Rasschaert, A.K. Cardozo, E. Bellomo, L. Overbergh, C. Mathieu, R. Lupi, T. Hai, A. Herchuelz, P. Marchetti, G.A. Rutter, D.L. Eizirik, and M. Cnop. 2008. Initiation and execution of lipotoxic ER stress in pancreatic beta-cells. *J Cell Sci*. 121:2308-2318.

Dalla Vestra, M., A. Masiero, A.M. Roiter, A. Saller, G. Crepaldi, and P. Fioretto. 2003. Is podocyte injury relevant in diabetic nephropathy? Studies in patients with type 2 diabetes. *Diabetes*. 52:1031-1035.

Diakogiannaki, E., H.J. Welters, and N.G. Morgan. 2008. Differential regulation of the endoplasmic reticulum stress response in pancreatic beta-cells exposed to long-chain saturated and monounsaturated fatty acids. *The Journal of endocrinology*. 197:553-563.

Ding, G., K. Reddy, A.A. Kapasi, N. Franki, N. Gibbons, B.S. Kasinath, and P.C. Singhal. 2002. Angiotensin II induces apoptosis in rat glomerular epithelial cells. *Am J Physiol Renal Physiol*. 283:F173-180.

Endlich, N., and K. Endlich. 2006. Stretch, tension and adhesion - adaptive mechanisms of the actin cytoskeleton in podocytes. *Eur J Cell Biol*. 85:229-234.

Foley, R.N., P.S. Parfrey, and M.J. Sarnak. 1998. Epidemiology of cardiovascular disease in chronic renal disease. *J Am Soc Nephrol*. 9:S16-23.

Fraser, D.A., J. Thoen, A.C. Rustan, O. Forre, and J. Kjeldsen-Kragh. 1999. Changes in plasma free fatty acid concentrations in rheumatoid arthritis patients during fasting and their effects upon T-lymphocyte proliferation. *Rheumatology (Oxford)*. 38:948-952.

Groop, L.C., R.C. Bonadonna, S. DelPrato, K. Ratheiser, K. Zyck, E. Ferrannini, and R.A. DeFronzo. 1989. Glucose and free fatty acid metabolism in non-insulin-dependent diabetes mellitus. Evidence for multiple sites of insulin resistance. *The Journal of clinical investigation*. 84:205-213.

Guo, W., S. Wong, W. Xie, T. Lei, and Z. Luo. 2007. Palmitate modulates intracellular signaling, induces endoplasmic reticulum stress, and causes apoptosis in mouse 3T3-L1 and rat primary preadipocytes. *American journal of physiology*. 293:E576-586.

Hagenfeldt, L., J. Wahren, B. Pernow, and L. Raf. 1972. Uptake of individual free fatty acids by skeletal muscle and liver in man. *J Clin Invest*. 51:2324-2330.

Hardy, S., W. El-Assaad, E. Przybytkowski, E. Joly, M. Prentki, and Y. Langelier. 2003. Saturated fatty acid-induced apoptosis in MDA-MB-231 breast cancer cells. A role for cardiolipin. *J Biol Chem*. 278:31861-31870.

Haugen, E.N., A.J. Croatt, and K.A. Nath. 2000. Angiotensin II induces renal oxidant stress in vivo and heme oxygenase-1 in vivo and in vitro. *Kidney Int*. 58:144-152.

Hihi, A.K., L. Michalik, and W. Wahli. 2002. PPARs: transcriptional effectors of fatty acids and their derivatives. *Cell Mol Life Sci*. 59:790-798.

Holzer, R.G., E.J. Park, N. Li, H. Tran, M. Chen, C. Choi, G. Solinas, and M. Karin. 2011. Saturated fatty acids induce c-Src clustering within membrane subdomains, leading to JNK activation. *Cell*. 147:173-184.

Hotamisligil, G.S. 2010. Endoplasmic reticulum stress and the inflammatory basis of metabolic disease. *Cell*. 140:900-917.

Huber, T.B., B. Hartleben, J. Kim, M. Schmidts, B. Schermer, A. Keil, L. Egger, R.L. Lecha, C. Borner, H. Pavenstadt, A.S. Shaw, G. Walz, and T. Benzing. 2003. Nephrin and CD2AP associate with phosphoinositide 3-OH kinase and stimulate AKT-dependent signaling. *Molecular and cellular biology*. 23:4917-4928.

Iwabu, M., T. Yamauchi, M. Okada-Iwabu, K. Sato, T. Nakagawa, M. Funata, M. Yamaguchi, S. Namiki, R. Nakayama, M. Tabata, H. Ogata, N. Kubota, I. Takamoto, Y.K. Hayashi, N. Yamauchi, H. Waki, M. Fukayama, I. Nishino, K. Tokuyama, K. Ueki, Y. Oike, S. Ishii, K. Hirose, T. Shimizu, K. Touhara, and T. Kadowaki. 2010. Adiponectin and AdipoR1 regulate PGC-1alpha and mitochondria by Ca(2+) and AMPK/SIRT1. *Nature*. 464:1313-1319.

Jefferson, J.A., S.J. Shankland, and R.H. Pichler. 2008. Proteinuria in diabetic kidney disease: a mechanistic viewpoint. *Kidney international*. 74:22-36.

Jeon, S.M., N.S. Chandel, and N. Hay. 2012. AMPK regulates NADPH homeostasis to promote tumour cell survival during energy stress. *Nature*. 485:661-665.

Jiang, T., X.X. Wang, P. Scherzer, P. Wilson, J. Tallman, H. Takahashi, J. Li, M. Iwahashi, E. Sutherland, L. Arend, and M. Levi. 2007. Farnesoid X receptor modulates renal lipid metabolism, fibrosis, and diabetic nephropathy. *Diabetes*. 56:2485-2493.

Kaufman, R.J. 2002. Orchestrating the unfolded protein response in health and disease. *J Clin Invest*. 110:1389-1398.

Kefas, B.A., Y. Cai, K. Kerckhofs, Z. Ling, G. Martens, H. Heimberg, D. Pipeleers, and M. Van de Casteele. 2004. Metformin-induced stimulation of AMP-activated protein kinase in beta-cells impairs their glucose responsiveness and can lead to apoptosis. *Biochem Pharmacol*. 68:409-416.

Keller, H., C. Dreyer, J. Medin, A. Mahfoudi, K. Ozato, and W. Wahli. 1993. Fatty acids and retinoids control lipid metabolism through activation of peroxisome proliferator-activated receptor-retinoid X receptor heterodimers. *Proc Natl Acad Sci U S A*. 90:2160-2164.

Kharroubi, I., L. Ladriere, A.K. Cardozo, Z. Dogusan, M. Cnop, and D.L. Eizirik. 2004. Free fatty acids and cytokines induce pancreatic beta-cell apoptosis by different mechanisms: role of nuclear factor-kappaB and endoplasmic reticulum stress. *Endocrinology*. 145:5087-5096.

Kimmelstiel P, W.C. 1936. Intercapillary lesions in the glomeruli of the kidney. *Am J Pathol*. 12:82-97.

Kimmelstiel, P., and C. Wilson. 1936. Intercapillary Lesions in the Glomeruli of the Kidney. *The American journal of pathology*. 12:83-98 87.

Kriz, W. 2003. The pathogenesis of 'classic' focal segmental glomerulosclerosis-lessons from rat models. *Nephrol Dial Transplant*. 18 Suppl 6:vi39-44.

Kume, S., T. Uzu, S. Araki, T. Sugimoto, K. Isshiki, M. Chin-Kanasaki, M. Sakaguchi, N. Kubota, Y. Terauchi, T. Kadowaki, M. Haneda, A. Kashiwagi, and D. Koya. 2007. Role of altered renal lipid metabolism in the development of renal injury induced by a high-fat diet. *Journal of the American Society of Nephrology : JASN*. 18:2715-2723.

Laybutt, D.R., A.M. Preston, M.C. Akerfeldt, J.G. Kench, A.K. Busch, A.V. Biankin, and T.J. Biden. 2007. Endoplasmic reticulum stress contributes to beta cell apoptosis in type 2 diabetes. *Diabetologia*. 50:752-763.

Lee, H.S., J.S. Lee, H.I. Koh, and K.W. Ko. 1991. Intraglomerular lipid deposition in routine biopsies. *Clin Nephrol*. 36:67-75.

Lennon, R., D. Pons, M.A. Sabin, C. Wei, J.P. Shield, R.J. Coward, J.M. Tavare, P.W. Mathieson, M.A. Saleem, and G.I. Welsh. 2009. Saturated fatty acids induce insulin resistance in human podocytes: implications for diabetic nephropathy. *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association*. 24:3288-3296.

Listenberger, L.L., X. Han, S.E. Lewis, S. Cases, R.V. Farese, Jr., D.S. Ory, and J.E. Schaffer. 2003. Triglyceride accumulation protects against fatty acid-induced lipotoxicity. *Proc Natl Acad Sci U S A*. 100:3077-3082.

Listenberger, L.L., and J.E. Schaffer. 2002. Mechanisms of lipoapoptosis: implications for human heart disease. *Trends Cardiovasc Med*. 12:134-138.

Locatelli, F., P. Pozzoni, and L. Del Vecchio. 2004. Renal replacement therapy in patients with diabetes and end-stage renal disease. *J Am Soc Nephrol*. 15 Suppl 1:S25-29.

Ma, Y., and L.M. Hendershot. 2001. The unfolding tale of the unfolded protein response. *Cell*. 107:827-830.

Maeda, S., M.A. Kobayashi, S. Araki, T. Babazono, B.I. Freedman, M.A. Bostrom, J.N. Cooke, M. Toyoda, T. Umezono, L. Tarnow, T. Hansen, P. Gaede, A. Jorsal, D.P. Ng, M. Ikeda, T. Yanagimoto, T. Tsunoda, H. Unoki, K. Kawai, M. Imanishi, D. Suzuki, H.D. Shin, K.S. Park, A. Kashiwagi, Y. Iwamoto, K. Kaku, R. Kawamori, H.H. Parving, D.W. Bowden, O. Pedersen, and Y. Nakamura. 2010. A single nucleotide polymorphism within the acetyl-coenzyme A carboxylase beta gene is associated with proteinuria in patients with type 2 diabetes. *PLoS Genet*. 6:e1000842.

Marshall, C.B., and S.J. Shankland. 2006. Cell cycle and glomerular disease: a minireview. *Nephron Exp Nephrol*. 102:e39-48.

Martinez, S.C., K. Tanabe, C. Cras-Meneur, N.A. Abumrad, E. Bernal-Mizrachi, and M.A. Permutt. 2008. Inhibition of Foxo1 protects pancreatic islet beta-cells against fatty acid and endoplasmic reticulum stress-induced apoptosis. *Diabetes*. 57:846-859.

Meyer, T.W., P.H. Bennett, and R.G. Nelson. 1999. Podocyte number predicts long-term urinary albumin excretion in Pima Indians with Type II diabetes and microalbuminuria. *Diabetologia*. 42:1341-1344.

Mundel, P., and W. Kriz. 1995. Structure and function of podocytes: an update. *Anat Embryol (Berl)*. 192:385-397.

Mundel, P., J. Reiser, A. Zuniga Mejia Borja, H. Pavenstadt, G.R. Davidson, W. Kriz, and R. Zeller. 1997. Rearrangements of the cytoskeleton and cell contacts induce process formation during differentiation of conditionally immortalized mouse podocyte cell lines. *Exp Cell Res*. 236:248-258.

Muoio, D.M., and C.B. Newgard. 2008. Fatty acid oxidation and insulin action: when less is more. *Diabetes*. 57:1455-1456.

Nakamura, T., C. Ushiyama, S. Suzuki, M. Hara, N. Shimada, I. Ebihara, and H. Koide. 2000. Urinary excretion of podocytes in patients with diabetic nephropathy. *Nephrol Dial Transplant*. 15:1379-1383.

Navarro-Gonzalez, J.F., A. Jarque, M. Muros, C. Mora, and J. Garcia. 2009. Tumor necrosis factor-alpha as a therapeutic target for diabetic nephropathy. *Cytokine Growth Factor Rev*. 20:165-173.

Nolan, C.J., and C.Z. Larter. 2009. Lipotoxicity: why do saturated fatty acids cause and monounsaturates protect against it? *Journal of gastroenterology and hepatology*. 24:703-706.

Ntambi, J.M., and M. Miyazaki. 2003. Recent insights into stearoyl-CoA desaturase-1. *Curr Opin Lipidol*. 14:255-261.

Olson, D.P., T. Pulinilkunnil, G.W. Cline, G.I. Shulman, and B.B. Lowell. 2010. Gene knockout of Acc2 has little effect on body weight, fat mass, or food intake. *Proceedings of the National Academy of Sciences of the United States of America*. 107:7598-7603.

Ostrander, D.B., G.C. Sparagna, A.A. Amoscato, J.B. McMillin, and W. Dowhan. 2001. Decreased cardiolipin synthesis corresponds with cytochrome c release in palmitate-induced cardiomyocyte apoptosis. *J Biol Chem*. 276:38061-38067.

Ozcan, U., Q. Cao, E. Yilmaz, A.H. Lee, N.N. Iwakoshi, E. Ozdelen, G. Tuncman, C. Gorgun, L.H. Glimcher, and G.S. Hotamisligil. 2004. Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. *Science*. 306:457-461.

Pagtalunan, M.E., P.L. Miller, S. Jumping-Eagle, R.G. Nelson, B.D. Myers, H.G. Rennke, N.S. Coplon, L. Sun, and T.W. Meyer. 1997. Podocyte loss and progressive glomerular injury in type II diabetes. *J Clin Invest*. 99:342-348.

Patrakka, J., and K. Tryggvason. 2010. Molecular make-up of the glomerular filtration barrier. *Biochem Biophys Res Commun*. 396:164-169.

Piro, S., M. Anello, C. Di Pietro, M.N. Lizzio, G. Patane, A.M. Rabuazzo, R. Vigneri, M. Purrello, and F. Purrello. 2002. Chronic exposure to free fatty acids or high glucose induces apoptosis in rat pancreatic islets: possible role of oxidative stress. *Metabolism*. 51:1340-1347.

Piwkowska, A., D. Rogacka, M. Jankowski, M.H. Dominiczak, J.K. Stepinski, and S. Angielski. 2010. Metformin induces suppression of NAD(P)H oxidase activity in podocytes. *Biochem Biophys Res Commun*. 393:268-273.

Qaseem, A., L.L. Humphrey, D.E. Sweet, M. Starkey, and P. Shekelle. 2012. Oral pharmacologic treatment of type 2 diabetes mellitus: a clinical practice guideline from the American College of Physicians. *Ann Intern Med*. 156:218-231.

Rasheva, V.I., and P.M. Domingos. 2009. Cellular responses to endoplasmic reticulum stress and apoptosis. *Apoptosis*. 14:996-1007.

Reidy, K., and F.J. Kaskel. 2007. Pathophysiology of focal segmental glomerulosclerosis. *Pediatr Nephrol*. 22:350-354.

Reiser, J., W. Kriz, M. Kretzler, and P. Mundel. 2000. The glomerular slit diaphragm is a modified adherens junction. *Journal of the American Society of Nephrology : JASN*. 11:1-8.

Ricchi, M., M.R. Odoardi, L. Carulli, C. Anzivino, S. Ballestri, A. Pinetti, L.I. Fantoni, F. Marra, M. Bertolotti, S. Banni, A. Lonardo, N. Carulli, and P. Loria. 2009. Differential effect of oleic and palmitic acid on lipid accumulation and apoptosis in cultured hepatocytes. *Journal of gastroenterology and hepatology*. 24:830-840.

Ryu, M., S.R. Mulay, N. Miosge, O. Gross, and H.J. Anders. 2012. Tumour necrosis factor-alpha drives Alport glomerulosclerosis in mice by promoting podocyte apoptosis. *J Pathol*. 226:120-131.

Savage, D.B., C.S. Choi, V.T. Samuel, Z.X. Liu, D. Zhang, A. Wang, X.M. Zhang, G.W. Cline, X.X. Yu, J.G. Geisler, S. Bhanot, B.P. Monia, and G.I. Shulman. 2006. Reversal of diet-induced hepatic steatosis and hepatic insulin resistance by antisense oligonucleotide inhibitors of acetyl-CoA carboxylases 1 and 2. *The Journal of clinical investigation*. 116:817-824.

Schiffer, M., M. Bitzer, I.S. Roberts, J.B. Kopp, P. ten Dijke, P. Mundel, and E.P. Bottinger. 2001. Apoptosis in podocytes induced by TGF-beta and Smad7. *J Clin Invest*. 108:807-816.

Shankland, S.J. 2006. The podocyte's response to injury: role in proteinuria and glomerulosclerosis. *Kidney Int*. 69:2131-2147.

Shankland, S.J., J.W. Pippin, J. Reiser, and P. Mundel. 2007. Podocytes in culture: past, present, and future. *Kidney Int*. 72:26-36.

Sharma, K., S. Ramachandrarao, G. Qiu, H.K. Usui, Y. Zhu, S.R. Dunn, R. Ouedraogo, K. Hough, P. McCue, L. Chan, B. Falkner, and B.J. Goldstein. 2008. Adiponectin regulates albuminuria and podocyte function in mice. *The Journal of clinical investigation*. 118:1645-1656.

Sieber, J., M.T. Lindenmeyer, K. Kampe, K.N. Campbell, C.D. Cohen, H. Hopfer, P. Mundel, and A.W. Jehle. 2010. Regulation of podocyte survival and endoplasmic reticulum stress by fatty acids. *Am J Physiol Renal Physiol*. 299:F821-829.

Sieber, J., A. Weins, K. Kampe, S. Gruber, M.T. Lindenmeyer, C.D. Cohen, J.M. Orellana, P. Mundel, and A.W. Jehle. 2013. Susceptibility of Podocytes to Palmitic Acid Is Regulated by Stearoyl-CoA Desaturases 1 and 2. *The American journal of pathology*. 183:735-744.

Siskind, L.J. 2005. Mitochondrial ceramide and the induction of apoptosis. *J Bioenerg Biomembr*. 37:143-153.

Smoyer, W.E., and P. Mundel. 1998. Regulation of podocyte structure during the development of nephrotic syndrome. *J Mol Med (Berl)*. 76:172-183.

Song, B., D. Scheuner, D. Ron, S. Pennathur, and R.J. Kaufman. 2008. Chop deletion reduces oxidative stress, improves beta cell function, and promotes cell survival in multiple mouse models of diabetes. *J Clin Invest*. 118:3378-3389.

Steffes, M.W., D. Schmidt, R. McCrery, and J.M. Basgen. 2001. Glomerular cell number in normal subjects and in type 1 diabetic patients. *Kidney Int*. 59:2104-2113.

Sun, L., N. Halaihel, W. Zhang, T. Rogers, and M. Levi. 2002. Role of sterol regulatory element-binding protein 1 in regulation of renal lipid metabolism and glomerulosclerosis in diabetes mellitus. *J Biol Chem*. 277:18919-18927.

Susztak, K., A.C. Raff, M. Schiffer, and E.P. Bottinger. 2006. Glucose-induced reactive oxygen species cause apoptosis of podocytes and podocyte depletion at the onset of diabetic nephropathy. *Diabetes*. 55:225-233.

Tang, S.C., V.T. Leung, L.Y. Chan, S.S. Wong, D.W. Chu, J.C. Leung, Y.W. Ho, K.N. Lai, L. Ma, S.C. Elbein, D.W. Bowden, P.J. Hicks, M.E. Comeau, C.D. Langefeld, and B.I. Freedman. 2010. The acetyl-coenzyme A carboxylase beta (ACACB) gene is associated with nephropathy in Chinese patients with type 2 diabetes. *Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association*. 25:3931-3934.

Tao, J.L., Y.B. Wen, B.Y. Shi, H. Zhang, X.Z. Ruan, H. Li, X.M. Li, W.J. Dong, and X.W. Li. 2012. Endoplasmic reticulum stress is involved in podocyte apoptosis induced by saturated fatty acid palmitate. *Chin Med J (Engl)*. 125:3137-3142.

Tejada, T., P. Catanuto, A. Ijaz, J.V. Santos, X. Xia, P. Sanchez, N. Sanabria, O. Lenz, S.J. Elliot, and A. Fornoni. 2008. Failure to phosphorylate AKT in podocytes from mice with early diabetic nephropathy promotes cell death. *Kidney Int*. 73:1385-1393.

USRDS. 2011. The United States Renal Data System 2011 Annual Data Report: Atlas of Chronic Kidney Disease and End-Stage Renal Disease in the United States. Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD, 2011.

Wang, Z., T. Jiang, J. Li, G. Proctor, J.L. McManaman, S. Lucia, S. Chua, and M. Levi. 2005. Regulation of renal lipid metabolism, lipid accumulation, and glomerulosclerosis in FVBdb/db mice with type 2 diabetes. *Diabetes*. 54:2328-2335.

Wei, Y., D. Wang, C.L. Gentile, and M.J. Pagliassotti. 2009. Reduced endoplasmic reticulum luminal calcium links saturated fatty acid-mediated endoplasmic reticulum stress and cell death in liver cells. *Molecular and cellular biochemistry*.

Wei, Y., D. Wang, F. Topczewski, and M.J. Pagliassotti. 2006. Saturated fatty acids induce endoplasmic reticulum stress and apoptosis independently of ceramide in liver cells. *American journal of physiology*. 291:E275-281.

White, K.E., and R.W. Bilous. 2000. Type 2 diabetic patients with nephropathy show structural-functional relationships that are similar to type 1 disease. *J Am Soc Nephrol*. 11:1667-1673.

White, K.E., and R.W. Bilous. 2004. Structural alterations to the podocyte are related to proteinuria in type 2 diabetic patients. *Nephrol Dial Transplant*. 19:1437-1440.

White, K.E., R.W. Bilous, S.M. Marshall, M. El Nahas, G. Remuzzi, G. Piras, S. De Cosmo, and G. Viberti. 2002. Podocyte number in normotensive type 1 diabetic patients with albuminuria. *Diabetes*. 51:3083-3089.

Yuan, Y., S. Huang, W. Wang, Y. Wang, P. Zhang, C. Zhu, G. Ding, B. Liu, T. Yang, and A. Zhang. 2012. Activation of peroxisome proliferator-activated receptor-gamma coactivator 1alpha ameliorates mitochondrial dysfunction and protects podocytes from aldosterone-induced injury. *Kidney international*. 82:771-789.

Zang, M., A. Zuccollo, X. Hou, D. Nagata, K. Walsh, H. Herscovitz, P. Brecher, N.B. Ruderman, and R.A. Cohen. 2004. AMP-activated protein kinase is required for the lipid-lowering effect of metformin in insulin-resistant human HepG2 cells. *J Biol Chem*. 279:47898-47905.

Zinszner, H., M. Kuroda, X. Wang, N. Batchvarova, R.T. Lightfoot, H. Remotti, J.L. Stevens, and D. Ron. 1998. CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. *Genes & development*. 12:982-995.

Ziyadeh, F.N. 2004. Mediators of diabetic renal disease: the case for tgf-Beta as the major mediator. *J Am Soc Nephrol*. 15 Suppl 1:S55-57.

Ziyadeh, F.N., B.B. Hoffman, D.C. Han, M.C. Iglesias-De La Cruz, S.W. Hong, M. Isono, S. Chen, T.A. McGowan, and K. Sharma. 2000. Long-term prevention of renal insufficiency, excess matrix gene expression, and glomerular mesangial matrix expansion by treatment with monoclonal antitransforming growth factor-beta antibody in db/db diabetic mice. *Proc Natl Acad Sci U S A*. 97:8015-8020.

7. ACKNOWLEDGEMENTS

First and foremost, I would like to express my sincere gratitude to my supervisor PD Dr. Andreas Jehle for giving me the opportunity to work in his lab. I am indebted to him for his unrestricted support and enthusiastic guidance all through my PhD journey, it was joy to work with him and hope to continue our relationship in coming years too.

It would not be possible to start my PhD project smoothly and continue steadily without the support of my colleague Dr. Jonas Sieber for his constant support all through my PhD. I would like to thank my other colleague Jana Orellana for the support and for bringing joyful environment to the lab.

I take this opportunity to express my deepest gratitude to Prof. Ed Palmer and Prof. Marc Donath for accepting to be the members of my PhD committee. Thank you very much for your support and for your valuable suggestions all through.

I would like to thank Prof. Marc Donath group for giving me the opportunity to be part of excellent progress report meetings. Special thanks to Dr. Nadine Sauter for introducing me to work with mice and it was excellent to work with her.

I am grateful to my friends Afzal, Hrishikesh, Pankaj, Sanjay, Vijay who made me feel homely even though I am away from home. Special thanks to, Pankaj for the all non-scientific talks during our PhD days and Vijay for helping me to establish lentiviral system in the lab.

I would like to dedicate my PhD thesis to my beloved parents Dr. Anjappa and Dr. Aruna, without their sacrifice and unconditional love I would not have reached this far. They are great inspiration to me. I am thankful to my brother Abhiram for his constant support all through my life and to Preethi and Aditi for bringing joy to the family.

Last but not the least I would like to thank my love, my better-half, Poojitha for the support and love, without which I could have never balanced my life.

7.1 Acknowledgements for micro-array data

We thank all participating centers of the European Renal cDNA Bank-Kroener-Fresenius biopsy bank (ERCB-KFB) and their patients for their cooperation. Active members at the time of the study: Clemens David Cohen, Holger Schmid, Michael Fischereder, Lutz Weber, Matthias Kretzler, Detlef Schlöndorff, Munich/Zurich/AnnArbor/New York; Jean Daniel Sraer, Pierre Ronco, Paris; Maria Pia Rastaldi, Giuseppe D'Amico, Milano; Peter Doran, Hugh Brady, Dublin; Detlev Mönks, Christoph Wanner, Würzburg; Andrew Rees, Aberdeen; Frank Strutz, Gerhard Anton Müller, Göttingen; Peter Mertens, Jürgen Floege, Aachen; Norbert Braun, Teut Risler, Tübingen; Loreto Gesualdo, Francesco Paolo Schena, Bari; Jens Gerth, Gunter Wolf, Jena; Rainer Oberbauer, Dontscho Kerjaschki, Vienna; Bernhard Banas, Bernhard Krämer, Regensburg; Moin Saleem, Bristol; Rudolf Wüthrich, Zurich; Walter Samtleben, Munich; Harm Peters, Hans-Hellmut Neumayer, Berlin; Mohamed Daha, Leiden; Katrin Ivens, Bernd Grabensee, Düsseldorf; Francisco Mampaso(†), Madrid; Jun Oh, Franz Schaefer, Martin Zeier, Hermann-Joseph Gröne, Heidelberg; Peter Gross, Dresden; Giancarlo Tonolo; Sassari; Vladimir Tesar, Prague; Harald Rupprecht, Bayreuth; Hermann Pavenstädt, Münster; Hans-Peter Marti, Bern.

APPENDIX

American Journal of Physiology, Renal Physiology Article

Am J Physiol Renal Physiol 306: F401–F409, 2014.
First published December 11, 2013; doi:10.1152/ajprenal.00454.2013.

CALL FOR PAPERS | *Novel Mechanisms and Role of Glomerular Podocytes*

Susceptibility of podocytes to palmitic acid is regulated by fatty acid oxidation and inversely depends on acetyl-CoA carboxylases 1 and 2

Kapil Kampe,[1] Jonas Sieber,[1,2] Jana Marina Orellana,[1] Peter Mundel,[2] and Andreas Werner Jehle[1,3]

[1]*Molecular Nephrology, Department of Biomedicine, University Hospital, Basel, Switzerland;* [2]*Harvard Medical School and Division of Nephrology, Massachusetts General Hospital, Boston, Massachusetts; and* [3]*Department of Internal Medicine, Transplantation, Immunology, and Nephrology, University Hospital, Basel, Switzerland*

Submitted 12 August 2013; accepted in final form 5 December 2013

Kampe K, Sieber J, Orellana JM, Mundel P, Jehle AW. Susceptibility of podocytes to palmitic acid is regulated by fatty acid oxidation and inversely depends on acetyl-CoA carboxylases 1 and 2. *Am J Physiol Renal Physiol* 306: F401–F409, 2014. First published December 11, 2013; doi:10.1152/ajprenal.00454.2013.— Type 2 diabetes is characterized by dyslipidemia with elevated free fatty acids (FFAs). Loss of podocytes is a hallmark of diabetic nephropathy, and podocytes are susceptible to saturated FFAs, which induce endoplasmic reticulum (ER) stress and podocyte death. Genome-wide association studies indicate that expression of acetyl-CoA carboxylase (ACC) 2, a key enzyme of fatty acid oxidation (FAO), is associated with proteinuria in type 2 diabetes. Here, we show that stimulation of FAO by aminoimidazole-4-carboxamide-1β-D-ribofuranoside (AICAR) or by adiponectin, activators of the low-energy sensor AMP-activated protein kinase (AMPK), protects from palmitic acid-induced podocyte death. Conversely, inhibition of carnitine palmitoyltransferase (CPT-1), the rate-limiting enzyme of FAO and downstream target of AMPK, augments palmitic acid toxicity and impedes the protective AICAR effect. Etomoxir blocked the AICAR-induced FAO measured with tritium-labeled palmitic acid. The beneficial effect of AICAR was associated with a reduction of ER stress, and it was markedly reduced in ACC-1/-2 double-silenced podocytes. In conclusion, the stimulation of FAO by modulating the AMPK-ACC-CPT-1 pathway may be part of a protective mechanism against saturated FFAs that drive podocyte death. Further studies are needed to investigate the potentially novel therapeutic implications of these findings.

diabetic nephropathy; AMPK; apoptosis; β-oxidation; palmitic acid

DIABETIC NEPHROPATHY (DN) has become the primary cause of end-stage renal disease (ESRD), and most affected patients have type 2 diabetes (1, 15). Podocyte injury and loss are critical events in the course of DN (31) and precede albuminuria (6, 17, 21). Type 2 diabetes mellitus is characterized by hyperglycemia and dyslipidemia with increased plasma levels of free fatty acids (FFAs) (24). In the kidneys of diabetic humans, intraglomerular lipid deposits were already described in 1936 by Kimmelstiel and Wilson (14). However, the potential role of FFAs and fatty acid metabolism in the pathogenesis of DN is only emerging.

Previously, we reported that podocytes are highly susceptible to the saturated FFA palmitic acid but not to monounsaturated FFAs (MUFAs), such as oleic acid, which attenuate palmitic acid-induced lipotoxicity (27). Mechanistically, palmitic acid-induced podocyte death is linked to endoplasmic reticulum (ER) stress involving the proapoptotic transcription factor C/EBP homologous protein (CHOP) (27). In addition, we reported that the gene expression of key enzymes of fatty acid metabolism is altered in glomeruli of type 2 diabetic patients with DN (29). Specifically, we found that stearoyl-CoA desaturases 1 (SCD-1), the enzyme converting saturated FFAs to MUFAs, is upregulated in podocytes. Functionally, stimulation of Scds by the liver X receptor (LXR) agonists TO901317 (TO) and GW3965 (GW) as well as overexpression of Scd-1 were shown to be protective for palmitic acid-induced podocyte death. Importantly, the previously reported changed glomerular gene expression pattern (29) also suggests disposition for increased fatty acid β-oxidation (FAO) as all three isoforms of carnitine palmitoyltransferase (CPT)-1, the rate-limiting enzyme for FAO, were upregulated and acetyl-CoA carboxylase (ACC) 2, which catalyzes the formation of the CPT-1 inhibitor malonyl-CoA, was downregulated (29).

In humans and rodents, there are two ACC isoforms, ACC1 (ACC α) and ACC2 (ACC β) (25), which share considerable sequence identity and the same domain structure responsible for enzyme activity (25). In contrast to ACC1, ACC2 has an extra N-terminal hydrophobic domain, which facilitates its localization to the mitochondrial membrane (2), where it preferentially regulates local malonyl-CoA levels and CPT-1 activity. In contrast, cytosolic ACC1 is classically thought to regulate malonyl-CoA synthesis for incorporation into fatty acids in lipogenic tissues. However, more recently this classical view has been challenged, and at least in some cell types, e.g., hepatocytes, both isoforms have been shown to regulate CPT-1 activity synergistically (25).

A key regulator of FAO is the low-energy sensor AMP-activated protein kinase (AMPK). Increased levels of AMP lead to AMPK activation, which finally triggers ATP production (10). AMPK directly targets and inactivates ACC by phosphorylation (18). The inactivation of ACC prevents the formation of malonyl-CoA (19) and thereby disinhibits CPT-1.

Importantly, two recent genome-wide association studies in type 2 diabetic patients found a polymorphism in a noncoding region of ACC2 with a strong association with proteinuria (16, 30). The DN-risk single nucleotide polymorphism of ACC2 results in a higher ACC2 expression (16) potentially leading to decreased FAO and accumulation of toxic FFAs and their deleterious metabolites.

Address for reprint requests and other correspondence: A. W. Jehle, Dept. of Biomedicine, Molecular Nephrology, Rm. 303, Univ. Hospital Basel, Hebelstrasse 20, 4031 Basel, Switzerland (e-mail: andreas.jehle@unibas.ch).

a

The objective of the present study was to investigate the effect of FAO on the susceptibility of podocytes to palmitic acid. Stimulation of FAO by the AMPK agonist aminoimidazole-4-carboxamide-1β-D-ribofuranoside (AICAR) was shown to protect from palmitic acid-induced cell death whereas inhibition of FAO by the CPT-1 inhibitor etomoxir enhanced the toxicity of palmitic acid. In addition, the functional role of the AMPK-ACC-CPT-1 pathway was assessed by gene silencing of ACC1 and ACC2.

MATERIALS AND METHODS

Materials. Palmitic acid (P9767), fatty acid free-BSA (A8806), etomoxir (E1905), compound C (P5499), and β-actin antibody (A5441) were purchased from Sigma (St. Louis, MO). Recombinant murine IFN-γ (CTK-358-2PS) was from MoBiTec (Goettingen, Germany). Type 1 collagen was from BD Biosciences. Annexin V (A23204) and propidium iodide (PI; P3566) were from Invitrogen (Eugene, OR). AICAR (no. 9944) and pAMPK (no. 2531), AMPK (no. 2532), pACC (no. 3661), ACC (no. 3676), and immunoglobulin heavy chain binding protein (BiP; no. 3183) antibodies were from Cell Signaling Technology. CHOP (sc-7351) antibody was from Santa Cruz Biotechnology (Dallas, TX). Adiponectin (no. 4902-100) was purchased from BioVision (Milpitas, CA). The horseradish peroxidase-conjugated secondary antibodies for rabbit and mouse were from Dako. Tritium-labeled palmitic acid (NET043001MC) was from PerkinElmer (Schwerzenbach, Switzerland).

Cell culture, free fatty acid preparation, and apoptosis assay. Murine podocytes were cultured as described before (27). Podocytes were differentiated for at least 11 days before the start of experiments. All experiments were carried out in six-well plates except for isolating protein or RNA for which 10-cm dishes were used. Free fatty acid preparations were done as described previously (27). The palmitic acid concentration used in this study was 200 μM complexed to BSA (0.2%), which is within the reported physiological range of 120–340 μM (7–9). Endotoxin concentration was equal or less than 0.5 ng/ml, as determined by a kit (no. L00350) from Genscript (Piscataway, NJ). Annexin V and PI stainings were performed as reported earlier (27). Flow cytometry was carried out with a CyAn ADP Analyzer (Beckman Coulter), and 20,000 cells were counted. Data from flow cytometry were analyzed by the FLOWJO (Tree Star, Ashland, OR) software program. Annexin V-positive/PI-negative podocytes were considered apoptotic, whereas annexin V-positive/PI-positive podocytes were considered (late apoptotic) necrotic cells (27).

Plasmids, RNA-mediated interference, and viruses. ACC1 and ACC2 genes were silenced by employing the following short hairpin (sh) RNA sequences (12): ACC1, 5′-GCAGATTGCCAACATCCTAGA-3′ and ACC2, 5′-GTGGTGACGGGACGAGCAA-3′. A

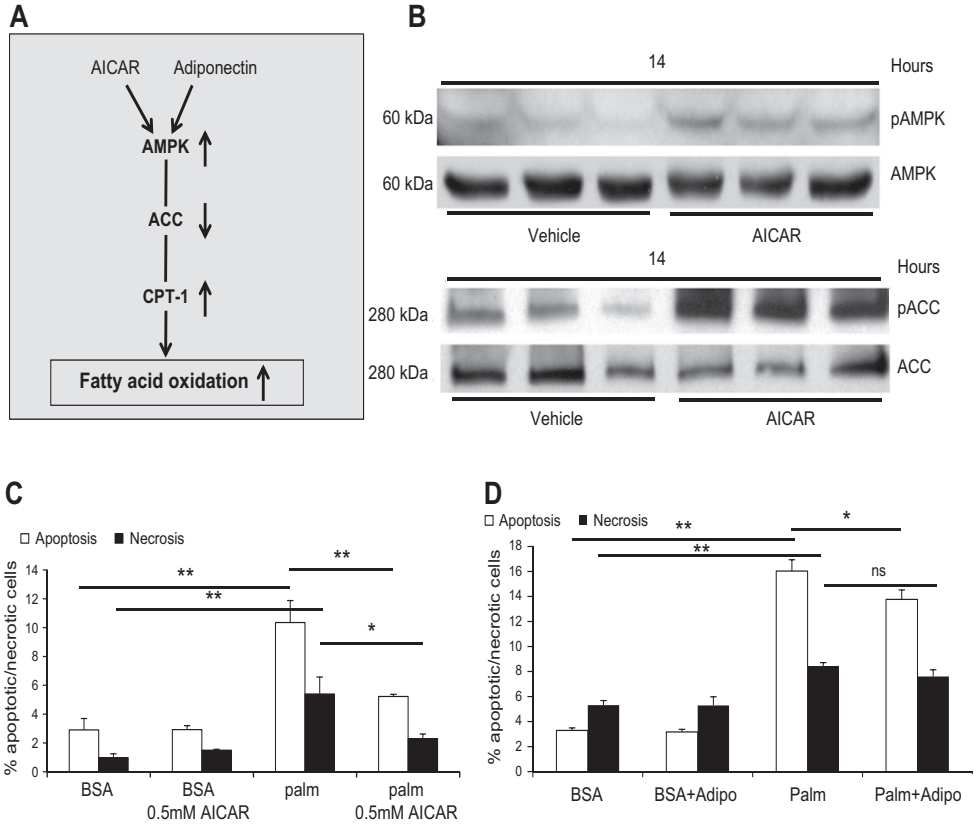


Fig. 1. Aminoimidazole-4-carboxamide-1β-D-ribofuranoside (AICAR) and adiponectin protect podocytes from palmtic acid-induced cell death. *A*: metabolic pathway activated by AICAR and adiponectin, which results in stimulation of fatty acid oxidation. CPT-1, carnitine palmitoyltransferase. *B*: immunoblot showing phosphorylation of AMPK and acetyl-CoA carboxylase (ACC) after incubation of podocytes with either (PBS) vehicle or 0.5 mM AICAR for 14 h. Total AMPK and total ACC served as loading controls. *C*: AICAR attenuated palmitic acid-induced cell death. Values are mean percentages ± SD of apoptotic and necrotic cells after 48 h ($n = 3$, $^*P < 0.05$, $^{**}P < 0.01$). *D*: 0.5 μg/ml adiponectin decreased palmitic acid-induced apoptosis and necrosis of podocytes. NS, not significant. Values are mean percentages ± SD of apoptotic and necrotic cells after 48 h ($n = 3$, $^*P < 0.05$).

b

21-nt scrambled sequence (5′-GACCGCGACTCGCCGTCTGCG-3′) (29) served as a control. ACC1, ACC2, and scrambled shRNA sequences were cloned into a pSIH-H1 lentiviral expression plasmid. A four-plasmid lentiviral system was used with following helper plasmids: pRSV-REV (Rev expression vector), pMDLg/pRRE (Gag-Pol expression vector), and pMD2.G (VSV-G expression vector). All four plasmids were transfected to HEK cells via the FuGene HD (Promega, Madison, WI) transfection agent, and the medium was changed after 12 h. Forty-eight hours posttransfection, the supernatant enriched with lentiviral particles was harvested, spun at 780 *g* for 5 min, and filtered through 0.45-μm filter. Transduction of podocytes was done by pretreating podocytes with 5 μg/ml polybrene (Sigma). All functional experiments were started after 4 days of transduction.

Western blotting. Western blotting was done as described before (27). Antibodies against pAMPK, AMPK, pACC, ACC, CHOP, BiP, and β-actin were employed at 1:250, 1:1,000, 1:500, 1:1,000, 1:1,000, 1:500, and 1:100,000 dilutions, respectively. Secondary antibodies for rabbit and mouse were employed at 1:1,600 and 1:4,000 dilutions, respectively.

Measuring oxidation of palmitic acid (β-oxidation). For these experiments, podocytes were pretreated with AICAR for 1 h. Podocytes were incubated with 200 μM palmitic acid in a serum starvation medium (0.2% FBS, 5 mM glucose) supplemented with 0.5% FFA-free BSA along with 0.5 μCi/ml ^{3}H-palmitic acid. Supernatants were collected followed by chloroform/methanol/5 N HCl (2:1:0.05, vol/vol) extraction. Four hundred microliters of the aqueous phase (containing ^3H_2O) was added to 2 ml of scintillation buffer before measurement of radioactivity.

Statistical analysis. All experiments were performed at least four times, and a representative experiment is shown. Individual experiments were performed in triplicate if not otherwise indicated. Data are expressed as means ± SD unless otherwise indicated. ANOVA and Bonferroni *t*-tests were used for statistical analysis. The Prism 6 program was used for this analysis, and differences were considered significant when the *P* value was <0.05.

RESULTS

AICAR protects from palmitic acid-induced podocyte death. To investigate whether stimulation of FAO plays a protective role in palmitic acid-treated podocytes, we took advantage of the AMPK activator AICAR. AICAR acts by phosphorylating AMPK, which in turn phosphorylates and inhibits ACC, resulting in disinhibition of CPT-1 (see Fig. 1*A*). Phosphorylation of AMPK and ACC by AICAR in podocytes was examined by Western immunoblotting (Fig. 1*B*). As shown in Fig. 1*C*, AICAR significantly prevented palmitic acid-induced podocyte death assessed by flow cytometry after staining for annexin V and PI. Specifically, AICAR reduced palmitic acid-induced apoptosis (annexin V single-positive cells) and necrosis (annexin V/PI double-positive cells) by 50.5 ± 1.5 ($P < 0.01$) and 42.5 ± 6.1% ($P < 0.05$), respectively. Similarly, the physiological AMPK agonist adiponectin (26) also reduced palmitic acid-induced podocyte death, although to a lesser

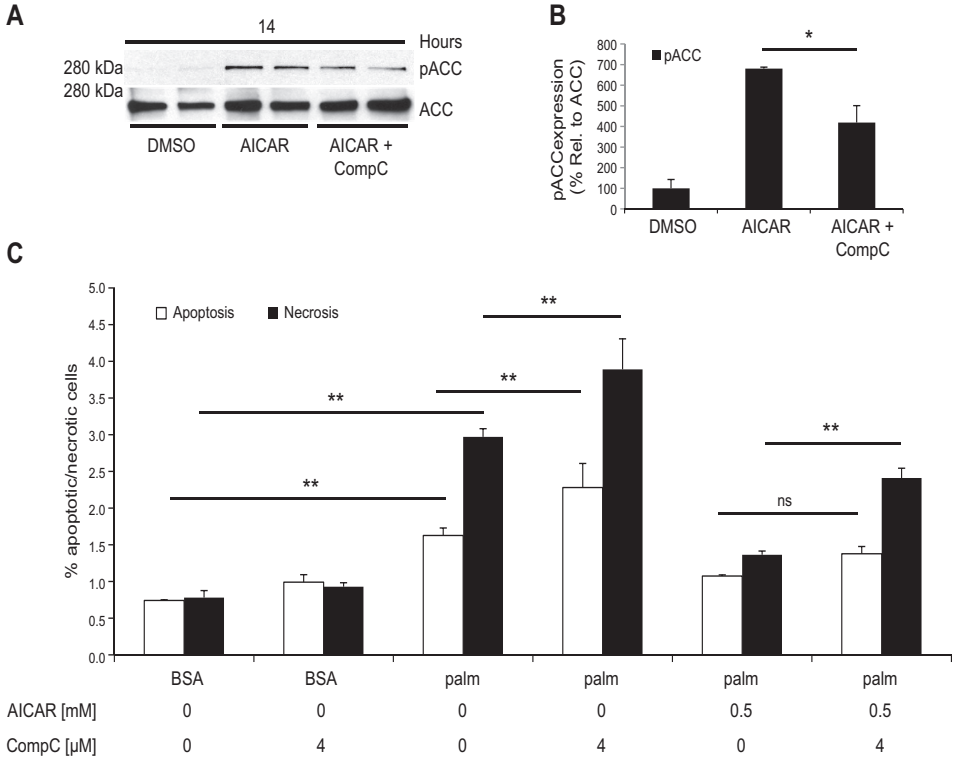


Fig. 2. Compound C aggravates palmitic acid-induced podocyte death and partially reverses the protective AICAR effect. *A*: podocytes were incubated for 14 h with DMSO (control), 0.5 mM AICAR, or 0.5 mM AICAR and 4 μM compound C. pACC and ACC levels were analyzed by Western immunoblotting. *B*: quantification of pACC and ACC by densitometry. Values are mean relative expression ± SD (* $P < 0.05$). DMSO-treated controls were set to 100%. *C*: compound C increased palmitic acid-induced podocyte death and partially reversed the protection of AICAR. Values are mean percentages ± SD of apoptotic and necrotic cells after 24 h ($n = 3$, ** $P < 0.01$).

extent than AICAR. Figure 1*D* shows that adiponectin decreased both apoptosis and necrosis by 14.1 ± 4.7 ($P < 0.05$) and 9.9 ± 6.3% (NS), respectively. To see the protective effect of adiponectin, podocytes were kept at a high glucose concentration of 22 mmol/l, which is known to reduce phosphorylation of AMPK (26) and increase the susceptibility of podocytes to AMPK activation (26).

Compound C aggravates palmitic acid-induced podocyte death and partially reverses the protective AICAR effect. To further explore the role of AMPK we used the AMPK inhibitor compound C. Compound C was used at a low concentration of 4 μM, and incubation time was limited to 24 h maximally, as higher concentrations and longer exposure times were toxic; i.e., podocyte death was markedly increased for BSA control (data not shown). The AICAR-induced ACC phosphorylation was significantly reduced by compound C (Fig. 2, *A* and *B*, $P < 0.05$). Compound C increased palmitic acid-induced apoptosis by 140.1 ± 20.1% ($P < 0.01$) and necrosis by 130.9 ± 14.0% ($P < 0.01$) (Fig. 2*C*). In agreement with the partial reduction of the AICAR-induced ACC phosphorylation, compound C compared with AICAR alone only moderately increased palmitic acid-induced podocyte death; i.e., apoptosis was increase by 128.2 ± 9.3 (NS) and necrosis by 176.7 ± 9.7% ($P < 0.01$), respectively.

Etomoxir aggravates palmitic acid-induced podocyte death and reverses the protective AICAR effect. To further investigate the impact of FAO on palmitic acid-induced podocyte death, we made use of the CPT-1 inhibitor etomoxir (Fig. 3*A*). Etomoxir exacerbated palmitic acid-induced podocyte death (Fig. 3*B*). Specifically, apoptosis was increased by 184.3 ± 6.0 ($P < 0.01$) and necrosis by 185.1 ± 16.3% ($P < 0.01$), respectively. Moreover, etomoxir reversed the protective effect of AICAR (Fig. 3*C*). Of note, this effect could already be seen at a very low etomoxir concentration (10 μM), which by itself had no significant effect on palmitic acid-induced podocyte death (data not shown). Compared with podocytes treated with AICAR alone, the presence of 10 μM etomoxir increased palmitic acid-mediated apoptosis by 131.1 ± 5.0 ($P < 0.05$) and necrosis by 127.3 ± 10.7% ($P < 0.05$), respectively. At 200 μM, etomoxir completely reversed the protective effect of AICAR (Fig. 3*C*). The experiments using AICAR, compound C, and etomoxir suggest an important role of the AMPK-ACC-CPT-1 pathway for regulating the susceptibility of podocytes exposed to palmitic acid.

AICAR increases and etomoxir inhibits oxidation of palmitic acid in podocytes. To directly measure the effect of AICAR on palmitic acid oxidation, we treated podocytes with 200 μM palmitic acid along with 0.5 μCi/ml tritiated palmitic acid in

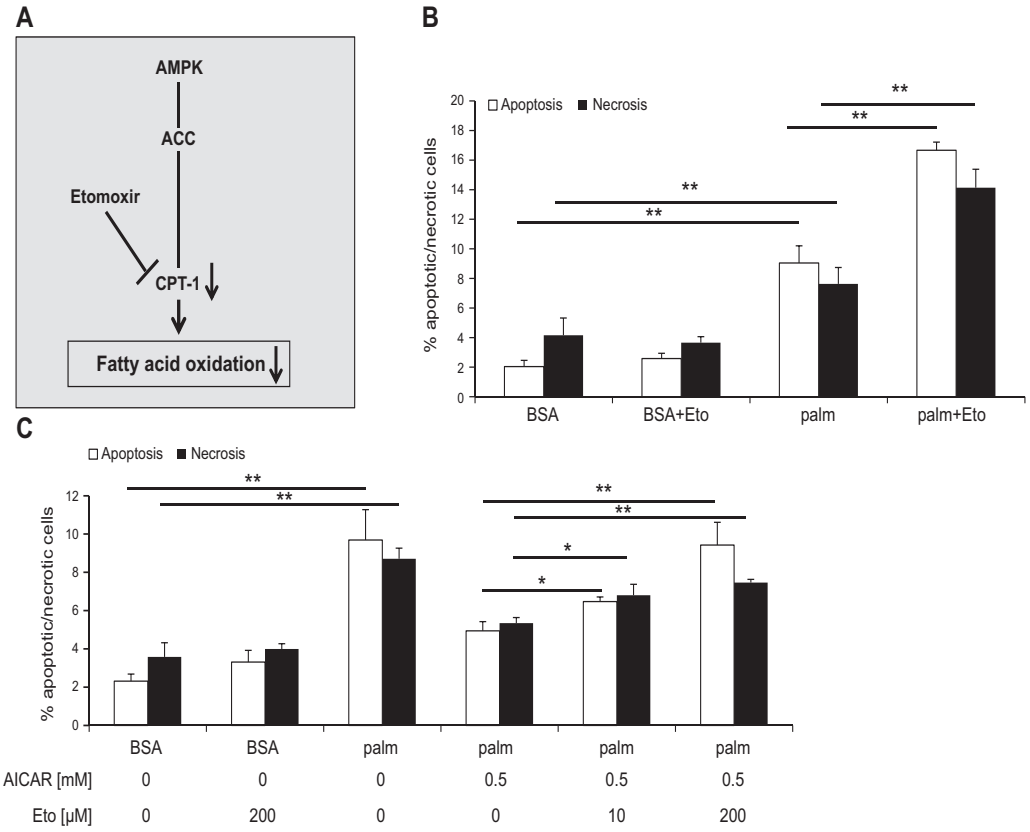


Fig. 3. Etomoxir exacerbates palmitic acid-induced podocyte death and reverses the protective effect of AICAR. *A*: mechanism of action of etomoxir, which inhibits CPT-1 and thereby fatty acid oxidation. *B*: etomoxir aggravated palmitic acid-induced podocyte death after 48 h. Values are mean percentages ± SD of apoptotic and necrotic cells ($n = 3$, $^{**}P < 0.01$). *C*: etomoxir reversed the protection of AICAR in palmitic acid-induced podocyte death. Values are mean percentages ± SD of apoptotic and necrotic cells after 48 h ($n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$).

d

the absence or presence of AICAR. As a direct readout of palmitic acid β-oxidation, we measured tritiated water released in the supernatants of podocytes. As expected, the release of tritiated water gradually increased from 1 to 3 h (Fig. 4*A*). The stimulation of podocytes with AICAR significantly increased the formation of tritiated water (146.6 ± 22.0%, $P < 0.05$, Fig. 4*A*), reflecting the stimulatory effect of AICAR on palmitic acid oxidation. Importantly, and as shown in Fig. 4*B*, the effect of AICAR could be completely prevented by etomoxir.

AICAR significantly reduces ER stress and upregulation of the proapoptotic transcription factor CHOP. As palmitic acid-induced podocyte death involves ER stress and as CHOP gene silencing attenuates palmitic acid-induced podocyte death (27), we next investigated the effect of AICAR on the ER chaperone BiP and the proapoptotic transcription factor CHOP. AICAR strongly suppressed the upregulation of BiP and CHOP (Fig. 5, *A* and *B*).

Combined gene silencing of ACC1 and ACC2 protects from palmitic acid-induced podocyte death. Two recent genome-wide association studies (16, 30) found a single nucleotide polymorphism in ACC2, leading to increased ACC2 expression (16), to be associated with proteinuria in type 2 diabetic patients. To investigate further the role of both ACC isoforms in podocytes, we generated cells deficient in ACC1, ACC2, or both by lentiviral infection using specific shRNAs. Knockdown of ACC1 but not ACC2 strongly reduced the band corresponding to both isoforms. The residual band seen in ACC1 single-knockdown podocytes was almost completely gone in ACC1/ACC2 double-knockdown cells. (Fig. 6*A*). These data suggest that the expression level of ACC1 is much higher than ACC2 in podocytes. Functionally, only double knockdown of both isoforms significantly reduced palmitic acid-induced podocyte death. Specifically, in ACC1/ACC2 double-knockdown podocytes, palmitic acid-induced apoptosis and necrosis were reduced by 57.4 ± 3.9 ($P < 0.01$) and 72.1 ± 7.5% ($P < 0.05$), respectively, compared with podocytes transfected with scrambled shRNA (Fig. 6*B*), whereas single knockdown of ACC1 or ACC2 had no significant effect (Fig. 6*C*). These results are in complete agreement with results in hepatocytes, which have shown that both ACC isoforms regulate FAO (25). Of note, a residual protective effect of AICAR was seen in ACC1/ACC2 double-silenced podocytes (Fig. 6*B*).

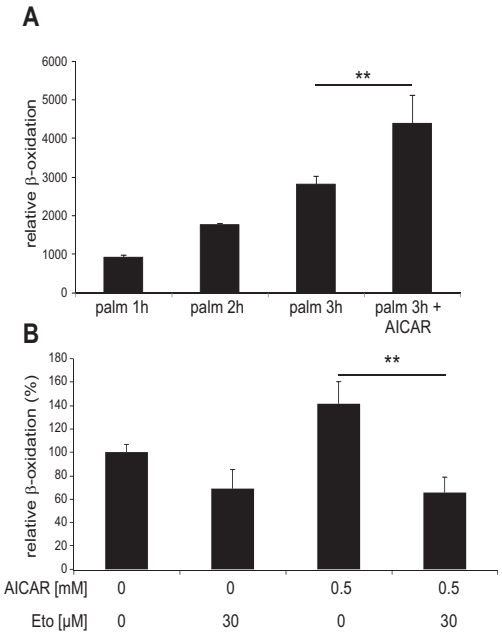


Fig. 4. AICAR increases and etomoxir decreases oxidation of palmitic acid in podocytes. *A*: AICAR upregulated oxidation of palmitic acid. Podocytes were treated with 0.5% free fatty acid (FFA)-free BSA with 200 μM palmitic acid in the presence of 0.5 μCi/ml [^{3}H]-palmitic acid for indicated time points either with or without 0.5 mM AICAR (pretreatment for 1 h). Values are mean relative β-oxidation levels ± SD ($n = 3$, $^{**}P < 0.01$). *B*: etomoxir prevented the AICAR-induced stimulation of β-oxidation (pretreatment with vehicle or AICAR and/or etomoxir for 1 h). Values are mean relative β-oxidation levels (%) ± SD after 3 h ($n = 3$, $^{**}P < 0.01$).

DISCUSSION

The present study uncovered that regulation of FAO critically determines the susceptibility of podocytes exposed to palmitic acid. Our findings are of clinical interest, and they relevantly amend recent clinical and experimental studies indicating a potentially important role of FFAs and FFA metabolism in the pathogenesis of DN.

Several lines of evidence indicate that regulation of FAO and interference with the AMPK-ACC-CPT-1 pathway affects podocytes exposed to palmitic acid. Specifically, the AMPK agonist AICAR, which significantly stimulates FAO in podocytes, reduces palmitic acid-induced podocyte death. Conversely, the AMPK inhibitor compound C increased palmitic acid-induced cell death. Furthermore, the CPT-1 inhibitor etomoxir, which completely prevents the AICAR-induced increase in FAO in podocytes, potentiates the toxicity of palmitic acid and dose dependently reverses the protective effect of AICAR. Moreover, gene silencing of ACC1/ACC2 markedly reduced palmitic acid-induced cell death.

Adiponectin, a physiological activator of AMPK in podocytes (26), also reduced palmitic acid-induced podocyte death. Although its protective effect was relatively small compared with pharmacological activation by AICAR, the sustained action of adiponectin in vivo may still be relevant for the protection of podocytes from lipotoxicity. Activation of AMPK by adiponectin or AICAR is also reported to suppress oxidative stress and the NADPH oxidase Nox4 (26). As neither tempol, a membrane-permeable radical scavenger, nor the antioxidant N-acetylcysteine reduce palmitic acid-induced podocyte death (unpublished observations, Sieber J.), the modulation of oxidative stress through the AMPK pathway related to lipotoxicity needs further investigation.

To further address the role of AMPK in palmitic acid-induced podocyte death, we additionally used the AMPK inhibitor compound C, which increased the toxicity of palmitic acid. In addition, compound C reduced the AICAR-induced phosphorylation of ACC and partially prevented the protective AICAR effect. Together, these findings suggest that the susceptibility of podocytes exposed to palmitic acid can be greatly modulated by AMPK.

The present results indicating an important role of FAO and the AMPK-ACC-CPT-1 pathway in the susceptibility of podocytes exposed to toxic FFAs extend and potentially explain the

e

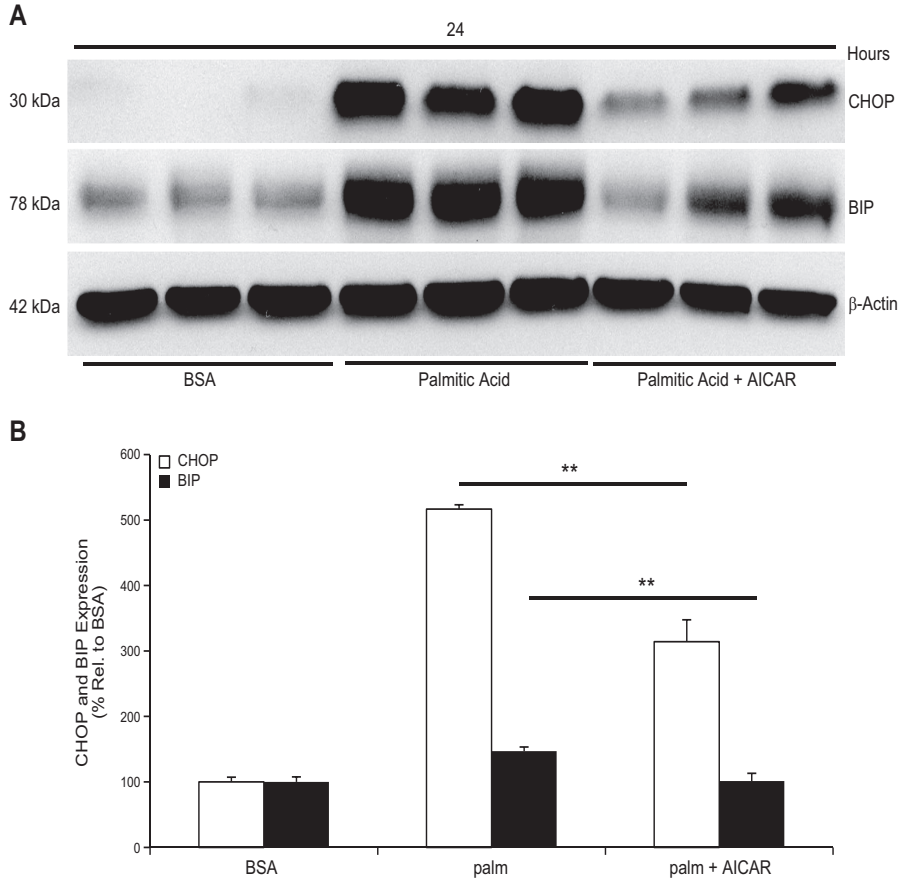


Fig. 5. AICAR mitigates palmitic acid-induced ER stress. *A*: AICAR attenuated palmitic acid-induced induction of C/EBP homologous protein (CHOP) and immunoglobulin heavy chain binding protein (BiP) after 24 h. CHOP and BiP levels were analyzed by Western immunoblotting. β-Actin served as a loading control. *B*: quantification of CHOP and BiP levels. BSA-treated controls were set to 100%. Values are mean expression levels ± SD ($n = 3$, $^{**}P < 0.01$).

results of recent genome-wide associations studies which found a SNP in ACC2 with significant enhancer activity, resulting in increased ACC2 expression associated with proteinuria in type 2 diabetic patients (16, 30). Moreover, the results of this study suggest that the recently published observation of decreased expression of ACC2 and increased expression of all CPT-1 isoforms in glomerular extracts of type 2 diabetic patients (29) reflects an adaptive, protective mechanism against toxic FFAs in DN.

The differential role of ACC1 and ACC2 in the regulation of FAO is under debate (20). We found that only double knockdown of ACC1 and ACC2 has a protective effect on palmitic acid-induced cell death. This indicates that both isoforms contribute to the inhibition of CPT-1 in podocytes, as previously suggested for hepatocytes and skeletal muscle cells (20, 25).

Interestingly, AICAR showed a small residual protective effect in ACC1/ACC2 double-knockdown podocytes. This may be due to residual expression of ACC isoforms or an additional ACC-independent effect. Activation of AMPK stimulates peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α (11), for example, which has been shown to be important for mitochondrial function in podocytes (32). Finally, AMPK-independent off-target effects of AICAR cannot be excluded. Future studies are needed to confirm or refute this hypothesis.

The biguanide metformin is widely used to treat type 2 diabetes (23). Its mechanism of action is not fully established but is reported to involve indirect activation of the AMPK-ACC-CPT-1 pathway via inhibition of complex I of the respiratory chain and a consequent increase in the AMP:ATP ratio, which results in AMPK activation (33). Despite this potential mode of action, preliminary experiments showed that metformin from 0.5 to 2 mM displays no protection from palmitic acid-induced lipotoxicity in podocytes (data not shown). Previously, undesired effects of metformin leading to cell death have been reported for pancreatic β cells (13). Of interest, a potential beneficial effect of metformin was shown in podocytes exposed to a high glucose concentration of 30 mM by decreasing ROS production through reduction of NAD(P)H oxidase activity (22). Clearly, more studies are required to reassess the short- and long-term effects of metformin on podocytes.

Interestingly, AICAR significantly reduces the induction of CHOP in podocytes exposed to palmitic acid, which likely contributes to the protective AICAR effect, as gene silencing

f

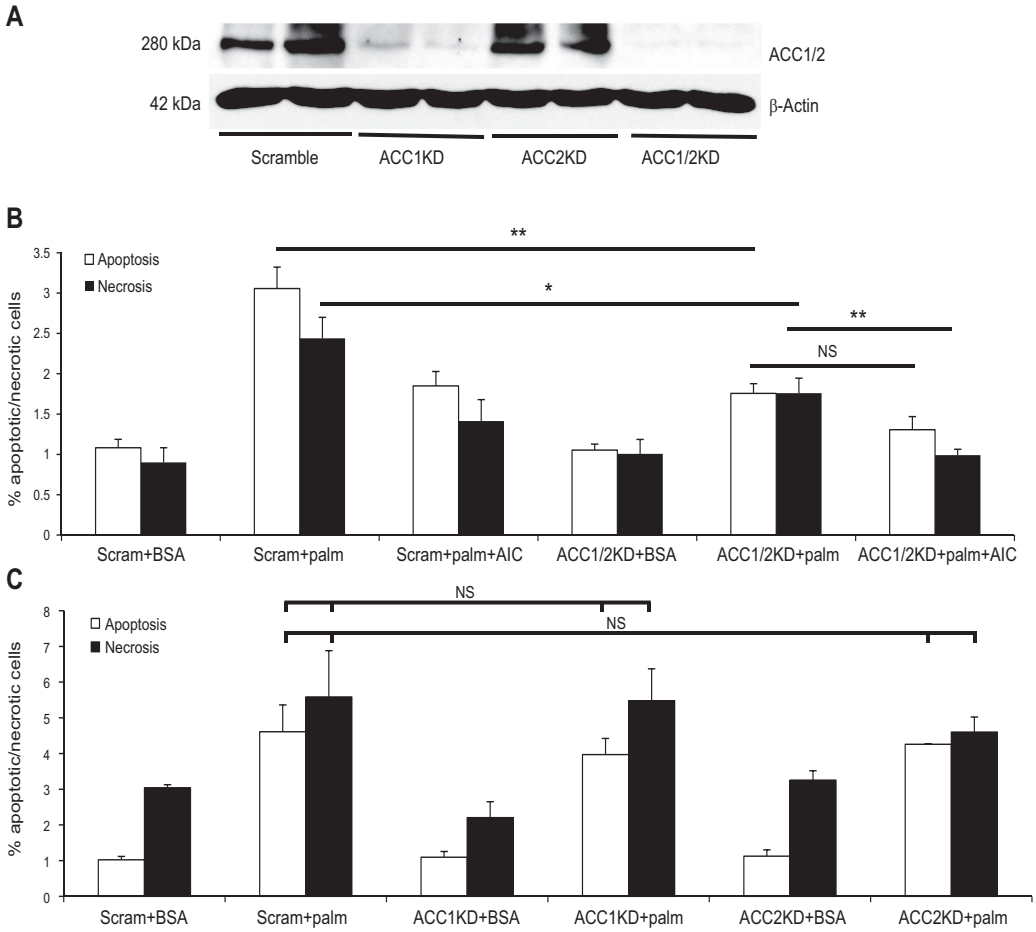


Fig. 6. ACC1 or ACC2 single knockdown is not protective, whereas combined silencing of ACC1 and ACC2 protects from palmtitic acid-induced podocyte death. *A*: ACC1, ACC2, or both were knocked down, and an immunoblot was done with an antibody recognizing both isoforms. β-Actin served as a loading control. *B*: combined knockdown of ACC1 and ACC2 protected podocytes from palmitic acid-induced cell death. Values are mean percentages ± SD of apoptotic or necrotic cells ($n = 3$, $^*P < 0.05$, $^{**}P < 0.01$). AICAR modestly further decreased podocyte death in ACC1/2 double-knockdown cells ($^{**}P < 0.01$). *C*: silencing of either ACC1 or ACC2 was not protective for palmitic acid-induced cell death. Values are show mean percentages ± SD of apoptotic or necrotic cells ($n = 3$).

of CHOP attenuates palmitic acid-induced cell death (27). The action of AICAR on the AMPK-ACC-CPT-1 pathway may indicate that increased FAO reduces palmitic acid-derived toxic metabolites and therefore suppresses the induction of ER stress. However, the basic unanswered question is how palmitic acid and its metabolites trigger ER stress. Some reports indicated that palmitic acid rapidly increases the saturated lipid content of the ER, leading to compromised ER morphology and integrity (4). In pancreatic β cells, palmitic acid depletes ER Ca^{2+} and slows ER Ca^{2+} uptake (5), which leads to accumulation of unfolded proteins. However, the detailed molecular mechanisms are not well known (3). Clearly, more experiments are needed to understand these principle mechanisms and to study whether FAO and ER stress are causatively related.

In conclusion, our results suggest that modulation of FFA metabolism and stimulation of FAO by activation of the AMPK-ACC-CPT-1 pathway critically influence the susceptibility of podocytes exposed to toxic FFAs. Together with our previous studies, the following working model is suggested (Fig. 7). Palmitic acid increases the generation of toxic metabolites, which leads to ER stress and podocyte death (27). Oleic acid or induction of Scd-1/-2 expression by the LXR-agonists TO and GW shift palmitic acid and its metabolites into a "safe lipid pool" containing triglycerides (TG), which reduces injurious metabolites and prevents podocyte death (29). In addition, activation of the AMPK-ACC-CPT-1 pathway by AICAR reduces palmitic acid-induced podocyte death by increasing FAO and reducing palmitic acid and its metabolites. Our findings may explain the results of recent genome-wide association studies indicating that modulation of FFA metabolism and specifically modulation of FAO directly affects the susceptibility to DN. In addition, our results have potentially important therapeutic implications for the prevention and treatment of DN in type 2 diabetic patients.

g

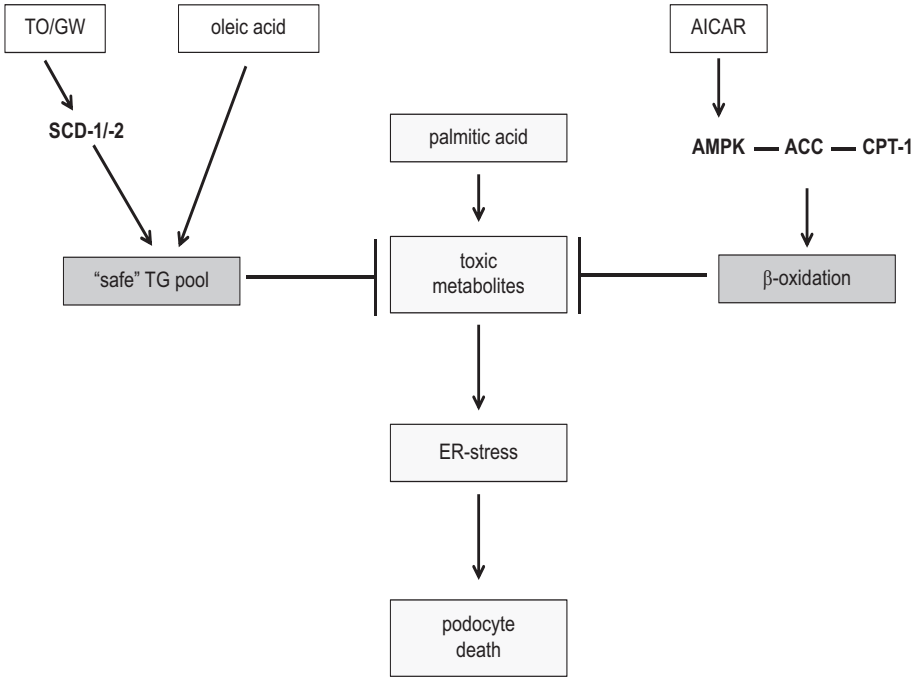


Fig. 7. Working model for the AICAR activated AMPK-ACC-CPT-1 pathway and the prosurvival effects of oleic acid and Scd-1/-2 on palmitic acid-induced podocyte death. Palmitic acid increases the generation of toxic metabolites, which leads to ER stress and podocyte death. The liver X receptor (LXR) agonists TO901317 (TO) and GW3965 (GW) increase Scd-1/2 expression, which in turn elevates the triglyceride (TG) safe pool and reduces injurious metabolites and subsequent podocyte death. Similarly, oleic acid ameliorates palmitic acid-induced podocyte death by shifting palmitic acid and toxic metabolites to the TG safe pool. Additionally, AICAR reduces palmitic acid-induced podocyte death by stimulation of the AMPK-ACC-CPT-1 pathway and subsequently β-oxidation, thus decreasing toxic metabolites and eventually podocyte death.

ACKNOWLEDGMENTS

The lentiviral system and expression plasmids were kindly provided by the Markus Heim laboratory (University Hospital, Basel, Switzerland).

GRANTS

This study was supported by Swiss National Science Foundation Grants 31003A-119974, and 31003A-144112/1 (A. W. Jehle), Swiss Diabetes Foundation (A. W. Jehle), Fondation Sana, Switzerland (A. W. Jehle), Swiss National Science Foundation Fellowship PBBSP3-144160 (J. Sieber), and National Institutes Health Grants DK62472 and DK57683 (P. Mundel).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: K.K., J.S., and A.W.J. provided conception and design of research; K.K. performed experiments; K.K., J.S., and A.W.J. analyzed data; K.K., J.S., and A.W.J. interpreted results of experiments; K.K. prepared figures; K.K. drafted manuscript; K.K., J.S., J.M.O., P.M., and A.W.J. approved final version of manuscript; J.S., J.M.O., P.M., and A.W.J. edited and revised manuscript.

REFERENCES

1. **Anonymous.** USRDS: the United States Renal Data System. *Am J Kidney Dis* 42: 1–230, 2003.
2. **Abu-Elheiga L, Matzuk MM, Kordari P, Oh W, Shaikenov T, Gu Z, Wakil SJ.** Mutant mice lacking acetyl-CoA carboxylase 1 are embryonically lethal. *Proc Natl Acad Sci USA* 102: 12011–12016, 2005.
3. **Back SH, Kang SW, Han J, Chung HT.** Endoplasmic reticulum stress in the beta-cell pathogenesis of type 2 diabetes. *Exp Diabetes Res* 2012: 618396, 2012.
4. **Borradaile NM, Han X, Harp JD, Gale SE, Ory DS, Schaffer JE.** Disruption of endoplasmic reticulum structure and integrity in lipotoxic cell death. *J Lipid Res* 47: 2726–2737, 2006.
5. **Cunha DA, Hekerman P, Ladriere L, Bazarra-Castro A, Ortis F, Wakeham MC, Moore F, Rasschaert J, Cardozo AK, Bellomo E, Overbergh L, Mathieu C, Lupi R, Hai T, Herchuelz A, Marchetti P, Rutter GA, Eizirik DL, Cnop M.** Initiation and execution of lipotoxic ER stress in pancreatic beta-cells. *J Cell Sci* 121: 2308–2318, 2008.
6. **Dalla Vestra M, Masiero A, Roiter AM, Saller A, Crepaldi G, Fioretto P.** Is podocyte injury relevant in diabetic nephropathy? Studies in patients with type 2 diabetes. *Diabetes* 52: 1031–1035, 2003.
7. **Fraser DA, Thoen J, Rustan AC, Forre O, Kjeldsen-Kragh J.** Changes in plasma free fatty acid concentrations in rheumatoid arthritis patients during fasting and their effects upon T-lymphocyte proliferation. *Rheumatology (Oxf)* 38: 948–952, 1999.
8. **Groop LC, Bonadonna RC, DelPrato S, Ratheiser K, Zyck K, Ferrannini E, DeFronzo RA.** Glucose and free fatty acid metabolism in non-insulin-dependent diabetes mellitus. Evidence for multiple sites of insulin resistance. *J Clin Invest* 84: 205–213, 1989.
9. **Hagenfeldt L, Wahren J, Pernow B, Raf L.** Uptake of individual free fatty acids by skeletal muscle and liver in man. *J Clin Invest* 51: 2324–2330, 1972.
10. **Hardie DG, Ross FA, Hawley SA.** AMPK: a nutrient and energy sensor that maintains energy homeostasis. *Nat Rev Mol Cell Biol* 13: 251–262, 2012.
11. **Iwabu M, Yamauchi T, Okada-Iwabu M, Sato K, Nakagawa T, Funata M, Yamaguchi M, Namiki S, Nakayama R, Tabata M, Ogata H, Kubota N, Takamoto I, Hayashi YK, Yamauchi N, Waki H, Fukayama M, Nishino I, Tokuyama K, Ueki K, Oike Y, Ishii S, Hirose K, Shimizu T, Touhara K, Kadowaki T.** Adiponectin and AdipoR1 regulate PGC-1alpha and mitochondria by Ca^{2+} and AMPK/SIRT1. *Nature* 464: 1313–1319, 2010.

h

12. **Jeon SM, Chandel NS, Hay N.** AMPK regulates NADPH homeostasis to promote tumour cell survival during energy stress. *Nature* 485: 661–665, 2012.
13. **Kefas BA, Cai Y, Kerckhofs K, Ling Z, Martens G, Heimberg H, Pipeleers D, Van de Casteele M.** Metformin-induced stimulation of AMP-activated protein kinase in beta-cells impairs their glucose responsiveness and can lead to apoptosis. *Biochem Pharmacol* 68: 409–416, 2004.
14. **Kimmelstiel P, Wilson C.** Intercapillary lesions in the glomeruli of the kidney. *Am J Pathol* 12: 83–98 87, 1936.
15. **Locatelli F, Pozzoni P, Del Vecchio L.** Renal replacement therapy in patients with diabetes and end-stage renal disease. *J Am Soc Nephrol* 15, *Suppl* 1: S25–S29, 2004.
16. **Maeda S, Kobayashi MA, Araki S, Babazono T, Freedman BI, Bostrom MA, Cooke JN, Toyoda M, Umezono T, Tarnow L, Hansen T, Gaede P, Jorsal A, Ng DP, Ikeda M, Yanagimoto T, Tsunoda T, Unoki H, Kawai K, Imanishi M, Suzuki D, Shin HD, Park KS, Kashiwagi A, Iwamoto Y, Kaku K, Kawamori R, Parving HH, Bowden DW, Pedersen O, Nakamura Y.** A single nucleotide polymorphism within the acetyl-coenzyme A carboxylase beta gene is associated with proteinuria in patients with type 2 diabetes. *PLoS Genet* 6: e1000842, 2010.
17. **Meyer TW, Bennett PH, Nelson RG.** Podocyte number predicts long-term urinary albumin excretion in Pima Indians with Type II diabetes and microalbuminuria. *Diabetologia* 42: 1341–1344, 1999.
18. **Munday MR.** Regulation of mammalian acetyl-CoA carboxylase. *Biochem Soc Trans* 30: 1059–1064, 2002.
19. **Muoio DM, Newgard CB.** Fatty acid oxidation and insulin action: when less is more. *Diabetes* 57: 1455–1456, 2008.
20. **Olson DP, Pulinilkunnil T, Cline GW, Shulman GI, Lowell BB.** Gene knockout of Acc2 has little effect on body weight, fat mass, or food intake. *Proc Natl Acad Sci USA* 107: 7598–7603, 2010.
21. **Pagtalunan ME, Miller PL, Jumping-Eagle S, Nelson RG, Myers BD, Rennke HG, Coplon NS, Sun L, Meyer TW.** Podocyte loss and progressive glomerular injury in type II diabetes. *J Clin Invest* 99: 342–348, 1997.
22. **Piwkowska A, Rogacka D, Jankowski M, Dominiczak MH, Stepinski JK, Angielski S.** Metformin induces suppression of NAD(P)H oxidase activity in podocytes. *Biochem Biophys Res Commun* 393: 268–273, 2010.
23. **Qaseem A, Humphrey LL, Sweet DE, Starkey M, Shekelle P.** Oral pharmacologic treatment of type 2 diabetes mellitus: a clinical practice guideline from the American College of Physicians. *Ann Intern Med* 156: 218–231, 2012.
24. **Randle PJ, Garland PB, Hales CN, Newsholme EA.** The glucose fatty-acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. *Lancet* 1: 785–789, 1963.
25. **Savage DB, Choi CS, Samuel VT, Liu ZX, Zhang D, Wang A, Zhang XM, Cline GW, Yu XX, Geisler JG, Bhanot S, Monia BP, Shulman GI.** Reversal of diet-induced hepatic steatosis and hepatic insulin resistance by antisense oligonucleotide inhibitors of acetyl-CoA carboxylases 1 and 2. *J Clin Invest* 116: 817–824, 2006.
26. **Sharma K, Ramachandrarao S, Qiu G, Usui HK, Zhu Y, Dunn SR, Ouedraogo R, Hough K, McCue P, Chan L, Falkner B, Goldstein BJ.** Adiponectin regulates albuminuria and podocyte function in mice. *J Clin Invest* 118: 1645–1656, 2008.
27. **Sieber J, Lindenmeyer MT, Kampe K, Campbell KN, Cohen CD, Hopfer H, Mundel P, Jehle AW.** Regulation of podocyte survival and endoplasmic reticulum stress by fatty acids. *Am J Physiol Renal Physiol* 299: F821–F829, 2010.
29. **Sieber J, Weins A, Kampe K, Gruber S, Lindenmeyer MT, Cohen CD, Orellana JM, Mundel P, Jehle AW.** Susceptibility of podocytes to palmitic acid is regulated by stearoyl-CoA desaturases 1 and 2. *Am J Pathol* 183: 735–744, 2013.
30. **Tang SC, Leung VT, Chan LY, Wong SS, Chu DW, Leung JC, Ho YW, Lai KN, Ma L, Elbein SC, Bowden DW, Hicks PJ, Comeau ME, Langefeld CD, Freedman BI.** The acetyl-coenzyme A carboxylase beta (ACACB) gene is associated with nephropathy in Chinese patients with type 2 diabetes. *Nephrol Dial Transplant* 25: 3931–3934, 2010.
31. **Wolf G, Chen S, Ziyadeh FN.** From the periphery of the glomerular capillary wall toward the center of disease: podocyte injury comes of age in diabetic nephropathy. *Diabetes* 54: 1626–1634, 2005.
32. **Yuan Y, Huang S, Wang W, Wang Y, Zhang P, Zhu C, Ding G, Liu B, Yang T, Zhang A.** Activation of peroxisome proliferator-activated receptor-gamma coactivator 1alpha ameliorates mitochondrial dysfunction and protects podocytes from aldosterone-induced injury. *Kidney Int* 82: 771–789, 2012.
33. **Zang M, Zuccollo A, Hou X, Nagata D, Walsh K, Herscovitz H, Brecher P, Ruderman NB, Cohen RA.** AMP-activated protein kinase is required for the lipid-lowering effect of metformin in insulin-resistant human HepG2 cells. *J Biol Chem* 279: 47898–47905, 2004.