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Stimulation of fat accumulation in hepatocytes by PGE_2-dependent repression of hepatic lipolysis, β-oxidation and VLDL-synthesis

Janin Henkel[1], Katja Frede[1], Nancy Schanze[1], Heike Vogel[2], Annette Schürmann[2], Astrid Spruss[3], Ina Bergheim[3] and Gerhard P Püschel[1]

Hepatic steatosis is recognized as hepatic presentation of the metabolic syndrome. Hyperinsulinaemia, which shifts fatty acid oxidation to *de novo* lipogenesis and lipid storage in the liver, appears to be a principal elicitor particularly in the early stages of disease development. The impact of PGE_2, which has previously been shown to attenuate insulin signaling and hence might reduce insulin-dependent lipid accumulation, on insulin-induced steatosis of hepatocytes was studied. The PGE_2-generating capacity was enhanced in various obese mouse models by the induction of cyclooxygenase 2 and microsomal prostaglandin E-synthases (mPGES1, mPGES2). PGE_2 attenuated the insulin-dependent induction of SREBP-1c and its target genes glucokinase and fatty acid synthase. Nevertheless, PGE_2 enhanced incorporation of glucose into hepatic triglycerides synergistically with insulin. This was most likely due to a combination of a PGE_2-dependent repression of (1) the key lipolytic enzyme adipose triglyceride lipase, (2) carnitine–palmitoyltransferase 1, a key regulator of mitochondrial β-oxidation, and (3) microsomal transfer protein, as well as (4) apolipoprotein B, key components of the VLDL synthesis. Repression of PGC1α, a common upstream regulator of these genes, was identified as a possible cause. In support of this hypothesis, overexpression of PGC1α completely blunted the PGE_2-dependent fat accumulation. PGE_2 enhanced lipid accumulation synergistically with insulin, despite attenuating insulin signaling and might thus contribute to the development of hepatic steatosis. Induction of enzymes involved in PGE_2 synthesis in *in vivo* models of obesity imply a potential role of prostanoids in the development of NAFLD and NASH.

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INTRODUCTION

With the rising prevalence of overweight, obesity and ensuing metabolic syndrome or type 2 diabetes, non-alcoholic fatty liver disease has become one of the most common liver pathologies.[1–3] The spectrum ranges from reversible fat accumulation (steatosis), which may be complicated by inflammation in steatohepatitis, to liver fibrosis, cirrhosis and ultimately organ loss. Although the exact mechanisms that determine the course of the disease remain elusive, hyperinsulinaemia and peripheral insulin resistance resulting in elevated levels of circulating free fatty acids appear to be among the principal causes of the initial steatosis.[1] Steatosis and inflammation seem to influence each other mutually: steatosis rendering the liver more susceptible to proinflammatory cytokines and cytokines in turn impairing the regulation of lipid metabolism in hepatocytes. In addition to cytokines, small molecular mass mediators, including prostaglandins[4] are released from non-parenchymal liver cells and infiltrating inflammatory cells. PGE_2 biosynthesis is upregulated by the induction of cyclooxygenase 2 (COX2) and microsomal prostaglandin E synthases 1 and 2 (mPGES1, mPGES2).[5,6] Prostaglandins may affect hepatocyte metabolism directly[7] or they can modulate the regulation of hepatocyte metabolism by hormones[8,9] or cytokines.[10,11] There are conflicting reports about the impact of prostaglandins on hepatic lipid metabolism. Although some studies indicate that prostaglandins might favor fat accumulation in hepatocytes and hence the development of

[1]Department of Nutritional Biochemistry, University of Potsdam, Nuthetal, Germany; [2]Department of Experimental Diabetology, German Institute of Human Nutrition, Nuthetal, Germany and [3]Department of Nutritional Medicine, University of Hohenheim, Stuttgart, Germany
Correspondence: Professor GP Püschel, MD, Universität Potsdam, Institut für Ernährungswissenschaft, Abteilung Biochemie der Ernährung, Arthur-Scheunert-Allee 114-116, D-14558 Nuthetal, Germany.
E-mail: gpuesche@rz.uni-potsdam.de

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hepatic steatosis,[12] others provide evidence that PGE_2 rather might suppress *de novo* lipogenesis[13] or that PGE_2 does not affect lipogenesis but attenuate triglyceride incorporation into VLDL.[14] So far, the impact of prostaglandin E_2 on the insulin-dependent changes in hepatic lipid metabolism was not studied. This is of importance because hyperinsulinaemia contributes to the pathogenesis of hepatic steatosis.[2,3] As we have previously shown that PGE_2 can attenuate insulin signaling,[8] we addressed the question whether PGE_2 is capable of attenuating insulin-dependent changes in hepatic lipid metabolism.

In accordance with such a hypothesis, PGE_2 attenuated the insulin-dependent induction of liponeogenic enzymes in *in vitro* studies with primary hepatocytes. At variance with the expectations, however, PGE_2 enhanced the incorporation of glucose into lipids synergistically with insulin, most likely by a combination of inhibiting β-oxidation and reducing lipoprotein secretion. In support of the *in vivo* relevance of such a mechanism, expression of inducible PGE_2-generating enzymes was increased in livers of various models for diet-induced obesity as well as of monogenic and polygenic obesity.

MATERIALS AND METHODS

Materials

Materials were from the following sources: Narcoreen, Merial GmbH (Hallbergmoos, Germany); Percoll, D-[U-^{14}C] glucose, GE Healthcare (Freiburg, Germany); PGE_2, PD98059, Alexis (Grünberg, Germany); monoclonal anti-phospho-Akt (Ser473), anti-phospho-p44/42 MAPK (Thr202/Tyr204), anti-FoxO1 antibodies, polyclonal anti-Akt, anti-p44/42 MAPK, anti-phospho-FoxO1 (Thr24)/FoxO3a (Thr32), anti-phospho-ACC (Ser79) and anti-ACC antibodies, Cell Signaling Technology (Frankfurt, Germany); anti PGC1α, Santa Cruz Biotechnology (Heidelberg, Germany); and SuperSignal West Pico Chemiluminescent Substrate, Pierce (Rockford, IL, USA). The plasmid for eukaryotic expression of PGC1α was kindly provided by Stephan Herzig (DKFZ, Heidelberg, Germany).

Animals and Treatments

C3H/HouJ (Jackson Laboratories, Bar Harbor, ME, USA) mice had free access to standard diet (ssniff, Soest, Germany) and tap water containing 30% fructose or plain tap water for 8 weeks.[15] C57BL/6J, ob/ob (B6.V-Lep^{ob}/J) (Charles River, Sulzfeld, Germany) and NZO mice (NZO/HIBomDife: Dr R Kluge, German Institute of Human Nutrition, Nuthetal, Germany) were kept on standard diet containing or high-fat diet (Altromin, Lage, Germany), as described previously,[16,17] 8 or 22 weeks at a temperature of 22 °C with a 12-h light/dark cycle. Mice were killed and portions of liver were snapfrozen.

Male Wistar rats (200–300 g) (Charles River) were kept on a 12-hour light/dark cycle with free access to water and a standard 1326 rat diet (Altromin).

Treatment of the animals followed the German animal protection laws and all experiments were approved by the ethics committee of the State Agency of Environment, Health and Consumer Protection (State of Brandenburg, Germany) and the principles of laboratory animal care.

Hepatocyte Cultivation

Density-gradient purified hepatocytes prepared without the use of collagenase and cultured in M199 (1×10^6 cells/35-mm plate) as described previously.[18,19] Unless otherwise stated, hepatocytes were incubated for 330 min with $\pm 10\,\mu$M PGE_2, stimulated with ± 10 nM insulin for the time indicated, washed with ice-cold PBS and snapfrozen. Hepatocytes were transfected immediately after plating with the plasmids indicated employing a modified calcium phosphate method described previously.[20]

Measurement of ^{14}C-Glucose Incorporation into Triglycerides

Hepatocytes were preincubated $\pm 10\,\mu$M PGE_2 for 330 min supplemented with 1 μCi/ml ^{14}C-glucose, containing either 10 mM or 25 mM glucose. Subsequently, hepatocytes were incubated ± 10 nM insulin for 6 h, washed with ice-cold PBS and lysed in 150 μl methanol. After adding 300 μl chloroform and sonication for 10 min, 90 μl of 0.05% $CaCl_2$ were added. After vigorous mixing, phases were separated by centrifugation at 5000 *g* for 10 min at room temperature. The chloroform phase was collected and the aqueous phase was re-extracted twice with chloroform as above. The combined chloroform phases were dried under vacuum and redissolved in 120 μl chloroform. Radioactivity in an aliquot of the extract was quantified by β-counting.

Staining of Lipid Droplets

Hepatocytes were incubated $\pm 10\,\mu$M PGE_2 with or without 10 nM insulin for 24 h. Oil Red-*O* staining: cells were fixed in 4% paraformaldehyde for 20 min, stained with Oil Red-*O* (working solution 0.3% Oil Red-*O* (Sigma Aldrich, Deisenhofen, Germany) in isopropanol: water (3:2)) for 30 min, washed and mounted. BODIPY staining: living cells were washed with 1 × PBS, stained with BODIPY 493/503 (working solution 1 μg/ml BODIPY 493/503 (Life Technologies, Darmstadt, Germany) in 1 × PBS) for 30 min, washed and subsequently analyzed by fluorescence microscopy (BODIPY: excitation wavelength 480 nm, emission wavelength 509 nm; CFP: excitation wavelength 434 nm, emission wavelength 479 nm). Staining of fat droplets was quantified with Image J 1.45 (Wayne Rasband, NIH, USA, http://imagej.nih.gov/ij).

Measurement of Acetoacetate Formation

After incubation, hepatocytes were immediately lysed in 50 mM Tris/HCl pH 7.5 containing 50 mM sucrose, 4 mM $MgCl_2$, 40 mM KCl, 2 mM EDTA; 10 mM NaF with protease inhibitors and 1 mM sodium orthovanadate (buffer 1). Crude mitochondria, which were prepared by centrifugation of the homogenates at 500 *g* at 4 °C and the supernatant for 10 min at 5000 *g* at 4 °C, were resuspended in buffer 1 and

Table 1 qPCR primer sequences

Gene (Acc. Nr.)	Forward primer	Reverse primer
COX2 (NM_011198)	5′-GGCCCTTCCTCCCGTAGCAG-3′	5′-CATCAGACCAGGCACCAGACCAA-3′
mPGES1 (NM_022415)	5′-GCGCTGAAACGTGGAGGCCT-3′	5′-GCGAAGGCGTGGGTTCAGCT-3′
mPGES2 (NM_133783)	5′-GGCACCAGCGTTCCCAGGAC-3′	5′-GCCCACTGCCGCCACTTCAT-3′
SREBP-1c (XM_213329)	5′-ACGACGGAGCCATGGATTG-3′	5′-TTTGATTCCAGGCCCAGGGG-3′
FAS (NM_017332)	5′-GGCCTGGACTCGCTCATGGG-3′	5′-TGGGCCTGCAGCTGGGAGCA-3′
ATGL (NM_001108509)	5′-AGCGGTTCCTGGGTCCCCTG-3′	5′-AACCATGGGCTCTGGCGGGA-3′
CPT-1a (NM_031559)	5′-CCAAGCTGTGGCCTTCCAGT-3′	5′-GGACGCCGCTCACAATGTTC-3′
MTP (NM_001107727)	5′-AGACTCCAGCCTCACTGGAA-3′	5′-TGCAGCCTTCATTCTGACAC-3′
ApoB (NM_019287)	5′-CAGGCTGATGCTGTTTTGAA-3′	5′-CTGAGGGATTTGGGATCAGA-3′
PGC1α (NM_031347)	5′-ACCGCAGTCGCAACATGCTCA-3′	5′-GGTGGAGTGGCTGCCTTGGG-3′
glucokinase (NM_012565)	5′-GCCGTGCCTGTGAAAGCGTGTC-3′	5′-CCACCCGTAGCAGCAGAATAGGTC-3′
PCK (NM_198780)	5′-GCAGAGCATAAGGGCAAGGTCA-3′	5′-CCAGCACGCGGGAGTTCT-3′
***β*-actin** (NM_031144)	5′-CCCTAAGGCCAACCGTGAAAAGATG-3′	5′-AGGTCCCGGCCAGCCAGGTCCAG-3′

incubated for 60 min at 37 °C in the same buffer containing additionally 100 μM palmitate, 1 mM ATP and 1 mM D,L-Carnitine. The reaction was stopped by adding 1/10th reaction volume 30% TCA. Acetoacetate was determined in the deproteinized supernatants colorimetrically.[21]

Western Blot Analysis and Quantitative PCR

Proteins and phosphoproteins were quantified by western blot with phosphospecific antibodies as previously described.[8,22] RNA was isolated with the Eurx Gene Matrix Universal RNA Purification Kit (Roboklon, Berlin, Germany) and from liver samples using peqGOLD TriFas (peqlab, Erlangen, Germany) or TRIzolReagent (Invitrogen, Darmstadt, Germany). cDNA was synthesized from purified, DNase-treated RNA as described previously.[8,22] Hot start real-time RT-PCR for the quantification of mRNAs was carried out in triplicates in a reaction mixture of 2 × Maxima SYBR Green qPCR Master Mix (MBI Fermentas GmbH, St. Leon Rot, Germany), 250 nM forward and reverse oligonucleotides (Table 1, MWG BiotechAG, Ebersberg, Germany), and 0.3 μl cDNA in a total volume of 10 μl. qPCR program: initial enzyme activation at 95 °C for 3 min, 52 × 95 °C, 20 s; 57–60 °C, 20 s; 72 °C, 30 s), 10 s at 95 °C; CFX Thermal Cycler (Bio-Rad, München, Germany). Gene of interest RNA expression normalized to β-actin was given by the formula: N-fold induction = $2^{(\text{CT gene of interest (control)} - \text{CT gene of interest (stimulated sample)})} / 2^{(\text{CT } \beta\text{-actin (control)} - \text{CT } \beta\text{-actin (stimulated sample)})}$.

RESULTS

Increased Expression of PGE_2-Generating Enzymes in Obesity Models

In inflamed tissues local levels of PGE_2 are increased as a consequence of LPS- and cytokine-dependent induction of COX2 and mPGES1.[5,6] Although mPGES2 is expressed constitutively, its expression can be enhanced in the course of inflammation.[23] Therefore, the mRNA expression of COX2, mPGES1 and mPGES2 was analyzed in the liver of mouse models, in which obesity was induced either by diet (Figure 1, left panel), age (middle panel) or leptin deficiency (right panel). In all these models development of obesity is accompanied by severe hepatic steatosis.[15–17] The respective controls are lean and devoid of hepatic pathology. In C3H/HouJ-mice fed with standard diet and tap water containing 30% fructose (the pathological model), mRNA expression of mPGES1 was 17-fold induced and expression of COX2 and mPGES2 were moderately increased by factor 1.5 in comparison with control mice fed with plain tap water (Figure 1). Similar results were obtained in old (22 week, pathological model) *versus* young (8 week, control) NZO mice, a polygene obesity model for (human) metabolic syndrome,[16] fed with standard diet. COX2 was induced about two-fold, mPGES1 six-fold in the older, steatotic animals. Similarly, both COX2 (four-fold) and mPGES1 (7-fold) were increased in response to leptin deficiency when comparing the ob/ob (B6.V-Lep^{ob}/J) with the corresponding healthy control strain C57BL/6J at 8 weeks of age when both strains are fed with standard diet (Figure 1). Data from further models of genetically or diet-induced obesity models yielded similar results (Supplementary Table S1, see Supplementary Information).

Attenuation of Insulin-Induced Induction of Transcription Factors and Enzymes Involved in *De Novo* Lipogenesis

Fatty acid synthesis in hepatocytes is increased by insulin and high glucose concentration. Incubation of primary cultures of rat hepatocytes with PGE_2 before a subsequent stimulation with insulin attenuated the insulin-dependent phosphoryla-

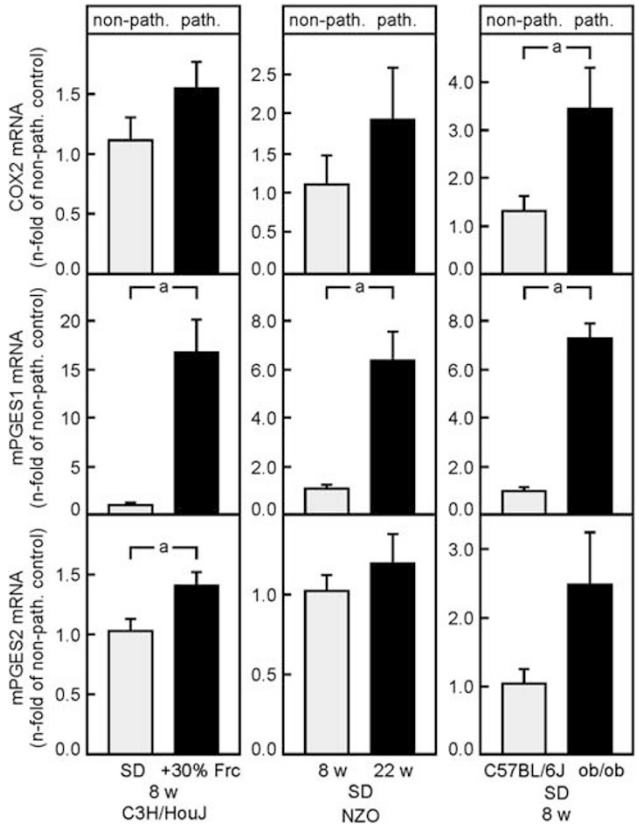

Figure 1 Increased expression of PGE_2-generating enzymes COX2, mPGES1 and mPGES2 in pathological obesity models. Healthy male mice were compared with their pathological counterpart, as indicated. COX2, mPGES1 and mPGES2 mRNA levels in liver samples were determined (see methods section). Values are means ± s.e.m. of four to seven animals per group. Statistics: Student's *t*-test for unpaired samples, a: two-sided $P<0.05$.

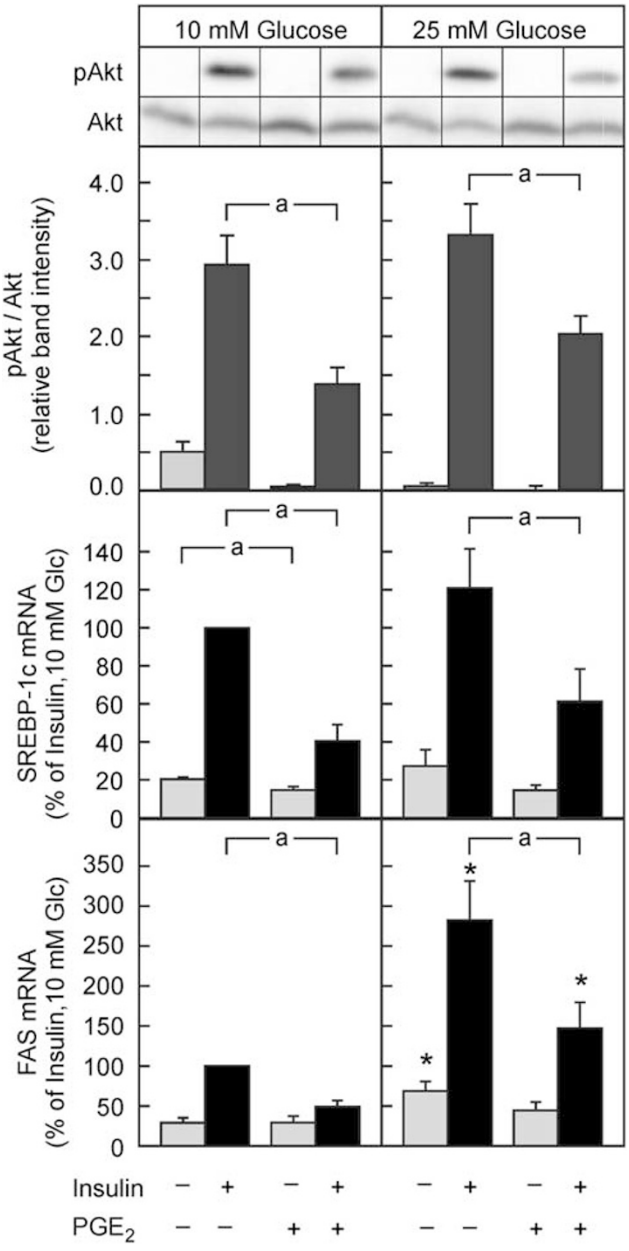

Figure 2 Inhibition of insulin-stimulated Akt phosphorylation and induction of Akt targets by PGE_2. Hepatocytes incubated in the presence of ± 10 μM PGE_2 for 330 min and subsequently stimulated in the same medium with 10 nM insulin for 15 min (Akt kinase) or 6 h (SREBP-1c, FAS). Phosphorylation of Akt kinase was determined by western blot analysis with phosphospecific antibodies. Values are means ± s.e.m. of five independent experiments. SREBP-1c and FAS mRNA levels were determined by RT-qPCR with β-actin as a reference gene (see methods section). Copy number in cells cultured in presence of 10 mM glucose and treated with insulin was set 100%. Values are means ± s.e.m. of six independent experiments. Statistics: Student's *t*-test for unpaired samples, a: two-sided $P<0.05$. *: different to corresponding sample with 25 mM glucose ($P<0.05$).

tion of the Akt kinase, which correlates with Akt activation, by about 50% (Figure 2), independently of the glucose concentration in the culture medium. As shown previously,[8] this was due to a sustained PGE_2-dependent activation of ERK1/2 (see Supplementary Figure S1). Inhibition of ERK1/2 by chemical inhibitors abrogated the PGE_2-dependent inhibition of the insulin-dependent Akt-phosphorylation (see Supplementary Figure S1). In addition to acutely regulating enzyme activities of key metabolic enzymes Akt kinase mediates the insulin-dependent induction of the transcription factor SREBP-1c and its targets. It was assumed that the insulin-dependent induction of SREBP-1c and its targets might be attenuated by prior incubation of hepatocytes with PGE_2. As expected, insulin-dependent five-fold induction of SREBP-1c was reduced about 60% by PGE_2 (Figure 2). PGE_2 also reduced the basal SREBP-1c expression by about 30%. Glucose slightly induced basal SREBP-1c mRNA. Insulin induced SREBP-1c about four-fold above the level reached by incubation with 25 mM glucose alone, resulting in mRNA levels that were about seven-fold higher than under control conditions with 10 mM glucose. The insulin-dependent induction in presence of 25 mM glucose was attenuated by PGE_2 to a similar extent as in hepatocytes cultured in a medium containing 10 mM glucose.

Glucokinase and fatty acid synthase (FAS)[24] are important downstream targets of SREBP-1c. Glucokinase was induced roughly 80-fold by insulin. PGE_2 reduced the insulin-dependent induction of glucokinase by about 80% (see Supplementary Figure S2). FAS was induced about four-fold by insulin under low-glucose conditions (Figure 2). This induction was largely attenuated by prior incubation of hepatocytes with PGE_2. In contrast to SREBP-1c, the basal expression of FAS was not affected by PGE_2 treatment. Raising glucose concentration to 25 mM also induced FAS. Stimulation with insulin further induced FAS, resulting in a 10-fold induction in comparison with the control at 10 mM glucose. The insulin-dependent induction of FAS at high glucose was inhibited by prior treatment of the hepatocytes with PGE_2 by about 50%.

Incorporation of ^{14}C-Glucose into Lipids in Hepatocytes

FAS is a key enzyme in *de novo* lipacidogenesis. Therefore, it was assumed that the attenuation of the insulin-dependent induction of FAS would be reflected in an attenuation of the insulin-induced incorporation of ^{14}C-glucose into hepatic triglycerides. At variance with expectations PGE_2 increased glucose incorporation into triglycerides in the absence of insulin both at low (10 mM) and at high (25 mM) glucose concentrations (Figure 3a). At 10 mM glucose, PGE_2 further enhanced the insulin-stimulated incorporation of glucose into triglycerides while it did not affect the insulin-stimulated incorporation at 25 mM glucose. In accordance with this finding, deposition of fat droplets was seen in Oil Red-*O*-stained hepatocytes both after treatment with insulin and PGE_2 (Figure 3b and c). The stimulation of lipid accumulation by PGE_2 and insulin appeared to be additive.

An acute stimulation of lipacidogenesis by PGE_2-dependent covalent modification of the key regulatory lipacidogenic enzyme acetyl-CoA-carboxylase (ACC), which is activated by dephosphorylation of Ser_{79}, most likely is not responsible for this enhanced lipid accumulation. PGE_2 did not affect its insulin-dependent dephosphorylation and only slightly but not significantly reduced phosphorylation by itself (see Supplementary Figure S3). Similarly, PGE_2 did not modulate the phosphorylation and activation of AMP-dependent kinase, an upstream regulator of ACC (not shown). Therefore, additional potential mechanisms were examined.

PGE_2-Dependent Inhibition of Lipolysis and Mitochondrial β-Oxidation

Lipid accumulation in hepatocytes represents an equilibrium between *de novo* synthesis on one side balanced by lipolysis and *β*-oxidation or secretion in the form of VLDL on the other. In the liver, the rate-controlling step in triglyceride breakdown is catalyzed by adipose triglyceride lipase (ATGL).[25,26] As previously shown for adipocytes,[27] insulin decreased ATGL expression in hepatocytes both at low (10 mM) or high (25 mM) glucose concentration (Figure 4a). Unexpectedly, PGE_2 also decreased ATGL expression. At low-glucose concentrations, the PGE_2-dependent repression was additive to the insulin-dependent repression. Fatty acids released from intrahepatic triglycerides may enter *β*-oxidation in the hepatocyte. The key regulatory step in mitochondrial *β*-oxidation is the initiation of the transport of activated fatty acids into the mitochondrium via the carnitin–palmitoyltransferase 1 (CPT-1). In addition to allosteric regulation, CPT-1 activity is regulated on the transcriptional level. In accordance with previous studies, insulin decreased CPT-1 expression about five-fold (Figure 4a). Unexpectedly, PGE_2 repressed CPT-1 to a similar extent as insulin, both a 10 mM and at 25 mM glucose. The PGE_2-dependent repression of CPT-1 expression was further enhanced by insulin.

The PGE_2-dependent repression of CPT-1 resulted in an inhibition of mitochondrial *β*-oxidation by PGE_2. As a consequence acetoacetate formation, which amounted to

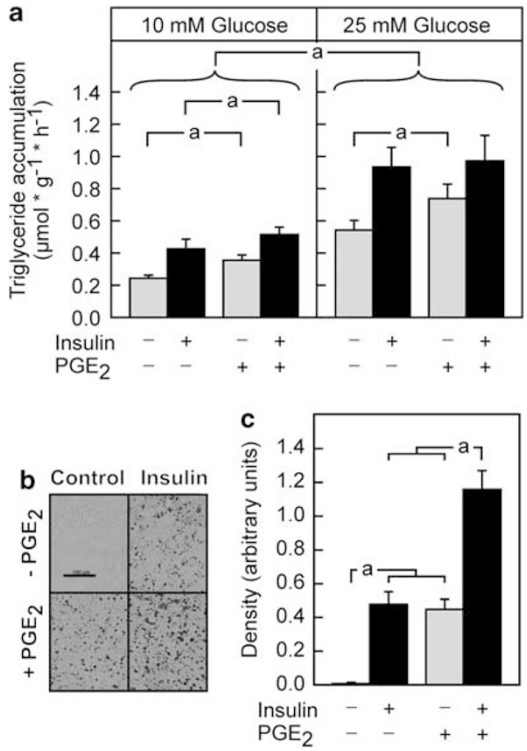

Figure 3 Stimulation hepatic triglyceride accumulation by insulin and PGE_2. (**a**) Stimulation of glucose incorporation into triglycerides: hepatocytes were incubated in the presence of ± 10 *μ*M PGE_2 for 330 min in a medium containing 1 *μ*Ci/ml ^{14}C-glucose and subsequently stimulated with insulin for 6 h in the same medium. Triglycerides were extracted and radioactivity in the organic phase was determined. Values are means ± s.e.m. of five independent experiments. Statistics: Student's *t*-test for unpaired samples a: $P<0.05$. (**b**) Histological determination of triglyceride accumulation: hepatocytes were treated as above in medium containing 10 mM glucose under the different conditions indicated for 24 h. Cells were fixed in 4% paraformaldehyde and then stained with Oil Red-*O*. Macrovesicular fat droplets that are absent from the controls can be seen in insulin- and PGE_2-treated hepatocytes and to a higher extend in cells treated with a combination of insulin and PGE_2. (**c**) Randomly chosen fields of Oil Red-*O*-stained hepatocyte cultures were subjected to image analysis to quantify the relative density of stained lipid droplets.

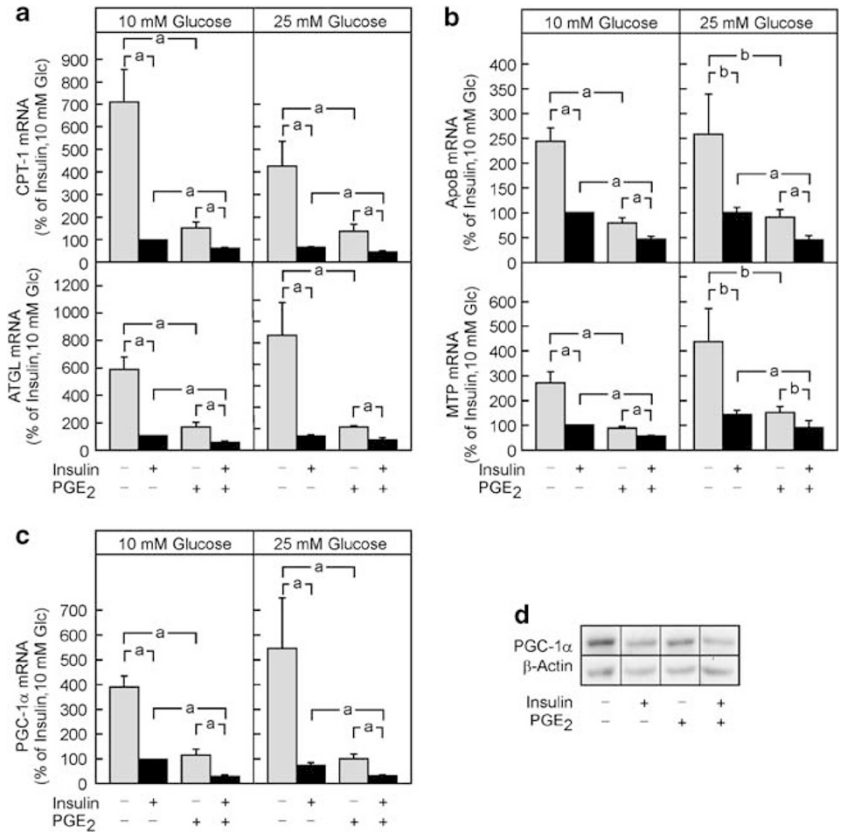

Figure 4 Synergistic repression of genes of proteins involved in triglyceride mobilization, fatty acid oxidation and VLDL synthesis by insulin and PGE_2. Hepatocytes were incubated in the presence of $\pm 10\,\mu M$ PGE_2 for 330 min and subsequently stimulated with insulin for 6 h. (**a**–**c**) ATGL, CPT-1, ApoB, MTP and PGC1α mRNA levels were determined by RT-qPCR with β-actin as a reference gene (see methods section). Copy number in cells cultured in the presence of 10 mM glucose and treated with insulin was set 100%. Values are means ± s.e.m. of five independent experiments. Statistics: Student's *t*-test for unpaired samples, a: $P<0.05$. (**d**) Protein expression of PGC1α and β-actin as a reference protein in cell lysates were determined by western blot analysis with specific antibodies. A representative blot is shown.

0.80 ± 0.37 nmol/g/h in control mitochondria, was reduced by 60 ± 9% and 53 ± 7% in mitochondria of insulin- or PGE_2-treated cells, but no additivity was observed.

Inhibition of VLDL Production by Insulin and PGE_2

Lipids are also released from the hepatocyte pool via secretion of VLDL containing apolipoprotein B (ApoB), which is cotranslationally and posttranslationally loaded with lipids. MTP (microsomal transfer protein) is crucial for this lipidation of ApoB. Insulin decreased both ApoB and MTP expression by about 50% to 60% at 10 mM glucose, respectively. PGE_2 decreased the expression to a similar extent as insulin (Figure 4b). Most notably, the PGE_2-dependent repression was further enhanced by insulin, indicating that the repression of MTP and ApoB by insulin and PGE_2 was additive. A similar pattern was observed in media containing 25 mM glucose.

Mechanism of the PGE_2-Dependent Repression of MTP and ApoB

FoxO1 phosphorylation is supposed to cause the insulin-dependent repression of MTP and ApoB. Insulin induced the phosphorylation of FoxO1 about four-fold (see Supplementary Figure S4). PGE_2 slightly enhanced FoxO1 phosphorylation but did not affect insulin-dependent FoxO1 phosphorylation making FoxO1 phosphorylation unlikely to account for the PGE_2-dependent repression of MTP and ApoB.

Another key regulator of MTP and ApoB and CPT-1 transcription is the PPARγ-coactivator-1α (PGC1α). PGE_2 reduced the PGC1α mRNA and protein level in hepatocytes cultured in presence of 10 mM glucose by more than 50% (Figure 4c and d). Similarly, treatment of hepatocytes with insulin reduced the PGC1α mRNA and protein level by about 80%. Notably, the insulin-dependent and PGE_2-dependent decrease in PGC1α were additive, combined treatment of hepatocytes with insulin and PGE_2 reduced the mRNA expression to about 8% of the control level. Similar results were obtained with hepatocytes cultured at higher glucose concentrations. Glucose itself did not affect PGC1α expression. The functional relevance of the PGC1α repression by PGE_2 and insulin is further corroborated by the synergistic repression of another PGC1α target gene, the gluconeogenic key regulatory enzyme phosphoenolpyruvat carboxykinase (PCK, see Supplementary Figure S5). To provide further

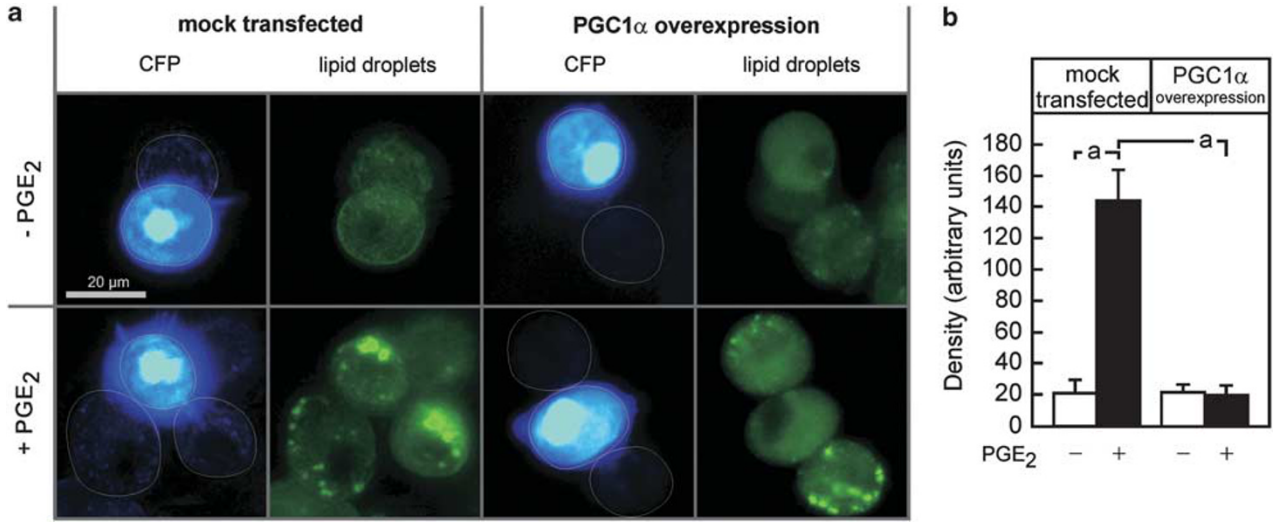

Figure 5 Repression of PGE_2-mediated lipid accumulation in PGC1α-overexpressing hepatocytes. (**a**) Hepatocytes were co-transfected with CFP and empty vector (mock-transfected) or a vector containing PGC1α after cell isolation. Cells were incubated in the presence of ± 10 µM PGE_2 for 24 h and subsequently stained with BODIPY 493/503. Fluorescence was detected with the respective exitation and emission wavelength for CFP and BODIPY (see methods). Co-transfected cells express ± PGC1α and, in addition, CFP (cyan fluorescence). The circumference of non fluorescent and hence untransfected cells, which serve as additional wild-type controls in the PGC1α-transfected cultures, is labeled with a white line. Lipid droplets (green fluorescence) are only visible in PGE_2-treated hepatocytes but are absent from PGE_2-treated cells overexpressing PGC1α. (**b**) CFP-fluorescent hepatocytes were subjected to image analysis (Image J, see methods section) to quantify the relative fluorescence intensity of stained lipid droplets. Statistics: Student's *t*-test for unpaired samples, a: $P<0.05$.

support of the hypothesis, the impact of forced overexpression of PGC1α in hepatocytes on the PGE_2-dependent lipid accumulation was tested. As transfection efficiency is very low in cultures of primary hepatocytes no changes in bulk lipid balance or gene expression can be expected. However, co-transfection with two plasmids encoding different fluorescent proteins showed that hepatocytes incorporated either both or none of the plasmids upon co-transfection (not shown). Hence, to allow identification of transfected cells, a plasmid encoding CFP was co-transfected together with either an empty mock vector or an expression vector for PGC1α. Lipid accumulation was determined by staining with the fluorescent dye BODIPY (Figure 5). PGE_2 enhanced lipid accumulation in non-transfected and mock-transfected hepatocytes. By contrast, the PGE_2-dependent lipid accumulation was completely blunted in cells overexpressing PGC1α. Similarly, the PGE_2-dependent decrease in expression of a reporter gene under the control of the PCK promoter was abolished by forced expression of PGC1α (Supplementary Figure S6).

DISCUSSION

In patients suffering from the metabolic syndrome, the exceedingly elevated plasma insulin concentration has been implicated in the development of hepatic steatosis by stimulating *de novo* lipogenesis and by inhibiting fatty acid oxidation and triglyceride export. A recent study[8] showed that PGE_2, which is produced in non-parenchymal liver cells, attenuated insulin signaling in hepatocytes (Figure 2) by a sustained activation of ERK1/2 (Supplementary Figure S1).[8] PGE_2 might thus alleviate the impact of insulin on the development of steatosis. In accordance with such a hypothesis, PGE_2 attenuated the insulin-dependent induction of SREBP-1c (Figure 2) and its target genes FAS (Figure 2) and glucokinase (Supplementary Figure S2). At variance with expectations PGE_2 nevertheless increased the net incorporation of glucose into hepatic triglycerides (Figure 3) most likely by a repression of ATGL-mediated lipolysis (Figure 4a), mitochondrial β-oxidation (Figure 4a) and VLDL synthesis (Figure 4b). A possible common underlying mechanism is the repression of PGC1α by PGE_2 (Figures 4c, d and 5). As a consequence, PGE_2 might exacerbate hepatocyte steatosis in the course of the metabolic syndrome rather than preventing it.

In Vivo support for a Role of Prostaglandin E_2 in the Development of NAFLD and NASH

The relevance of the current finding is supported by a number of *in vivo* studies. Local production of PGE_2 in the liver depends on phospholipase A2, which liberates arachidonic acid from phospholipids, cyclooxygenases to generate PGH_2, and PGE synthases, which isomerize PGH_2 to PGE_2.[6,23] Inducible COX2 and mPGES1, which are expressed after induction by proinflammatory stimuli, and mPGES2, which is induced in livers of LPS-treated mice and patients with hepatitis C infection,[5,23] are responsible for inflammation-elicited PGE_2 biosynthesis that is blunted in $mPGES1^{-/-}$ mice.[28,29] COX2, mPGES1 and mPGES2 were induced in

livers of diet-induced and genetic obesity mouse models (Figure 1, Supplementary Table S1). Similarly, COX2 was induced in livers of mice with methionine–choline-deficient diet-induced steatosis.[30,31] In these NASH models, inflammatory cells recruited into the liver might contribute to prostaglandin E_2 in addition to resident macrophages. Inhibition of prostanoid synthesis in a NASH mouse model with a selective COX2 inhibitor not only attenuated the liver damage and signs of inflammation but, most notably, also improved steatosis.[32] Surprisingly, this study detected COX2 immune reactivity not only in resident and infiltrating immune cells but also in hepatocytes, which normally express neither COX1 nor COX2 or mPGES1 and do not produce PGE_2. Although COX2-expression was not affected, mPGES1 was induced in cytokine-treated hepatocytes (Henkel *et al.*, unpublished data). If indeed the hepatocyte compartment additionally produces prostaglandins in the course of NASH development, the prostaglandin burden might actually be much higher than that would be expected if non-parenchymal cells and infiltrating mononuclear cells were the only source. In further support for a potential impact of eicosanoids on the development of steatosis, high-fat diet induced intrahepatic fat accumulation and signs of liver damage in high-fat diet-induced NAFLD was reduced by knockdown of the group IVA phospholipase A2, which furnishes arachidonic acid as substrate for COX2-dependent prostaglandin formation. These changes were paralleled by a pronounced decrease of the plasma PGE_2 concentration in these mice.[33,34]

Possible Mechanism Underlying PGE_2-Dependent Triglyceride Accumulation in Hepatocytes

One important pathway by which triglyceride accumulation in hepatocytes is entailed is *de novo* lipogenesis. In contrast to rodents on normal chow, lipogenesis is neglible in healthy humans but increases in the course of NAFLD development as a result of persistently elevated insulin and glucose plasma concentrations, which result in the induction of key lipogenic enzymes via SREBP and ChREBP activation, respectively.[2] The increase in *de novo* lipogenesis actually precedes the development of steatosis. PGE_2 in the current study slightly reduced the mRNA levels of SREBP-1c and FAS and attenuated their insulin-dependent induction. Therefore, a PGE_2-dependent stimulation of *de novo* lipogenesis can be excluded as the reason for the enhanced incorporation of glucose in to triglycerides. Rather, glucose probably furnished the activated glycerol for the glycerol backbone of triglycerides. The triglyceride accumulation would then be a result of redirecting fatty acids from combustion to esterification and the redistribution for triglycerides between secreted lipoproteins and intracellular stores. The PGE_2-dependent repression of ATGL (Figure 4a), CPT-1 (Figure 4a), the inhibition of ketogenesis and the repression of ApoB and MTP (Figure 4b) support this view. Overexpression of ATGL has previously been shown to counteract hepatic steatosis in obese mice.[35] The central role of CPT-1 in the development of hepatic steatosis has also been shown both *in vivo* and *in vitro*: moderate overexpression[36] or induction of CPT-1 has been shown to improve diet-induced steatosis of the liver in different NASH models.[37,38] By contrast, a reduction of CPT-1 activity along with other mitochondrial defects, which preceded the development of steatosis and NASH, has been implicated as leading cause for disease development in the Otsuka Long–Evans Tokushima Fatty rat model of NASH.[39] In further support of a contribution of impaired mitochondrial β-oxidation to steatosis, inhibition of CPT-1 has been identified as a possible underlying mechanism in drug-induced steatosis.[40] Development of steatosis was also favored by other defects of mitochondrial β-oxidation such as heterozygous deletion of mitochondrial trifunctional protein or knockdown of long chain acyl-CoA dehydrogenase.[41,42]

Similarly, impairment of VLDL synthesis has been implicated as a possible contributor to fat accumulation in hepatocytes in a polygenic mouse model of NASH.[43] As in the current study, lipid accumulation occurred in this mouse model despite reduction of SREBP-1c and FAS expression. Although a defect of hepatic mitochondrial β-oxidation reflected by a repression of CPT-1 might also be relevant in this model, a reduction of VLDL production due to low MTP levels was identified as the major cause. Steatosis and insulin resistance in this mouse model of NASH were largely improved by overexpression of MTP, which resulted in a rescue of VLDL production.[43] Notably, rescue of MTP expression not only improved steatosis in this model but also reduced signs of inflammation. In further support of the relevance of MTP in the development of steatosis, inhibition of MTP has been shown to contribute to drug- or alcohol-induced hepatic steatosis.[44,45]

Although development of steatosis, hepatic insulin resistance and NASH are closely correlated, the evidence for a causal interrelation is controversial. Efficient storage of fatty acids in triglycerides may actually protect the liver from adverse effects caused by high concentrations of circulating non-esterified fatty acids.[46,47] This could explain why in some *in vivo* experiments PGE_2 had beneficial effects on the development of NASH,[48,49] whereas others[32–34] (see above) rather support the view that PGE_2 has a negative impact on the development of steatosis.

The current study showed that PGE_2 increased triglyceride accumulation in hepatocytes most likely by a combined inhibition of β-oxidation due to CPT-1 repression and a reduced VLDL production due to repression of ApoB and MTP, all of which are downstream targets of PGC1α. PGC1α, which is known to be induced by an increase in cellular cAMP,[50] was also repressed by PGE_2, most likely by activation of the Gi-coupled EP3 receptor, which has previously been shown be responsible for the PGE_2-dependent repression of PCK in hepatocytes.[51] The hypothesis that the repression of PGC1α might be the underlying mechanism of the

PGE_2-induced lipid accumulation in hepatocytes is in accordance with studies showing that knockdown of PGC1α caused hepatic steatosis in 24 h fasted mice. Similarly, lipid accumulation was higher in oleate-exposed hepatocytes lacking PGC1α than in control hepatocytes.[52] Finally, forced overexpression of PGC1α completely blunted the PGE_2-dependent lipid accumulation (Figure 5), lending further support to this hypothesis.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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