Hyperinsulinemia Enhances Hepatic Expression of the Fatty Acid Transporter Cd36 and Provokes Hepatosteatosis and Hepatic Insulin Resistance*

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Background: Enhanced hepatic expression of the fatty acid transporter Cd36 correlates with liver fat accumulation, hepatosteatosis, insulin resistance, and hyperinsulinemia.

Results: Insulin increases hepatic Cd36 expression in a Pparγ-dependent manner.

Conclusion: Hyperinsulinemia stimulates hepatic Cd36 expression, which correlates with the development of hepatosteatosis, hepatic insulin resistance, and dysglycemia.

Significance: Hyperinsulinemia contributes to the development of hepatosteatosis and insulin resistance.

Hepatosteatosis is associated with the development of both hepatic insulin resistance and Type 2 diabetes. Hepatic expression of Cd36, a fatty acid transporter, is enhanced in obese and diabetic murine models and human nonalcoholic fatty liver disease, and thus it correlates with hyperinsulinemia, steatosis, and insulin resistance. Here, we have explored the effect of hyperinsulinemia on hepatic Cd36 expression, development of hepatosteatosis, insulin resistance, and dysglycemia. A 3-week sucrose-enriched diet was sufficient to provoke hyperinsulinemia, hepatosteatosis, hepatic insulin resistance, and dysglycemia in CBA/J mice. The development of hepatic steatosis and insulin resistance in CBA/J mice on a sucrose-enriched diet was paralleled by increased hepatic expression of the transcription factor Pparγ and its target gene Cd36 whereas that of genes implicated in lipogenesis, fatty acid oxidation, and VLDL secretion was unaltered. Additionally, we demonstrate that insulin, in a Pparγ-dependent manner, is sufficient to directly increase Cd36 expression in perfused livers and isolated hepatocytes. Mouse strains that display low insulin levels, i.e. C57BL6/J, and/or lack hepatic Pparγ, i.e. C3H/HeN, do not develop hepatic steatosis, insulin resistance, or dysglycemia on a sucrose-enriched diet, suggesting that elevated insulin levels, via enhanced Cd36 expression, provoke fatty liver development that in turn leads to hepatic insulin resistance and dysglycemia. Thus, our data provide evidence for a direct role for hyperinsulinemia in stimulating hepatic Cd36 expression and thus the development of hepatosteatosis, hepatic insulin resistance, and dysglycemia.

T2D is a current global epidemic, and similarly NAFLD, currently the most common chronic liver disease worldwide, is increasing globally with an estimated overall prevalence of ~30% and with a prevalence up to ~75% in obese individuals and Type 2 diabetics (1–3). NAFLD covers a spectrum of conditions associated with lipid accumulation in hepatocytes ranging from fatty liver, i.e. hepatosteatosis, to nonalcoholic steatohepatitis, advanced fibrosis, and cirrhosis (1, 3). Several independent factors, including (i) increased free fatty acid (FFA) uptake, (ii) de novo lipogenesis, (iii) decreased FA oxidation, and (iv) reduced VLDL secretion, may contribute to hepatic fat accumulation (2, 4, 5). The fatty acid transporter Cd36 alone increases FA uptake in mouse hepatocytes (6). Cd36 mRNA levels are drastically increased in livers of murine models of obesity and T2D (7), and Cd36 expression correlates with liver TG accumulation, insulin resistance, and hyperinsulinemia in human NAFLD (8, 9). Moreover, under normal, nonmetabolically challenged conditions forced expression of Cd36 alone increases FA uptake in mouse hepatocytes ex vivo and mouse liver TG content in vivo (10). Nonetheless, the factors provoking increased hepatic Cd36 expression remain unknown.

Hepatosteatosis is associated with development of hepatic insulin resistance and T2D and, vice versa, T2D is an established risk factor for the development of NAFLD (4, 11). Development of T2D is initially characterized by decreased insulin sensitivity, manifested by ensuing hyperglycemia and a compensatory response where pancreatic β-cells produce and secrete more insulin, which in turn results in hyperinsulinemia. Thus, hyperinsulinemia, which is associated with both T2D and NAFLD, is generally considered a consequence of insulin resis-

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1 The abbreviations used are: T2D, Type 2 diabetes; NAFLD, nonalcoholic fatty liver disease; SRD, sucrose-enriched diet; PPAR, peroxisome proliferator-activated receptor; FFA, free fatty acid; FA, fatty acid; GSIS, glucose-stimulated insulin secretion; GTT, glucose tolerance test; ITT, insulin tolerance test; qRT, quantitative RT; CD, Control Diet; 3w, 3 weeks; TG, triglyceride; HOMA-IR, homeostasis model for insulin resistance.
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In Vivo Insulin Signaling—To address hepatic insulin signaling, we performed intraperitoneal injections of insulin (0.1 milliunits/µl in 1× PBS) or equal volume control solution (1× PBS) in conscious mice (1.5 milliunits/kg). 20 min after injection, mice were killed, and livers were rapidly (within 30 s) dissected. Liver pieces were immediately put into liquid nitrogen and then stored at −80 °C for Western blot analysis.

Liver Perfusion Assay—Livers of 10-week-old male B6 or C3H mice were perfused with a 37 °C control (EBSS, E3024, Sigma, + 0.1% BSA) or insulin solution (10 nm insulin, Actrapid, in EBSS + 0.1% BSA) at flow rate of 100 ml/h by inserting a 27-gauge syringe in the portal vein and cutting open the right atrium of the heart. After 1 h, the liver was dissected into pieces that were immediately frozen in liquid nitrogen and stored at −80 °C for Western blot analysis.

Primary Hepatocyte Isolation, Culture, and Stimulation with Insulin—Primary hepatocytes were isolated from CBAXB6 F1 mice, following the protocol described previously (29). Isolated primary hepatocytes were seeded on 12-l or 6-well collagen I-coated plates (BD Biosciences) and cultured in a 37 °C/5% CO2 incubator. Primary hepatocytes were incubated for 4 h in DMEM supplemented with 10% (v/v) FCS, 1× GlutaMAX (Life Technologies, Inc.), and penicillin/streptomycin to promote cell adhesion to the wells, followed by a change of media to serum-free Medium 199 (Gibco, Life Technologies, Inc.) supplemented with 10 nm dexamethasone and penicillin/streptomycin, and incubated overnight. The hepatocytes were then exposed to fresh media with or without 10 nm insulin (Actrapid) and incubated for 5 and 45 min. At the end time point, the hepatocytes were harvested for Western blot analysis. In each biological replicate, the experiment for the 45-min time point was performed in duplicate/triplicate wells.

Western Blot—Liver tissue whole cell lysates containing an equal amount of protein were run on 4–15% polyacrylamide gels. For PKCε translocation experiment, 100 mg of liver tissue was used as starting material, and subcellular fractionation was performed with the subcellular protein fractionation kit for tissues (Pierce). Antibodies used for immunoblotting analysis are listed in Table 1. Semi-quantitative densitometry analysis of signals was performed using ImageJ software (National Institutes of Health). Quantification values were normalized against GAPDH (cytoplasmic fractions and whole liver lysates) or Na/K-ATPase (membrane proteins), and the relative mean protein expression ratio of control samples was arbitrarily set to 1. For PKCε translocation, the membrane (normalized to Na/K-ATPase)/cytosolic (normalized to GAPDH) fraction ratio was calculated (arbitrary units) (11), and the mean ratio of CD samples was arbitrarily set to 1.

qRT-PCR—qRT-PCR was performed as described previously (15), using as template the cDNAs prepared separately from each biological replicate. In all cases, reactions were run in duplicate and TATA-binding protein was used as a reference gene. The comparative threshold cycle method (∆Ct method) was used to determine relative amounts of mRNAs in all samples. For all genes, relative mRNA expression in CD samples was arbitrarily set to 1. Primers used for qRT-PCR are listed in Table 2.

Experimental Procedures

Animals—CBA/J mice were purchased from Scanbur, Denmark. C3H/HeN and C57BL/6J mice (stock number 000656, Jax mice) were purchased from Taconic. Male animals were used in all experiments. Animals were fed standard diet (CRM (E), Special Diet Service) and maintained on a 12:12-h light/dark cycle in a temperature/humidity-controlled (22 °C/50% humidity) room. For the sucrose-rich diet (SRD) regime, 10-week-old CBA/J, C57BL/6J, or C3H/HeN mice were given regular chow and 32% sucrose (Sigma) drinking water. The animals were housed in a temperature/humidity (22 °C/50%) room. For the sucrose-rich diet (SRD) regime, 10-week-old CBA/J, C57BL/6J, or C3H/HeN mice were given regular chow and 32% sucrose (Sigma) drinking water. The animals were housed in a temperature/humidity (22 °C/50%) room. For the sucrose-rich diet (SRD) regime, 10-week-old CBA/J, C57BL/6J, or C3H/HeN mice were given regular chow and 32% sucrose (Sigma) drinking water. The animals were housed in a temperature/humidity (22 °C/50%) room.

Metabolic Parameters—Blood/plasma samples were collected from 6-h fasted CBA, B6, and C3H mice. Blood glucose was measured using glucometer (Ultra 2, One Touch), and plasma insulin was analyzed by an ultrasensitive mouse insulin ELISA kit (Crystal Chem Inc.). Glucose-stimulated insulin secretion (GSIS), glucose tolerance test (GTT), and insulin tolerance test (ITT) was performed as described in (15). Area under the curve was calculated according to the trapezoid rule. The homeostasis model for insulin resistance (HOMA-IR) was calculated from the fasting blood glucose (mmol/liter) × fasting plasma insulin (microunits/liter) divided by 22.5. Oil-red-O staining of liver sections and determination of liver triglycerides were performed as described previously (15).

In nondiabetic humans (14). We previously showed that levels are positively correlated with the development of NAFLD (12, 13). Additionally, it has been reported that high insulin resistance, and dysglycemia in CBA/J mice. We also show that high levels of insulin alone directly enhance Cd36 expression in liver-specific inactivation of Pparγ expression, hepatosteatosis, and insulin resistance were associated (15). The expression of Pparγ, an upstream regulator of Cd36 expression (16, 17), has also been linked to hepatosteatosis. Although hepatic Pparγ expression is relatively low under nonmetabolically stressed conditions (18), hepatic Pparγ expression is increased in obese and diabetic mouse models (19) as well as in human NAFLD (8, 20), and hepatic overexpression of Pparγ provokes fatty liver development in mice (21–25), whereas liver-specific inactivation of Pparγ in ob/ob and AZIP mice reduces hepatosteatosis (26–28). Here, we provide evidence that hyperinsulinemia triggers fatty liver development and insulin resistance by stimulating hepatic expression of Cd36. A 3w sucrose-enriched diet was sufficient to provoke hyperinsulinemia, increased hepatic Pparγ and Cd36 expression, hepatosteatosis, hepatic insulin resistance, and dysglycemia in CBA/J mice. We also show that high levels of insulin alone directly enhance Cd36 expression in perfused livers and isolated hepatocytes and that insulin stimulation of hepatic Cd36 expression is Pparγ-dependent. Our data suggest a direct and sufficient role for hyperinsulinemia in Pparγ-mediated enhancement of hepatic Cd36 expression and thus the subsequent development of hepatosteatosis.

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TABLE 1
Antibodies used in the study

| Antigen     | Species | Supplier                  | Dilution-conditions |
|-------------|---------|---------------------------|---------------------|
| Cd36        | Goat    | R&D Biosystems (AF2519)    | 1:1000 TBST + 5% BSA |
| Phospho-AKT(Ser473) | Rabbit | Cell Signaling (catalog no. 9271) | 1:1000 TBST + 5% BSA |
| GAPDH       | Rabbit  | Cell Signaling (catalog no. 4685) | 1:1000 TBST + 5% BSA |
| PKCε        | Rabbit  | Santa Cruz Biotechnology (C-15) (sc-214) | 1:1000 TBST + 5% BSA |
| Na+/K-ATPase| Rabbit  | Santa Cruz Biotechnology (E-8) (sc-7273) | 1:1000 TBST + 5% BSA |
| PPARγ       | Mouse   | Abcam (catalog no. 3672)    | 1:1000 5% dry milk   |

TABLE 2
Oligomers used for qRT-PCR analysis

Targets in boldface represent human genes. Primers against TBP recognize both human and mouse gene.

| Target          | Forward primer (5′ → 3′)   | Reverse primer (5′ → 3′)               |
|-----------------|----------------------------|---------------------------------------|
| Tbp             | GATTTGACTCCAGACCTTTCTAA   | AGTGCAGTCGTCCTGTCAGTT                |
| Cd36            | TCATTGTGTTCCCCATTCA       | GCCTTTACCAAAAGTGTGCCAGGT             |
| PPARγ2          | TGCTATAGCAGCCTACTAAGAAT  | CCGAGCTGTCGTTGCAGAGAA                |
| PPARγ1          | GCGCTGTCAGAAGTCACGT      | ACCCTCTTTCTTCAAATCTCTGT              |
| Mogat1          | TCTTCACTGGGTGTGATGCC     | CGGCTCTGAGTTGAGGTTC                 |
| Fabp4           | CGACAGCCAGAGAAGGTT       | AGGGCTCCGCCACT                      |
| Fatp2           | GTGCAAGCTGAGGACTCTG       | TCGACGCTCTAGACTGCTG                  |
| Fatps           | ACCACCTGAGCTCAAAACGCC    | AGGACAGCACTTGGCTACT                  |
| Fabps           | TCTTCACTGGGTGTGATGCC     | TCTGACGCTCTAGACTGCTG                  |
| Charbp          | ACTGCGAGCAGACCTAGCTT     | TGTGACGCTCTAGACTGCTG                  |
| Srebpl1         | TGACATATTGAAAGACATGCTC   | AGCATAGAGGGCGGCACAA                   |
| Srebpl1c        | TGACATATTGAAAGACATGCTC   | GCAGGCAATAGGGCGGCACAA                   |
| Fasn            | GGGTTTCTGCCAGCCGGTTA     | AGCAGACAGCCAGCAGCTG                  |
| Ppar            | TATCCAGAGCCTCAGGCT       | GGTGCAGTATCCCGAGGT                   |
| Acc-1           | TGGGATCTCTCCGCTTTACAGG   | AGCCACAGGCTGCTGACGTCTTC             |
| Srd-1           | GGGCTGTTAACGAGATCTAGCTG  | GGGCTGTTAACGAGATCTAGCTGAAATATCCGGAAAG |
| Elovb           | AGGCACCAACATTTGAGCACAA  | TGTCGTCGCTCGACGAGAA                  |
| Cpt-1a          | GCGACCTGCGACCTCAGCTCA   | GTGTAACGACGGAGGCAAGC                  |
| Mttn            | TGCGATAGGTGGACACCTGAG    | TCCGCTGTAGAAGGACCTTTC                |
| Lpk             | GGGAGGAGGTGGAGGTAGGAGG   | GCCAGGCTGCCAGACAA                    |
| Cd36            | AGTGGAGAAGACAGGCTGACATCT| TATGGAAGAAGACAGGCTGACATCT            |
| PPARγ           | AGGGCGAAGGCGCATCTTG      | ATCGTAAAGAGCTGCTGCTGACTGAGC          |

Cell Culture—Human liver hepatocellular carcinoma cell line HepG2 cells (ATCC-HB-8065) were maintained in a humidified incubator at 37 °C, 5% CO₂. HepG2 cells were cultured in DMEM with 1 g/liter glucose (catalog no. 21885-025, Gibco), supplemented with 10% FBS (catalog no. 10500-064, Gibco), 1% nonessential amino acids (catalog no. 11140-035, Gibco), 1% penicillin/streptomycin (catalog no. 15070-063, Gibco), and 0.05% gentamicin (catalog no. 15750-037, Gibco).

siRNA Transfection—siRNA oligomers were purchased from Sigma. PPARγ siRNA sequence was obtained from Ref. 21. This siRNA recognizes a sequence that is conserved within all PPARγ isoforms in both mice and humans. MISSION siRNA universal negative control 1 (Sigma) was used as control siRNA. HepG2 cells and primary hepatocytes were transfected using Lipofectamine RNAiMax (Life Technologies, Inc.) according to the reverse transcription protocol. HepG2 cells were transfected with control and PPARγ siRNA (final concentration 10 nm) and were seeded in 24-well plates (50,000 cells/well). Primary hepatocytes were transfected with control and PPARγ siRNA (final concentration 10 nm) and were seeded in 12-well collagen-coated plates (100,000 cells/well). 24 h after transfection, cell culture medium was replaced with either control medium or medium containing 100 μM insulin, and cells were incubated for 2 h (HepG2 cells) or 100 min (primary hepatocytes). 30 h after transfection, HepG2 cells were incubated for 2 h (HepG2 cells) or 100 min (primary hepatocytes). After this incubation period, cells were harvested for RNA preparation.

Statistical Analysis—All data are presented as mean ± S.E. In all experiments, n refers to the number of biological replicates. Data were analyzed and graphs were generated using Microsoft Excel and GraphPad Prism 6. A two-tailed Student’s t test was used for all analyses between the two groups or the two treatments, and the following p values were considered to be statistically significant: *, p < 0.05; **, p < 0.01; ***, p < 0.001.

Results

CBA/J, but Not C57BL6/J, Mice on a Sucrose-rich Diet Become Hyperinsulinemic and Glucose-intolerant—To investigate a potential correlation between hyperinsulinemia and development of hepatosteatosis, we established an in vivo model for early onset hyperinsulinemia by exposing CBA/J (CBA), i.e. high insulin secreting, mice to a standard diet (CD) or standard diet plus 32% sucrose in the drinking water (sucrose-rich diet (SRD)) during a 3w period. Under these regimes, fasted insulin levels were significantly increased in 3w SRD CBA as compared with 3w CD CBA mice (Fig. 1A), whereas fasted insulin levels were overall lower in B6 mice and did not increase on a sucrose-rich diet, not even at 3w (Fig. 1A). Thus, a 3w SRD provokes early onset hyperinsulinemia in CBA but not B6 mice. In both CBA and B6 mice on SRD, the fastest blood glucose levels were slightly increased during the 3w period (Fig. 1B).
SRD B6 mice showed a modest increase in body weight at 2 and 3w as compared with age-matched CD B6 mice, although there was no difference in body weight between the CD and SRD group of CBA mice (Fig. 1C). Nonetheless, total body fat content was increased already after 1 week in SRD CBA mice compared with CD CBA mice (Fig. 1D). In contrast, total body fat content was generally lower in B6 mice and did not increase in response to SRD (Fig. 1D). GTT confirmed the hyperinsulinemic phenotype of 3w SRD CBA mice; thus, in addition to fasting hyperinsulinemia, GSIS was significantly increased at 30 min following glucose injection (Fig. 1E). Consistent with the development of hyperinsulinemia and enhanced GSIS, CBA mice on 3w of SRD were glucose-intolerant (Fig. 1F). In contrast, 3w SRD B6 mice did not show enhanced GSIS or glucose intolerance during a GTT (Fig. 1, E and F). Taken together, these data show that, regardless of diet, nonstimulated, nonstimulated and stimulated insulin levels were markedly higher in CBA mice than in B6 mice. In addition, 3w of SRD provoked hyperinsulinemia, glucose intolerance, and increased body fat content in CBA mice. In contrast, B6 mice have lower basal levels of insulin compared with CBA mice and did not develop hyperinsulinemia nor glucose intolerance on increased body fat mass, in response to the 3w of SRD.

CBA Mice on SRD Develop Hepatosteatosis and Hepatic Insulin Resistance—The hyperinsulinemia in 3w SRD CBA mice was paralleled by hepatic lipid accumulation as evidenced by increased accumulation of fat droplets and increased TG levels (Fig. 2, A and B), providing evidence of hepatosteatosis. In contrast, hepatic TG content was not increased in B6 mice following 3w of SRD (Fig. 2, A and B). Notably, the TG content of CD B6 livers were considerably lower than that of CD CBA mice, thus paralleling the overall lower basal insulin levels of B6 mice.
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compared with CBA mice. Hepatic fat accumulation is frequently associated with hepatic insulin resistance (30), and the combined observation of hepatic steatosis, hyperinsulinemia, and glucose intolerance displayed by 3w SRD CBA mice suggested that they were insulin-resistant. ITT and homeostasis model assessment of insulin resistance (HOMA-IR) confirmed that 3w SRD CBA mice were insulin-resistant (Fig. 2, C and D).

Insulin stimulation of AKT phosphorylation (Ser\textsuperscript{473}) was markedly reduced (by \textasciitilde50%) in livers of 3w SRD CBA as compared with 3w CD CBA mice (Fig. 2E), providing molecular evidence that the SRD provoked hepatic insulin resistance in these mice. During fatty liver development, pathological accumulation of diacylglycerol stimulates the activation of PKC\varepsilon by enhancing its translocation to the plasma membrane, thereby impairing canonical insulin signaling that in turn leads to hepatic insulin resistance (11). We therefore next examined the subcellular localization of PKC\varepsilon in livers of 3w SRD CBA mice and observed a 2-fold increase in the membrane/cytosolic PKC\varepsilon ratio as compared with 3w CD CBA mice (Fig. 2F). Thus, 3w of SRD not only provoked hyperinsulinemia and hepatic steatosis in CBA mice but also resulted in development of hepatic insulin resistance, as illustrated by the reduced in vivo response to exogenous insulin during an ITT, increased HOMA-IR, reduced insulin-stimulated hepatic AKT phosphorylation, and increased hepatic PKC\varepsilon membrane translocation. Taken together, these data link hyperinsulinemia, hepatic fat accumulation, and hepatic insulin resistance and suggest that increased insulin levels promote hepatic fat accumulation and thus hepatic insulin resistance.

Insulin Enhances Hepatic Cd36 Expression—To elucidate the molecular mechanisms underlying increased hepatic fat content in CBA mice on SRD, we assessed the expression of key components of FA uptake, FA trafficking, FA synthesis, and FA oxidative pathways. mRNA and protein levels of the FA transporter Cd36 were markedly increased in livers of CBA mice at 3w of SRD (Fig. 3, A and B), as was the plasma membrane localization of Cd36 (Fig. 3C), thus coinciding with the increase in insulin levels. In contrast, hepatic mRNA levels of the FA transporters Fatp2 and Fatp5 and the FA-binding protein Fabp5 were largely unchanged in CBA mice at 3w of SRD (Fig. 3D). Expression of the key lipogenic transcription factors Srebp-1, Srebp-1c, and ChREBP, which regulate FA biosynthesis, was also unchanged as was the expression of the key enzymes for lipogenesis as follows: Fasn and Acc1, FA desaturation; Scd1, and FA elongation; Elovl6 (Fig. 3E). Moreover, the expression of the FA \(\beta\)-oxidation enzyme, Cpt1a (Fig. 3F), the rate-limiting enzyme of very low density lipoprotein (VLDL) secretion, Mttp (Fig. 3G), and the ChREBP target rate-limiting enzyme of glycolysis, Lpk (Fig. 3H), was unaltered in livers of CBA mice during the 3-week period of SRD. Thus, although we cannot exclude a role for de novo lipogenesis, reduced FA oxidation, or decreased VLDL secretion as contributors to the increased tri-glyceride content observed in CBA mice following 3w of SRD, our data imply a role for enhanced Cd36 expression in the development of hepatic steatosis in these mice.

Cd36 mRNA expression was not increased in livers of B6 mice at 3w of SRD (Fig. 4A), raising the notion that the elevated levels of insulin observed in 3w SRD CBA, but not B6 mice, increase hepatic Cd36 expression and thus likely hepatic FA uptake. If so, exposure of B6 livers to high insulin levels would result in enhanced Cd36 expression. To test this idea, we perfused livers of CD-fed B6 mice with 10 nM insulin. Perfusion of B6 livers with insulin not only resulted in robust Ser\textsuperscript{473} AKT phosphorylation (Fig. 4B), demonstrating that B6 livers were insulin responsive, but also in increased levels of Cd36 (Fig. 4B). Similarly, exposure of isolated primary mouse hepatocytes to 10 nM insulin also resulted in distinct Ser\textsuperscript{473} AKT phosphorylation and enhanced Cd36 levels (Fig. 4C). These results show that hyperinsulinemia not only correlates with enhanced hepatic Cd36 expression in vivo but that high levels of insulin are sufficient to directly increase hepatic Cd36 expression both in liver perfusion experiments and in cultured primary hepatocytes. Together, these findings provide evidence that hyperinsulinemia, by stimulating hepatic Cd36 expression, promotes hepatosteatosis in vivo.

Insulin-mediated Stimulation of Hepatic Cd36 Expression Is Ppar\textsuperscript{γ}-dependent—Several transcription factors, including Ppar\gamma, PXR, LXR, and KLF2, have been implicated in the regulation of Cd36 expression (16, 17, 31). Among these, the expression of the Ppar\gamma2 isoform is not only up-regulated under hepatosteatosis conditions (18, 19) but also stimulated by insulin in primary hepatocytes (32). Consistent with these findings and in agreement with the increased expression of Cd36, hepatic Ppar\gamma2 expression was up-regulated more than 2-fold in CBA, but not B6, mice on 3w SRD (compare Fig. 5, A and E), suggesting that hyperinsulinemia enhanced hepatic Ppar\gamma2 expression in 3w SRD CBA mice. There was also a nonsignificant tendency of increased hepatic Ppar\gamma1 expression in 3w SRD CBA mice as compared with 3w CD CBA mice (Fig. 5B). Alike Cd36, the expression of other Ppar target genes, such as the intracellular FA chaperone fatty acid-binding protein 4 (Fabp4) and monoacylglycerol acetyltransferase (Mogat1), which enhances hepatic fat accumulation by stimulating incorporation of FAs into TG via a FA biosynthesis-independent pathway (33, 34), was also increased in livers of CBA mice at 3w of SRD (Fig. 5, C and D), whereas hepatic Mogat1 and Fabp4 expression was unaltered in 3w SRD B6 mice (Fig. 5E). To directly investigate the role for Ppar\gamma in stimulation of hepatic Cd36 expression, we used siRNA to knock down Ppar\gamma in HepG2 cells and primary mouse hepatocytes. HepG2 cells and primary mouse hepatocytes in which Ppar\gamma expression had been knocked down failed to enhance Cd36 expression in response to insulin (Fig. 5, F and G). Taken together, these results provide evidence that insulin enhancement of hepatic Cd36 expression is Ppar\gamma-dependent.

In agreement with the notion that insulin enhancement of hepatic Cd36 expression is Ppar\gamma-dependent, exposure of C3H/HeN (C3H) mice, in which hepatic Ppar\gamma expression is deficient (Fig. 6A) (21), to the 3w SRD regime did not provoke an increase in hepatic Cd36 mRNA or protein levels (Fig. 6, B and C). Similarly, hepatic mRNA levels of the Mogat1 and Fabp4 were not increased in C3H mice after 3w of SRD (Fig. 6B). Moreover, neither PKC\varepsilon membrane translocation nor hepatic TG levels was increased in livers of 3w SRD C3H mice, providing evidence that these mice are protected against developing hepatosteatosis and liver insulin resistance under an SRD regime (Fig. 6, D and E). Although the normal glucose and insu-
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lin levels observed in 3w SRD C3H mice (Fig. 6, F and G) support the notion that these mice do not develop insulin resistance on an SRD, the normoinsulinemic levels preclude a direct assessment of the role for Ppar in insulin-stimulated enhancement of hepatic Cd36 expression in C3H mice. Thus, we next perfused livers of C3H mice with insulin. Although insulin potently stimulated Ser473 AKT phosphorylation in insulin-perfused C3H livers (Fig. 6H), it failed to increase Cd36 protein and mRNA expression (Fig. 6, H and I).

Discussion

Hyperinsulinemia is widely considered a consequence of insulin resistance where β-cells, as a compensatory response to the insulin-resistant state, produce and secrete increased levels of insulin, resulting in elevated basal levels of insulin but normoglycemia. Accumulating evidence suggests, however, that hyperinsulinemia and insulin resistance might be partly disconnected and that hyperinsulinemia per se is an independent risk factor for the development of perturbed glucose tolerance, T2D and NAFLD (13, 14, 35). Thus, it has been proposed that hyperinsulinemia is not only a consequence of insulin resistance but may also act as an independent driver that triggers, and sustains, insulin resistance and dysglycemia (12). Here, we present in vivo and ex vivo data providing evidence that increased circulating levels of insulin trigger fatty liver development and insulin resistance by stimulating hepatic expression of the FA transporter Cd36 in a Ppar-dependent manner. Our study shows that a 3-week SRD is sufficient to provoke hyperinsulinemia, hepatic steatosis, hepatic insulin resistance, and dysglycemia in CBA mice. The increase in insulin levels and hepatic fat accumulation in CBA mice was paralleled by an increase in the hepatic expression of Ppar and the Ppar target genes Cd36, Fabp4, and Mogat1, i.e., genes involved in FA uptake and TG synthesis. B6 mice, which are considered to be obesity- and
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![Representative Western blot of phosphorylated AKT and Cd36 protein expression in total protein lysates from vehicle and insulin perfused B6 livers. Quantification of Cd36 expression in B6 mouse liver lysates isolated from B6 mice after 3 weeks on CD (n = 6) and SRD (n = 6).](image)

**Figure 4. Insulin enhances Cd36 expression in perfused livers and primary hepatocytes.** A, real time PCR analyses of hepatic expression of Cd36 in liver lysates isolated from B6 mice after 3 weeks on CD (n = 6) and SRD (n = 6). B, representative Western blot of phosphorylated AKT and Cd36 protein expression in total protein lysates from vehicle and insulin perfused B6 livers. Quantification of Cd36 expression in B6 mouse liver lysates isolated from B6 mice after 3 weeks on CD (n = 12) and insulin (n = 9) perfused livers is shown in the diagram. C, representative Western blot of phosphorylated AKT and Cd36 expression in vehicle and insulin cultured primary hepatocytes. Quantification of Cd36 expression assessed by Western blot densitometry is shown in the diagram (n = 5). Data are presented as mean ± SE, where *p < 0.05.

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Insulin was shown to enhance Cd36 levels in skeletal muscle and cardiac muscle; under a short term hyperinsulinemic-euglycemic clamp, insulin stimulated Cd36 levels in skeletal muscle, and the increase in Cd36 levels were positively correlated to insulin resistance (44). Insulin has also been shown to directly enhance the expression of Cd36 in isolated rat cardiac myocytes and in perfused intact hearts (45). In agreement with these findings and in support for a potential general role for insulin in stimulating Cd36 expression, we here show that insulin directly enhances Cd36 expression in isolated primary hepatocytes and in a liver perfusion assay. Our data disagree however with the results reported by Aspichueta and co-workers (6) who did not observe increased Cd36 expression in insulin-exposed primary hepatocytes. The use of different concentrations of insulin and different experimental protocols and/or different methodologies used in the two studies may explain this discrepancy.

PPary, an upstream positive regulator of Cd36 expression (16, 17), is also linked to hepatic steatosis. PPary, and in particular PPary2, overexpression promotes hepatic fat accumulation (21–24) in an Srebp-1c independent way (25), whereas a liver-specific deletion of PPary attenuates hepatic steatosis, highlighting the important role for PPary in fatty liver development (26–28). In agreement with these observations, and in contrast to B6 mice that express PPary in the liver, insulin perfusion of C3H livers did not provoke an increase in Cd36 expression. However, we can not exclude that additional strain differences, *i.e.*, apart from hepatic PPary expression, between B6 and C3H mice contribute to the difference in stimulation of Cd36 expression following liver insulin perfusion. Nonetheless, the failure of insulin to increase Cd36 expression in primary hepatocytes and HepG2 cells, in which PPary had been knocked down, provides direct evidence for a role for PPary in mediating the insulin stimulatory effect on Cd36 expression. The increased expression of PPary2 and its target genes in the livers of obese mouse models and humans with NAFLD (8, 20, 46, 47), *i.e.*, under conditions of insulin resistance, might appear contradictory. However, PPary expression is in part regulated through a positive feedback loop, and additionally, PPary activity is enhanced by ligands, including natural ligands such as fatty acids (48, 49). Thus, our findings together with the observation of increased hepatic PPary2 expression in obese models and NAFLD provide evidence that insulin is sufficient to induce, but is not required for maintenance of, PPary2 expression. Additionally, PPary-mediated enhancement of Cd36 expression is increased and positively correlates with plasma insulin levels, insulin resistance, and the degree of steatosis in NAFLD patients (8, 9). Although Cd36 is not critically required for hepatic FFA uptake in humans (41), overexpression of Cd36 in human hepatoma cell lines as well as forced expression of Cd36 in isolated mouse hepatocytes resulted in increased FFA uptake (17, 42). In addition, forced expression of Cd36 in livers of normal fed, non-metabolically challenged mice caused hepatic steatosis (10), and an age-related increase in hepatic Cd36 expression is associated with increased susceptibility to NAFLD (43). Interestingly, insulin has been shown to enhance Cd36 levels in skeletal muscle; under a short term hyperinsulinemic-euglycemic clamp, insulin stimulated Cd36 levels in skeletal muscle, and the increase in Cd36 levels were positively correlated to insulin resistance (44). Insulin has also been shown to directly enhance the expression of Cd36 in isolated rat cardiac myocytes and in perfused intact hearts (45). In agreement with these findings and in support of a potential general role for insulin in stimulating Cd36 expression, we here show that insulin directly enhances Cd36 expression in isolated primary hepatocytes and in a liver perfusion assay. Our data disagree however with the results reported by Aspichueta and co-workers (6) who did not observe increased Cd36 expression in insulin-exposed primary hepatocytes. The use of different concentrations of insulin and different experimental protocols and/or different methodologies used in the two studies may explain this discrepancy.

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expression would increase hepatic uptake of fatty acids, thus providing increased availability of Ppar ligands that likely further enhance Ppar activity.

Insulin is known to also stimulate hepatic Srebp-1c expression, as well as Srebp-1c activation, thus promoting de novo lipogenesis in the liver that in turn likely contributes to fatty
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In summary, we show that increased intake of sucrose in CBA mice results in rapid development of hyperinsulinemia, hepatosteatosis, and insulin resistance and that insulin enhances hepatic expression of the FA transporter Cd36 in a Pparγ-dependent manner. Thus, our data suggest that diet-induced hyperinsulinemia is an early and potent inducer of hepatosteatosis, insulin resistance, and dysglycemia that may predispose individuals to T2D and NAFLD. Notably, a relatively recent prospective study involving a 24-year follow up of 515 individuals identified basal hyperinsulinemia in normoglycemic individuals as an independent risk factor for the development of dysglycemia (35). In conclusion, our observations support the notion of hyperinsulinemia as an initiator and driver of insulin resistance and dysglycemia. Additionally, our findings reinforce the view that determination of basal insulin levels may be clinically relevant for diagnosing early dysglycemia. Monitoring of insulin levels combined with therapeutic intervention and life style changes aimed at restoring normoinsulinemia in insulin-resistant individuals would likely help to impede a further deterioration of insulin sensitivity and glucose homeostasis and thus the development of overt T2D and NAFLD.

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