Increased Hepatic CD36 Expression Contributes to Dyslipidemia Associated with Diet-Induced Obesity

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Running Title: The Role of Hepatic CD36 in Dyslipidemia

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ABSTRACT:

Objective: The etiology of type 2 diabetes (T2D) often involves diet-induced obesity (DIO), which is associated with elevated plasma fatty acids (FA) and lipoprotein associated triglycerides (TG). Since aberrant hepatic FA uptake may contribute to this, we investigated whether increased expression of a FA transport protein (CD36) in the liver during DIO contributes to the dyslipidemia that precedes development of T2D.

Research design and methods: We determined the effect DIO has on hepatic CD36 protein expression as well as the functional consequence of this in terms of hepatic TG storage and secretion. In addition, in vivo adenoviral gene delivery of CD36 to the livers of lean mice was performed to determine if increased hepatic CD36 protein was sufficient to alter hepatic FA uptake, TG storage and secretion.

Results: CD36 protein levels in the liver are significantly elevated during DIO, which is correlated with increased hepatic TG storage and secretion. These alterations in liver lipid storage and secretion were also observed upon forced expression of hepatic CD36 in the absence of DIO and were accompanied with a marked rise in hepatic FA uptake in vivo, demonstrating that increased CD36 expression is sufficient to recapitulate the aberrant liver lipid handling observed in DIO.

Conclusions: Increased expression of hepatic CD36 protein in response to DIO is sufficient to exacerbate hepatic TG storage and secretion. As these CD36-mediated effects contribute to the dyslipidemia that often precedes the development of T2D, increased hepatic CD36 expression likely plays a causative role in the pathogenesis of T2D.
Type 2 diabetes (T2D) is currently a global epidemic and the number of individuals affected by this disease continues to rise (1). Much of this increase in the number of T2D individuals is correlated with the growing number of overweight or obese individuals (1). Although T2D has been correlated with obesity, the disease is multi-factorial and includes genetics factors, environmental factors, as well as a shift towards a more sedentary lifestyle (1). Ultimately, however, T2D develops as a direct result of insulin resistance (particularly in skeletal muscle and liver (2; 3)) and the eventual decrease in \(\beta\)-cell insulin secretory capacity (3). Although the exact cause of insulin resistance in skeletal muscle and liver is not known, one possible mechanism is increased fatty acid (FA) uptake in both of these organs (2-4). Indeed, elevated levels of plasma FAs induced by diets rich in FAs and the resulting skeletal muscle insulin resistance both contribute to hepatic insulin resistance (5), increased hepatic glucose output (6), hepatic steatosis (4), and increased hepatic triglyceride (TG) secretion in very low-density lipoproteins (VLDL) (7). Such dramatic FA-induced aberrant liver function further contributes to the dyslipidemia associated with the eventual development of T2D (8). While exposure of the liver to elevated levels of plasma FAs may have a number of deleterious effects on liver function, we investigated whether these profound changes in liver function are a result of molecular changes within the hepatocyte that alter hepatic FA uptake.

While previous reports have indicated that a deletion variant of \(Cd36\) may be a quantitative trait loci for insulin resistance in the spontaneously hypertensive rat (SHR) (9; 10), emerging evidence has suggested that increased \(Cd36\) expression actually contributes to insulin resistance in humans with T2D (11; 12) and that inhibition of \(Cd36\) at the level of the pancreatic \(\beta\)-cell and the skeletal muscle may prevent cellular lipotoxicity and subsequent peripheral insulin resistance (12; 13). Although \(Cd36\) is not believed to play a significant role in FA uptake in the liver (14-17), postprandial uptake of FA by the liver is increased in T2D patients indicating the existence of a regulatory mechanism controlling hepatic FA uptake (18). In addition, mouse models of high fat diet-induced steatohepatitis and genetic models of obesity have shown that \(Cd36\) transcript levels increase in the liver (19-21), suggesting that \(Cd36\) protein levels may also be increased. However, whether this translates into increased \(Cd36\) protein expression and the effects this may have on hepatic FA transport has not been explored.

RESULTS AND DISCUSSION:

To investigate if DIO, a risk factor for the development of T2D, can regulate hepatic \(Cd36\) expression, C57BL6 mice were fed a standard low fat chow diet (LF; 10% kcal from lard) or a high-fat diet (HF; 60% kcal from lard) for a period of 5 weeks. In accordance with the well-documented model of high fat feeding (22), mice fed a HF diet rapidly gained weight culminating in a significant increase in body weight in mice fed a HF diet compared to mice fed a LF diet (HF; 10.2 ± 0.9 vs LF; 1.8 ± 0.3 g, \(p<0.05\)). While fasted plasma glucose levels did not differ in mice fed either diet (LF; 6.4 ± 0.3 vs HF; 7.8 ± 0.6 mM, ns), mice fed a HF diet for 5 weeks did display delayed glucose clearance as determined by a glucose tolerance test (Fig. 1a, b). Although hepatic glucose output and insulin measurements were not performed on mice after 5 weeks of LF or HF diet, glucose and insulin tolerance tests indicated that early stages of insulin resistance did not develop in mice until approximately 10 weeks of HF diet feeding (data not shown) indicating that at 5 weeks of HF diet, mice are glucose intolerant. Consistent with dietary FA consumption, fasted plasma non-esterified fatty acid (NEFA) levels were significantly increased in mice fed a HF diet compared to mice fed a LF diet (HF; 6.4 ± 0.3 vs HF; 7.8 ± 0.6 mM, ns), mice fed a HF diet for 5 weeks did display delayed glucose clearance as determined by a glucose tolerance test (Fig. 1a, b). Although hepatic glucose output and insulin measurements were not performed on mice after 5 weeks of LF or HF diet, glucose and insulin tolerance tests indicated that early stages of insulin resistance did not develop in mice until approximately 10 weeks of HF diet feeding (data not shown) indicating that at 5 weeks of HF diet, mice are glucose intolerant. Consistent with dietary FA consumption, fasted plasma non-esterified fatty acid (NEFA) levels were significantly increased in mice fed a HF diet compared to mice fed a LF diet (HF; 2.0 ± 0.08 vs LF; 1.7 ± 0.07 mM, \(p<0.05\)). In addition, \(Cd36\) mRNA levels were significantly increased in livers from mice fed a HF diet compared to mice fed
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a LF diet (Fig. 1c), and when compared to an organ that expresses high levels of CD36 such as heart, hepatic CD36 mRNA levels increased from approximately 10% of what is present in the heart in the low fat group to approximately 28% of what is present in the heart in the high fat group (Fig. 1d). Furthermore, CD36 protein expression (Fig. 1e) was significantly increased in livers from mice fed a HF diet compared to mice fed a LF diet, indicating that DIO, or possibly elevated plasma NEFA levels, were sufficient to induce hepatic CD36 protein expression. Although previous studies have reported increased CD36 transcript levels in the liver of mice fed a HF diet occur via a peroxisome proliferator activated receptor gamma (PPARγ) mediated mechanism (19; 20), our findings demonstrate that this translates into increased CD36 protein levels and that this results in increased CD36 localized to the plasma membrane (data not shown). This finding is consistent with the plasma membrane localization of CD36 in heart and skeletal muscle (for review see (23)). Associated with the 2.6-fold increase in CD36 protein expression was a 1.7-fold increase in hepatic TG storage (Fig. 1f), suggesting a potential role for aberrant CD36 expression in enhanced liver FA uptake during DIO.

To confirm that elevated CD36 expression can increase hepatic FA uptake, primary cultures of mouse hepatocytes were infected with a recombinant adenovirus harboring mouse CD36 cDNA (Ad.CD36) or control adenovirus expressing no transgene (Ad.Null). Immunoblot analysis revealed that levels of total cellular CD36 (Fig. 2a) and plasma membrane associated CD36 (Fig. 2b) were significantly increased in Ad.CD36 infected hepatocytes compared to Ad.Null infected hepatocytes demonstrating that Ad.CD36 transduced cells had increased levels of properly localized CD36. Consistent with the role of CD36 as a fatty acid transport protein in other tissues such as skeletal muscle (24) and adipose tissue (14), FA uptake into Ad.CD36 infected hepatocytes was significantly elevated compared to Ad.Null infected hepatocytes as measured by the fluorescent fatty acid analog, BODIPY c16 (Fig. 2c). In addition, Ad.Null and Ad.CD36 infected hepatocytes were incubated in the presence of [3H]-palmitate for 2 hours, which was the time necessary to detect changes in 3H-TG levels. These primary mouse hepatocytes were used to determine 3H-TG cellular incorporation, 3H-TG secretion as well as 3H-palmitate oxidation. In addition, separate sets of hepatocytes were collected 16 hours after adenoviral infection to determine the levels of TG prior to the onset of the [3H]-palmitate experiments. Prior to treating with [3H]-palmitate, baseline TG levels in the two groups of cells were not different (Ad.Null; 29.2 ± 0.7 vs Ad.CD36; 28.5 ± 1.6 μg/mg protein) indicating that alterations in baseline TG did not influence label distribution. After the 2 hour incubation period, palmitate oxidation was not significantly different between the two groups of cells (Ad.Null; 45.8 ± 1.2 vs Ad.CD36; 49.0 ± 2.8 nmol/mg protein) and therefore an equal fraction of the [3H]-palmitate was lost to oxidation in the Ad.Null and Ad.CD36 infected cells. In contrast, there was a significant increase in 3H-TG cellular incorporation (Fig. 2d) and 3H-TG secretion (Fig. 2e) in Ad.CD36 infected hepatocytes compared to Ad.Null infected hepatocytes (Fig. 2d and e). However, when the amount of 3H-TG secreted (Fig. 2e) was normalized to the amount of 3H-palmitate incorporated into TG (Fig. 2d), there was no significant difference between Ad.Null and Ad.CD36 infected hepatocytes (Fig. 2f). This finding suggests that increased TG secretion was directly related to CD36-mediated FA uptake as opposed to CD36 being involved in channeling intracellular FAs to oxidation, secretory pathways and/or directly modifying the processes involved in hepatic TG secretion.

In order to assess if increased CD36 expression alone is sufficient to produce the liver-specific effects observed with DIO, lean mice maintained on a LF diet were administered a single systemic dose of either Ad.Null or Ad.CD36 via tail vein injection. Seven days after adenoviral administration, the levels of hepatic CD36 mRNA (Fig. 3a) and protein (Fig. 3b) expression were significantly increased in Ad.CD36 injected mice to
approximately the same levels as observed with the HF feeding model (Fig. 1c, e). There were no significant differences in body weight (Ad.Null; 21.3 ± 0.3 vs. Ad.CD36; 21.2 ± 0.4 g, ns) or liver weight/body weight ratio (Ad.Null; 4.5 ± 0.1 vs. Ad.CD36; 4.8 ± 0.1 g, ns), nor were there any significant changes in fasted plasma glucose levels (Ad.Null; 4.6 ± 0.4 vs. Ad.CD36; 5.3 ± 0.3 mM, ns) or tolerance to a glucose load (data not shown) between Ad.Null and Ad.CD36 injected mice, respectively.

Although previous reports have suggested that adenoviral administration can cause liver damage as evidenced by plasma levels of aspartate aminotransferase (ALT) higher than 200 U/l (25), mean plasma ALT levels in Ad.Null or Ad.CD36 injected mice were not different and ranged from 47-123 U/l (not shown), indicating an absence of liver damage. Moreover, and in agreement with low dietary FA intake, NEFA levels were unaltered in the two groups of mice (Ad.Null; 1.2 ± 0.04 vs. Ad.CD36; 1.3 ± 0.08 mM, ns) and were lower than mice fed a HF diet. In addition, single photon emission computed tomography (SPECT) imaging and direct, in vivo, fatty acid uptake measurements in Ad.Null and Ad.CD36 expressing mice using the radio-labeled fatty acid analogue, I-123-[p-iodophenyl]-3-methylpentadecanoic acid (BMIPP), indicates that hepatic fatty acid uptake was significantly elevated in Ad.CD36 expressing mice compared to Ad.Null expressing mice (Fig. 3c; sagittal and axial views (left) and quantified scintigraphs (right)). Consistent with these data, direct measurements of livers extracted from mice 1 hour post-injection of BMIPP also demonstrated a significant increase in FA uptake in Ad.CD36 expressing mice compared to Ad.Null expressing mice (Fig. 3d). However, there was no difference in FA uptake in other tissues that express high levels of CD36 such as heart (Fig. 3e), skeletal muscle (Fig. 3f) and adipose tissue (Fig. 3g), suggesting that peripheral organs/tissues are not changed with respect to FA clearance. Nonetheless, since the kinetics of FA uptake/clearance is very different from tissue uptake/clearance of TG (26), it remains to be determined whether VLDL-TG production or clearance is the major cause of the increased TG accumulation in the livers of Ad.CD36 expressing mice.

Despite normal levels of NEFA in Ad.Null and Ad.CD36 injected mice, hepatic CD36 expression was still able to significantly increase hepatic TG levels (Fig. 4a), which is consistent with the elevated in vivo FA uptake measurements in these mice (Fig. 3c). In addition, plasma TG levels (Fig. 4b) were increased accordingly in Ad.CD36 injected mice, which is consistent with our observations that TG secretion is related to CD36-mediated FA uptake in the isolated mouse hepatocytes (Fig. 2e, f). Although, we cannot rule out the possibility that altered VLDL-TG clearance contribute to the elevated levels of TG in the plasma of mice on a HF diet (27-29), this is unlikely in the Ad.CD36 mice given the liver-specific increase in CD36 in these mice.

In agreement with the hyperlipidemia observed in rodent models of DIO (30; 31) and in humans (32), the elevated plasma TG in Ad.CD36 injected mice appears to be primarily contained within the VLDL fraction (Fig. 4c). Hepatic cholesterol (Fig. 4d) and plasma cholesterol levels (Fig. 4e) were not significantly different in Ad.Null and Ad.CD36 injected mice and the amount of cholesterol in the high-density lipoprotein (HDL) fraction was almost identical (Fig. 4f). Furthermore, plasma cholesteryl ester (Fig. 4g) levels did not differ between Ad.Null and Ad.CD36 injected mice, however, increased expression of hepatic CD36 was able to promote cholesteryl ester formation within the liver (Fig. 4h) thereby emphasizing the secondary effects of CD36-mediated FA uptake on cholesteryl ester formation and VLDL secretion in the mouse liver. Finally, consistent with CD36 having a functional role in hepatic FA uptake resulting in increased hepatic TG storage, Ad.CD36 injected CD36 knockout (KO) mice had significantly elevated levels of hepatic TG compared to Ad.Null injected mice (Fig. 4i) despite equally high plasma TG levels in both groups (Fig. 4j). These data also show that increased CD36 expression in the liver can further increase FA uptake and subsequent
hepatic TG storage even when plasma FA and TG levels already significantly favor maximal hepatic FA uptake. These data also confirm that our method of expressing CD36 produces a protein that is functional in terms of FA transport. Interestingly, plasma TG levels did not increase in CD36 KO mice following Ad.CD36 injection, suggesting that alterations in plasma TG clearance may be more important in the control of plasma TG levels than hepatic VLDL-TG production in the CD36 KO mouse as previously reported (28; 33). However, since plasma TG levels are already extremely high in the CD36 KO mouse (28; 33), the contribution of hepatic VLDL-TG production in the CD36 KO mice infected with Ad.CD36 may be more difficult to detect than the contribution of hepatic VLDL-TG production to plasma TG levels in control mice.

CONCLUSIONS:
Elevated levels of plasma FAs induced by diets rich in FAs contribute to hepatic insulin resistance (5), increased hepatic glucose output (6), hepatic steatosis (4; 34), and increased hepatic TG secretion in VLDL (7). Such dramatic FA-induced aberrant liver function further contributes to the dyslipidemia associated with the eventual development of T2D (8; 35). While exposure of the liver to elevated levels of plasma FAs may have a number of deleterious effects on liver function, we show that increased hepatic CD36 protein expression is a regulatory mechanism that, in addition to what has been shown for FABPpm (36) and FATP5 (37; 38), also exists in the mouse liver to control FA uptake. In addition, we provide evidence that increased hepatic CD36 protein expression in response to diets rich in FAs and/or obesity (Fig. 1e) contributes to aberrant liver FA uptake and subsequent dyslipidemia (Fig. 1f). These findings are entirely consistent with those of Degrace et al. (39) where hepatic CD36 expression has been shown to increase in a mouse model of human hypercholesterolemia. Moreover, we show that elevations in hepatic CD36 protein expression in lean mice directly affect hepatic FA uptake, TG storage, and VLDL-TG secretion and therefore are sufficient to recapitulate the hepatic and plasma FA phenotype observed in DIO. Whether other genes involved in FA handling or lipoprotein assembly are altered in the liver secondary to increased CD36 expression also contribute to aberrant liver FA uptake and subsequent dyslipidemia is currently unknown. Nevertheless, these data show that increased expression of CD36 in the liver occurs in response to diets rich in FAs and that this increases hepatic FA uptake and exacerbates hepatic TG storage and secretion similar to what has been shown with FATP5 (40). While this increase in CD36 expression is likely an early adaptive process that may remove excess plasma FAs to protect extra-hepatic organs from insulin resistance, we propose that increased expression of CD36 in the liver eventually becomes maladaptive by promoting prolonged and more severe hepatic FA uptake and TG storage and exacerbating hepatic TG secretion.

While the extensive changes that occur at the cellular level in the liver as a result of DIO and insulin resistance makes identifying the effects of a single protein extremely difficult, we have been successful in identifying one specific protein that is abnormally expressed in the liver during the development of DIO, and this protein, by itself, is sufficient to cause dyslipidemia and may play a causative role in the pathogenesis of T2D. Based on our finding, we propose that specific inhibition of hepatic CD36 FA transport activity may prove to be a novel strategy to normalize hepatic FA uptake and TG secretion in individuals consuming a HF diet and thus may be useful in the prevention of insulin resistance and the eventual development of T2D and related diseases.

RESEARCH DESIGN AND METHODS:
Mice- 10-12 weeks of age male C57BL6 mice (Charles River Laboratories), and CD36 knockout (KO) male mice (41) were used in this study. All experiments involving mice were performed with the approval of The University of Alberta Animal Policy and Welfare Committee. In some instances, mice were
randomly divided into a LF diet group (D12450B, Research Diets, Inc) and a HF diet group (D12450B, Research Diets, Inc) and given free access to water and food unless otherwise stated.

**Glucose Tolerance Tests-** Following a 5 hour fast, mice were injected intraperitoneally with a 50% glucose solution (2g/kg). Glucose from blood collected from the tail prior to and following glucose injection (at 30, 60 and 90 min) was measured using an ACCU-CHEK Advantage glucometer (Roche Diagnostics).

**Primary Culture of Mouse Hepatocytes-** Primary hepatocytes were isolated by collagenase perfusion and plated as previously described (42). 2-4 h after plating, the cultures were transduced with Ad.CD36 or Ad.Null at a titer of 10 PFU/cell. 16 h post infection, hepatocytes were lysed and equal amounts of protein were subjected to SDS-PAGE and immunoblot analysis. In some instances, cell lysates were homogenized in a Potter-Elvejhem glass homogenizer, frozen in liquid nitrogen and the plasma membrane was isolated as previously described (43; 44) prior to SDS-PAGE and immunoblot analysis.

**Fatty Acid Uptake Experiments-** Stock solutions of 4,4-difluoro-5,7-dimethyl-4-bora-3α,4α-diaza-s-indacene-3-hexadecanoic acid (BODIPY-C16; Molecular Probes) were prepared in dimethyl sulfoxide (DMSO; Sigma). Hepatocytes were cultured on collagen coated microscope coverslips and 16 h post infection, the cells were serum-starved for 3 h and rinsed with 1 x PBS. Hepatocytes were incubated for 3 min in 1 x PBS supplemented with BODIPY-C16 to a final concentration of 100 mM, rinsed three times with ice-cold 1 x PBS and fixed in ice-cold 3.7% paraformaldehyde for 15 min. Coverslips were washed and mounted on microscope slides using the Prolong Antifade Kit from Molecular Probes. The fluorescence intensity of 90-100 cells was quantified using Image J (NIH) software and expressed as arbitrary units. In vivo hepatic FA uptake was determined directly using a gamma counter.

**13H-Palmitate Oxidation and Incorporation into Cellular and Secreted Lipids-** 16 hrs after adenoviral infection, hepatocytes were incubated for 2 h with 0.05 mM [3H] palmitate (5 μCi/ml) bound to BSA in a 3:1 molar ratio. Following treatment, an aliquot of medium was collected and utilized to determine palmitate oxidation essentially as previously described (45). In separate cellular isolations and treatments, the medium was collected, and cells scraped and sonicated in PBS (pH 7.4). Lipids from the medium and the cell lysates were extracted and separated by thin layered chromatography (TLC) as described (46). Additional sets of hepatocytes were collected 16 hrs after adenoviral infection to determine the levels of TG prior to the onset of the [3H]-palmitate experiments.

**Injection of Recombinant Adenoviruses-** A single dose of adenovirus (2.85 x 10^8 plaque forming-units (PFU)) harboring no transgene (Ad.Null) or mouse CD36 (Ad.CD36; a gift from F. de Beer, Lexington, KY, USA) were injected into male C57BL6 mice via the tail-vein. Six days post-injection, mice were fasted (16 h) and anesthetized with metofane. A large blood sample was drawn via cardiac puncture and livers were dissected immediately, frozen in liquid nitrogen, and stored at -80°C until further analysis was done. Plasma ALT levels were measured by the INFINITY ALT kit (ThermoTrace).
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**Determination of Liver and Plasma FFA, Triglycerides, Cholesterol and Cholesteryl Esters** - Crushed liver tissues from mice following a 16 hour fast were homogenized, the phospholipids were digested with phospholipase C (2 hrs, 30°C), lipids extracted and the amount of TG, cholesterol and cholesteryl ester was determined by gas-liquid chromatography as previously described (47). Plasma from mice following a 16 hour fast was collected in the presence of EDTA and immediately stored on ice to inhibit lipase activity without the use of chemical inhibition. An aliquot of plasma was collected and separated into lipoprotein fractions using high performance liquid chromatography (HPLC) with an Amersham Bioscience Superose column attached to a Beckman Systems Gold or Nouveau Gold apparatus. The remainder of the plasma samples were immediately frozen and stored at -80°C. In-line assays for total TG and cholesterol were performed as described (47), and plasma non-esterified fatty acids (NEFA) levels were measured using the NEFA C kit (Wako Chemicals GmbH).

**Liver Membrane Preparation** - Liver tissues were prepared in ice-cold sucrose homogenization buffer as previously described (48). In brief, tissues were homogenized on ice in a 20-sec burst and the homogenate was centrifuged at 1000 g for 20 min at 4°C to remove nuclei and cell debris. The supernatant was centrifuged at 100,000 x g for 30 min at 4°C and the subsequent pellet containing the final membrane fraction was suspended in ice-cold homogenation buffer.

**Immunoblot Analysis** - Equal amounts of protein were subjected to 10 % SDS-PAGE, transferred to nitrocellulose, immunoblotted with affinity-purified rabbit polyclonal antibody to CD36 and goat polyclonal anti-actin (I-19; Santa Cruz Biotechnology) and visualized using the Amersham Pharmacia enhanced chemiluminescence Western blotting detection system.

**Quantitative RT-PCR Analysis** - RNA extraction, and the cyclopholin and CD36-specific quantitative RT-PCR assay of samples was performed using previously described methods (49; 50). Gene expression data are represented as mRNA molecules per ng total RNA, which was not different when expressed as a ratio to the cyclopholin mRNA (data not shown).

**Statistical Analysis** - Data are expressed as mean ± SEM. Comparisons between groups were performed using the unpaired Student's two-tailed t-test or analysis of variance (ANOVA) with a Bonferroni post-hoc test of pairwise comparisons where appropriate. A probability value of <0.05 is considered significant.

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FIGURE LEGENDS:

Figure 1. Increased weight gain resulting from a high fat diet increases CD36 expression and hepatic triglyceride storage in mice. Glucose tolerance test (a) and area under the curve (AUC) of the glucose tolerance test (b) of mice fed a high fat diet (HF; closed circles) or a standard (low fat) diet (LF; open circles) for 5 weeks. Quantitative RT-PCR measurement of CD36 transcript levels in liver (c, d) and heart samples (d) were performed on RNA extracted from mice fed a LF or HF diet. Immunoblot analysis using anti-CD36 and anti-actin antibodies were performed on liver extracts (e) from mice fed a LF or HF diet for 5 weeks. Immunoblots were quantified by densitometry and normalized against actin as a control for protein loading (e). Triglyceride (TG) levels were determined in livers of mice fed a LF or HF diet for 5 weeks (f). Values are the mean ± S.E.M. of n = 4-5 mice in each group. * P<0.05 indicates comparisons performed between LF and HF fed mice.

Figure 2. Effects of increased CD36 expression on fatty acid uptake and triglyceride secretion in freshly isolated primary cultures of mouse hepatocytes. Extracts from mouse hepatocytes transduced with an empty adenovirus (Ad.Null) or with an adenovirus expressing CD36 (Ad.CD36) were subjected to immunoblot analysis using anti-CD36 antibody and anti-actin antibodies (a). Plasma membranes isolated from Ad.Null and Ad.CD36 transduced hepatocytes were also assessed using the same antibodies (b). Fatty acid transport into Ad.Null or Ad.CD36 transduced hepatocytes was determined by the uptake of the fatty acid analogue, BODIPY C16 (c; left panel, representative fluorescence image; quantified fluorescence intensity expressed as arbitrary units (AU), right panel) and by determining the amount of $^3$H-palmitate incorporated into intracellular TG (d). $^3$H-palmitate secreted as TG was also measured in Ad.Null or Ad.CD36 transduced hepatocytes (e) and normalized to the amount of $^3$H-palmitate incorporated into TG (f). Values are the mean ± S.E.M. of n = 3-4 independent hepatocyte isolations. * P<0.05 indicates comparisons performed within Ad.Null or Ad.CD36 infected hepatocytes.

Figure 3. Forced expression of CD36 in the liver via adenoviral gene delivery increases hepatic CD36 protein levels without altering plasma glucose or fatty acid levels and increases hepatic fatty acid uptake in mice fed a standard chow diet. 7-days following systemic injection of Ad.Null or Ad.CD36 into mice, liver homogenates were subjected to quantitative RT-PCR (a) and immunoblot analysis using anti-CD36 antibody and anti-actin antibodies (b). Immunoblots were quantified by densitometry and normalized against actin as a control for protein loading (b). A separate group of adenovirus treated mice were used to determine in vivo hepatic fatty acid uptake using $^1$-123 15 -(p-iodophenyl)-3-methyl pentadecanoic acid (BMIPP). Representative SPECT/CT dual-modality images of mice from Ad.Null and Ad.CD36 injected mice are shown in the sagittal view c; left side of image) and images of the livers from these mice in the axial views (c, right side of images). Quantification of hepatic scintigraphy is shown in µCi/cc (c, graph). Liver (d), heart (e), skeletal muscle (f) and adipose tissue (g) collected from sacrificed mice 1 hour post-BMIPP injection were quantified for the level of $^1$-123 incorporation using a gamma counter and expressed as µCi/ gram wet weight (gww). Values are the mean ± S.E.M. of n = 4-5 mice in each group. * P<0.05 indicates comparisons performed between Ad.Null and Ad.CD36 infected mice.

Figure 4. Forced expression of hepatic CD36 increases hepatic triglyceride storage, plasma triglyceride levels, and hepatic cholesteryl esters in mice fed a standard chow diet and exacerbates hepatic triglyceride storage in CD36 knockout mice. 7-days following systemic injection of Ad.Null or Ad.CD36 into mice, hepatic (a) and plasma (b) triglyceride (TG) levels were quantified and the fraction of TG contained within the VLDL and LDL fraction was determined (c).
Hepatic (d), plasma (e) cholesterol levels, the amount of cholesterol in the VLDL, LDL and HDL fraction (f) as well as plasma (g) and hepatic (h) cholesterol esters levels were determined in mice injected with Ad.Null or Ad.CD36. 7-days following systemic injection of Ad.Null or Ad.CD36 into CD36 knockout (KO) mice, hepatic TG storage was increased in Ad.CD36 injected CD36 KO mice (i) despite equally high plasma TG levels in both groups (j). Values are the mean ± S.E.M. of n = 5 mice in each group. * P<0.05 indicates comparisons performed between Ad.Null and Ad.CD36 infected mice.
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Figure 1

(a) Blood Glucose (mmol/L) over time (min) for LF and HF groups.

(b) Glucose Tolerance Test (mg/dL) for LF and HF groups.

(c) CD36 mRNA Expression (CD36/Gapdh ratio) for LF and HF groups.

(d) CD36 mRNA Expression for Liver and Heart tissues in LF and HF groups.

(e) Western Blot for CD36 and Actin in LF and HF groups.

(f) Hepatic TG (μg/mg protein) for LF and HF groups.
Figure 2

(a) CD36 Protein Expression (Actin:CD36 ratio)

(b) CD36 Plasma Membrane Protein Expression (CD36:Actin ratio)

(c) Fluorescence Intensity

(d) \[^{3}H\text{TG Synthesis (mmol/g) \times 1000}\]

(e) \[^{3}H\text{TG Secretion (mmol/g) \times 1000}\]

(f) \[^{3}H\text{TG Secretion (% of Total \[^{3}H\text{TG}\]}\]
Figure 3

(a) CD36 mRNA expression (CD36/total mRNA ratio) for Ad.Null and Ad.CD36.

(b) CD36 protein expression (CD36/Actin ratio) for Ad.Null and Ad.CD36.

(c) Image depicting hepatic FA uptake (μCl/100 g liver) for Ad.Null and Ad.CD36.

(d) FA uptake in liver for Ad.Null and Ad.CD36.

(e) FA uptake in heart for Ad.Null and Ad.CD36.

(f) FA uptake in skeletal muscle for Ad.Null and Ad.CD36.

(g) FA uptake in adipose tissue for Ad.Null and Ad.CD36.