VASP increases hepatic fatty acid oxidation by activating AMPK in mice

Short title: A Role of VASP on hepatic fatty acid oxidation

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Abstract

Activation of AMP-activated protein kinase (AMPK) signaling reduces hepatic steatosis and hepatic insulin resistance, however, its regulatory mechanisms are not fully understood. In this study, we sought to determine whether Vasodilator-stimulated phosphoprotein (VASP) signaling improves lipid metabolism in the liver and if so, whether VASP’s effects are mediated by AMPK.

We show that disruption of VASP results in significant hepatic steatosis as a result of significant impairment of fatty acid oxidation, VLDL-triglyceride secretion and AMPK signaling. Overexpression of VASP in hepatocytes increased AMPK phosphorylation, fatty acid oxidation, and reduced hepatocyte triglyceride (TG) accumulation, however, these responses were suppressed in the presence of an AMPK inhibitor. Restoration of AMPK phosphorylation by administration of 5-aminimidazole-4-carboxamide riboside (AICAR) in Vasp−/− mice reduced hepatic steatosis and normalized fatty acid oxidation and VLDL-TG secretion. Activation of VASP by the phosphodiesterase-5 (PDE5) inhibitor, sildenafil, in db/db mice reduced hepatic steatosis and increased phospho-AMPK and phospho-acetyl CoA carboxylase (ACC). In Vasp−/− mice, however, sildenafil treatment did not increase p-AMPK or reduce hepatic TG content.

These studies identify a role of VASP to enhance hepatic fatty acid oxidation by activating AMPK and to promote VLDL-TG secretion from the liver.
Introduction

Obesity is often associated with hepatic steatosis in a large proportion of obese patients, which leads to nonalcoholic fatty liver disease (NAFLD) and in some cases, to nonalcoholic steatohepatitis (NASH) (1, 2, 3). Hepatic lipid accumulation results from an imbalance between lipid availability (from circulating lipid uptake or de novo lipogenesis) and lipid disposal (via fatty acid oxidation or triglyceride-rich lipoprotein secretion).

In the liver, most fatty acids are metabolized through β-oxidation, which occurs mainly in mitochondria, but also in peroxisomes (2). During fasting, hepatic fatty acid oxidation and secretion of VLDL-TG are enhanced to respond to energy demand, while in the fed state, these responses are suppressed by insulin. Fasting increases free fatty acid (FFA) oxidation, by inducing the phosphorylation of AMPK, a critical energy sensor, which inactivates ACC and activates carnitine palmitoyltransferase 1a (CPT1a) (4), all important steps in the transport of long-chain fatty acids to the mitochondria. Peroxisome proliferator-activated receptor α (PPARα) participates in the regulation of fatty acid oxidation through altered transcription of several key enzymes such as acyl-CoA oxidase 1 (Acox1), uncoupling proteins 2 (UCP2), nuclear respiratory factor (NRF), and mitochondrial transcription factor (Tfam) (2).
Microsomal triglyceride transfer protein (MTP), an endoplasmic reticulum resident protein that acts as both a lipid transfer protein (5) and as a facilitator of apoB folding and translocation is a critical factor for hepatic VLDL production (6, 7). Recently, insulin-dependent suppression of MTP was shown to require forkhead transcription factor 1 (FoxO1) (8), yet molecular mechanisms regulating fatty acid oxidation and VLDL-TG secretion are not fully understood.

VASP belongs to the ENA/VASP family of adaptor proteins linking the cytoskeletal systems to signal transduction pathways and functions in cytoskeletal organization, fibroblast migration, platelet activation and axon guidance (9, 10). We have shown previously that endothelial nitric oxide (NO)/cGMP/VASP signaling attenuates high-fat (HF) mediated insulin resistance and inflammatory activation in hepatic tissue (11), whereas the absence of VASP increases NF-κB signaling in the liver, impairs insulin signaling and increases hepatic TG content (11). Hepatic inflammation and insulin resistance are commonly associated with an elevated hepatic triglyceride content. NO/cGMP is implicated with mitochondrial biogenesis (12), which influences fatty acid oxidation capacity (13). Therefore, in this study we sought to investigate whether VASP, a downstream target of NO/cGMP, reduces hepatic TG levels by enhancing fatty acid oxidation in the liver.
Research Design and Methods-

Animal Experiments

VASP+/G mice on a C57BL/6 background have been described previously (14). C57BL/6 wild-type (WT) mice were purchased from Jackson Laboratories. VASP+/G mice were crossed to obtain VASP+/+ and WT littermate control mice. Male db/db and lean controls, db/+m mice were purchased from Jackson Laboratories. To evaluate VLDL secretion, Triton WR 1339 (500 mg/kg) was injected (ip) after a 16 h fast and blood was sampled from the tail vein. AICAR (200 mg/kg) was injected (sc) daily for 5 days. Twelve week old db/db and db/+m (control) mice received daily oral administration of either vehicle or the PDE-5 inhibitor, sildenafil (30 mg/kg/day) for 4 weeks. For the obesity-induced hepatic steatosis study, WT and VASP+/+ mice were maintained on a HF (60 % saturated fat, D12450B; Research Diet, New Brunswick, NJ) diet for 8 weeks, and for the last 2 weeks of the diet, study mice received 30 mg/kg/day oral sildenafil or vehicle. In all experiments, male, age-matched groups were maintained in a temperature-controlled facility with a 12-hour light-dark cycle. All procedures were approved by the University of Washington Institutional Animal Care and Use Committee.

Quantitative RT-PCR Analyses

RNA was extracted using RNAase kit (Quiagen; Valencia, CA). For gene expression analysis, real-time RT-PCR reactions were conducted as described previously (15) using
TaqMan Gene Expression Analysis (Applied Biosystems; Foster City, CA).

**Western Blotting**

Cell lysis and tissue extraction were performed as described previously (16). All Western blots used equal amounts of total protein for each condition from individual experiments, and were performed as described previously (17).

**Materials**

Anti-VASP, anti-phosphorylated VASP (Ser239), anti-phosphorylated AMPKα (Thr172), anti-AMPKα, anti-phosphorylated ACC (Ser79), and anti-ACC were obtained from Cell Signaling (Denvers, MA). Anti-GAPDH rabbit polyclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Triton WR 1339 and STO-609 was purchased from Sigma (St Louis, MO). AICAR and compound c were purchased from Calbiochem (Darmstadt, Germany). Sildenafil was purchased from Pfizer (New York, NY). Oleic acid was purchased from NU-CHEK PREP (Elysian, MN).

**Measurement of metabolic parameter and hepatic triglyceride content**

Plasma insulin was measured by a mouse insulin ELISA kit (Crystal Chem, Downers Grove, IL). Plasma triglyceride and free fatty acid were measured enzymatically (L-Type TG M, Wako and NEFA-HR, Wako, respectively; Richmond, VA). Alanine aminotransferase (ALT) and serum albumin were measured using an autoanalyzer.
through the Nutrition Obesity Research Center (NORC) at the University of Washington. Determinations of body lean and fat mass were made in conscious mice using quantitative magnetic resonance (EchoMRI 3-in-1 body composition analyzer; Echo Medical Systems, Houston, TX) (NORC). Hepatic triglyceride content was enzymatically measured (L-Type TG M, Wako, Richmond, VA) in liver lysates as described previously (18).

**Cell culture**

AML12 hepatocytes were purchased from American Type Culture Collection (Manassas, VA) and cultured in DMEM/F-12 50/50 (10-090-CV, Cellgro, Manassas, VA) with 3.151mg/ml D-glucose, 0.005mg/ml insulin, 0.005mg/ml transferrin, 5ng/ml selenium, 40ng/ml dexamethasone, and 10% FBS as described previously (11). AML12 hepatocytes were treated with oleic acid (1.3mM BSA) or BSA (1.3mM) alone for 24 h, followed by homogenization with 2-propanol. The Supernatant were concentrated (Savant SpeedVac) and triglyceride content was measured enzymatically (L-Type TG M, Wako, Richmond, VA). A retroviral construct containing human wild-type VASP in the retroviral vector LXSN (14) was used for overexpression studies. AML12 cells were infected with LXSN or LXSN-wt-VASP virus. Multiple clones were selected, propagated, and maintained in the presence of G418 (0.6 mg/ml, Cellgro; Manassas,
VA). Primary hepatocyte cell culture was performed as described previously (11). In brief, after hepatic perfusion through the portal vein with liver digest medium (GIBCO) containing 0.05mg/ml of collagenase type 4 (Worthington), the liver was minced and transferred into a 50ml conical tube through a 70 µm cell strainer. After centrifugation (5 min at 50 g) primary hepatocytes (the cell pellet) were collected and cultured in a same media as for AML12 cells.

**Knockdown of AMPK α1 and α2 by siRNA in AML12 cells**

AML12 hepatocytes were transfected with either scramble siRNA (4390843, Ambion, Austin, TX) or siRNA for AMPK α1 (Prkaa1; s98535, Ambion, Austin, TX) and AMPK α2 (Prkaa2; s99117, Ambion, Austin, TX) using siPORT NeoFX Transfection Agent (Ambion, Austin, TX) according to the manufacturer’s protocol. Sequences of siRNA are as follows, *Prkaa1*; Sense: 5’ CGGGAUCCAUCAGCAACUATT 3’ Antisense: 5’

UAGGUUGCUAGGGAUCCCGAT 3’ Prkaa2; Sense; 5’

GGAUUUGCCCAGCUACCUTT 3’ Antisense; 5’

UAGGUAGCUGGGCAAAUCCCTG 3’.

**Fatty acid oxidation**

The production of acid soluble metabolites (ASM) was measured as described previously (19) with minor modification, and was used as an index of the β-oxidation of
fatty acids. Briefly, AML12 cells were incubated in DMEM + 0.5% fatty acid-free BSA with 0.05mM carnitine and 0.25 µCi [1-\(^{14}\)C] palmitate (GE Healthcare Life Sciences; Pittsburgh, PA) per well in 6-well plates. After 24 hours, 800 µl of the medium was harvested on ice, and 200 µl of ice-cold 70% perchloric acid was added in order to precipitate BSA:fatty acid complexes. The samples were centrifuged for 10 min at 14000g, and the radioactivity of the supernatant was determined by liquid scintillation (19).

**Statistical analysis**

In all experiments, densitometry measurements were normalized to controls incubated with vehicle and fold increase above the control condition was calculated. Analysis of the results was performed using the Graphpad statistical package. Data are expressed as means ± SEM, and values of \(p<0.05\) were considered statistically significant. A two-tailed \(t\) test was used to compare mean values in two-group comparisons. For four-group comparisons, two-way ANOVA, and the Bonferroni post hoc comparison test were used to compare mean values between groups.
Results

Mice lacking VASP develop hepatic steatosis on chow diet.

Since hepatic insulin resistance is associated with impaired hepatic lipid metabolism, we first asked whether an absence of VASP is associated with increased hepatic lipid content.

Chow-fed Vasp<sup>−/−</sup> and littermate control mice were sacrificed at 12 wk of age either in a fasting condition (overnight) or in a fed condition. VASP deficiency was not associated with significant differences in body weight or adiposity (Table 1), however, in Vasp<sup>−/−</sup> mice, enzymatic measurements of hepatic TG content, demonstrated a significant increase in hepatic lipid content compared to littermate controls (Figure 1A). No significant differences in changes in liver function tests (albumin, ALT) (Table 1) were noted. These results suggest that absence of VASP is associated with hepatic steatosis without obvious changes in synthetic liver function.

VASP deficiency is associated with an impairment of fatty acid oxidation and VLDL-related triglyceride secretion.

Hepatic triglyceride content is determined by a balance between circulating FFA, incorporation of FFA into the liver, lipogenesis, liver fatty acid oxidation, and VLDL-related triglyceride secretion. An imbalance of these lipid metabolism
factors is implicated in the development of hepatic steatosis (2, 3). Therefore, we sought
to determine whether VASP deficiency alters all or a combination of these factors
leading to increased hepatic steatosis. No differences were noted in plasma FFA levels
(Table 1), mRNA expression of hormone-sensitive lipase (Hsl) or adipose tissue
triglyceride lipase (Atgl), enzymes that regulate lipolysis in epididymal white adipose
tissue (Figure 1B), hepatic incorporation of FFA as measured by mRNA expressions of
Caveolin-1, Cd36, and Fatty acid transport protein 5 (Fatp5) expression (2) or in gene
expression of factors in the lipogenesis pathway [(sterol regulatory element binding
protein 1c (Srebp1c), fatty acid synthase (Fas), stearoyl-CoA desaturase 1 (Scd1), and
diacylglycerol acyltransferase 1 (Dgat1)] (Figure 1C).

Fasting increases fatty acid oxidation at the level of mitochondrial and
peroxisomal β-oxidation and this was observed in littermate wild-type control mice,
whereas in the Vasp<sup>−/−</sup> mice, fasting failed to increase fatty acid oxidation at the level of
Acox1, Ppara, Cpt1a, Ucp2, peroxisome proliferator-activated receptor-γ-coactivator-1 α
(Pgc1α), Nrf-1, and Tfam (2, 13, 20) (Figure 1C). In addition, mRNA of Mtp (involved in
VLDL secretion) (Figure 1C) and fasting TG levels were significantly lower in Vasp<sup>−/−</sup>
compared to littermate control mice (Table 1), suggesting an impairment of VLDL
secretion from the liver. To investigate this further, we treated fasted WT and Vasp<sup>−/−</sup> mice
with Triton WR1339, an agent known to inhibit lipoprotein lipase-mediated triglyceride removal from plasma. This results in a progressive increase in plasma TG levels, which provides a measure of the rate of VLDL secretion. Triton WR1339-dependent increase in plasma TG was inhibited in \( Vasp^-\) mice compared to \( WT \) mice, suggesting an impairment of VLDL secretion from the liver (Figure 1D). Collectively, these results suggest that development of hepatic steatosis in \( Vasp^-\) mice may be attributed to alterations in fatty acid oxidation and VLDL secretion.

**Overexpression of VASP in AML12 cells increases fatty acid oxidation.**

Since the absence of VASP signaling in mice reduces fatty acid oxidation and VLDL release, we next asked whether VASP signaling is sufficient to increase fatty acid oxidation directly in hepatocytes. Human VASP or empty vector control was overexpressed in AML12 hepatocytes, by retroviral gene transfer, and VASP overexpression was confirmed by Western blot (Figure 2A). VASP overexpression was associated with increased fatty acid oxidation and \( Mtp \) gene expression compared to control hepatocytes (Figure 2B) in the absence of significant changes in fatty acid uptake or lipogenic gene expression.

To determine the effect of VASP on fatty acid oxidation directly we next
assessed the rate of [1-\textsuperscript{14}C] palmitate incorporation into acid-soluble metabolites. Compared to the controls, VASP overexpressing AML12 cells demonstrated a higher rate of fatty acid oxidation (Figure 2C), consistent with fatty acid oxidation gene expression data.

To model the effect of hepatic steatosis in vitro, we treated AML12 hepatocytes with increasing doses of oleic acid, which leads to increased TG accumulation (21). VASP overexpression in hepatocytes decreased oleic acid-dependent TG accumulation compared to empty vector control hepatocytes (Figure 2D). These data collectively suggest a direct role of VASP in liver cells to enhance fatty acid oxidation and Mtp.

**VASP regulates hepatic AMPK signaling during fasting.**

During fasting, activation of AMPK (4) activates PPAR\(\alpha\) and co-factor PGC1\(\alpha\) (22-26), both key upstream transcription factors regulating fatty acid oxidation gene expression (\textit{Acox1, Ucp2, Nrf-1, Tfam}) (22, 27-31). Since VASP similarly regulates these responses (Figure 1C, 2B), we hypothesized that VASP-dependent effects on lipid metabolism are mediated by AMPK. To test this hypothesis we analyzed AMPK signaling in AML12 hepatocytes and from hepatic tissues of WT and \textit{Vasp}\(^{+/−}\) mice. Overexpression of VASP in AML12 hepatocytes was associated with increased
p-AMPK and p-ACC (Figure 3A) compared to empty vector-transduced hepatocytes, whereas primary hepatocytes isolated from Vasp^−/− mice exhibit reduced AMPK signaling and rate of fatty acid oxidation (Figure 3B). In vivo, overnight fasting increased hepatic levels of p-AMPK and p-ACC. However, this expected response was attenuated in Vasp^−/− mice (Figure 3C). Collectively, these data suggest roles of VASP on AMPK signaling and fatty acid oxidation in hepatocytes.

**AMPK is necessary for the protective effect of VASP on hepatic lipid metabolism**

To determine whether AMPK is necessary for the effect of VASP on hepatic FFA oxidation, we employed a RNAi mediated knockdown of AMPKα1 and AMPKα2, catalytic subunits of AMPK, both of which are important for hepatic fatty acid oxidation (4, 23) (Figure 4A). As expected, overexpression of VASP in AML12 hepatocytes increased peroxisomal and mitochondrial β-oxidation and VLDL-TG secretion, however in the presence of siRNA for AMPKα, these responses were significantly attenuated (Figure 4B). Furthermore, even though VASP overexpression reduced oleic acid-induced hepatic TG accumulation, inhibition of AMPK attenuated VASP’s protective responses (Figure 4C). Similar results were obtained using a pharmacological inhibitor of AMPK, compound c (Supplemental Figure 1A, B). These data collectively suggest that intact AMPK signaling is required for VASP to attenuate TG accumulation.
by enhancing fatty acid oxidation and upregulating $Mtp$ gene expression in the liver.

**Restoration of AMPK signaling in $Vasp^{−/−}$ mice attenuates fatty liver through a normalization of impaired fatty acid oxidation and VLDL secretion.**

Since reduced AMPK signaling is associated with the development of hepatic steatosis in $Vasp^{−/−}$ mice, we next determined whether restoration of AMPK signaling would restore fatty acid oxidation in $Vasp^{−/−}$ mice. Administration of an AMPK activator, AICAR, daily for 5-7 days has been shown to enhance fatty acid oxidation and ameliorate hepatic steatosis not only in rodent models but also in human subjects (31, 32, 33). $Vasp^{−/−}$ mice received daily AICAR injections (200 mg/kg) for 5 days and were then sacrificed after an overnight fast. Restoration of p-AMPK and p-ACC (Supplemental Figure 3 2) by the administration of AICAR did not change body weight (Supplemental Table 1), however, AICAR reduced hepatic triglyceride content and increased peroxisomal β-oxidation genes ($Acox1$ and $Ppara$), mitochondrial β-oxidation genes ($Cpt1a$, $Ucp2$, and $Pgc1α$) (Figure 4D, E) and $Mtp$ gene (Figure 4D) expression in $Vasp^{−/−}$ mice. Finally, triton-induced VLDL-TG secretion in $Vasp^{−/−}$ mice was normalized in response to AICAR treatment (Figure 4F). These data suggest that restoration of AMPK signaling in $Vasp^{−/−}$ mice restores hepatic fatty acid oxidation and VLDL-TG
secretion.

Activation of VASP by sildenafil reduces hepatic steatosis in \textit{db/db} mice and during diet-induced obesity.

We next determined whether activation of VASP signaling reduces hepatic steatosis in a genetic model of obesity. Since activation of VASP (phosphorylation of VASP at Ser239) is governed by cGMP-dependent protein kinase (PKG) (34), we first tested whether 8Br-cGMP, an analog of cGMP could directly regulate AMPK activation and fatty acid oxidation in hepatocytes. We found that cGMP enhances hepatic fatty acid oxidation and this was mediated by AMPK (Supplemental Figure 3A-C).

In order to investigate this further in vivo, we utilized the PDE5 inhibitor, sildenafil, which is known to increase intracellular cGMP levels, activate PKG and phosphorylate (serine 239) VASP (11, 34, 35). \textit{Db/db} and control \textit{db/+} mice received daily doses of vehicle or sildenafil (30 mg/kg) for 4 wk. At the end of the study, liver lysates were analyzed for VASP, AMPK, and ACC phosphorylation by Western blot. As expected, \textit{db/db} mice exhibited reduced levels of phospho-VASP, p-AMPK, and p-ACC in liver lysates compared to \textit{db/+} control mice, whereas sildenafil treatment in \textit{db/db} mice restored phospho-VASP (Ser239), p-AMPK, and p-ACC levels comparable to control
These observations were accompanied by a reduction of hepatic steatosis in the \( db/db \) mice (Figure 5B). Collectively, pharmacological activation of VASP by sildenafil reduces hepatic steatosis in \( db/db \) mice, through increased AMPK signaling and fatty acid oxidation in the liver.

**Sildenafil does not ameliorate hepatic steatosis in \( Vasp^{+/} \) mice.**

Finally, we sought to test whether VASP is required for sildenafil to enhance AMPK signaling and ameliorate hepatic steatosis. Previously we demonstrated that sildenafil reduces hepatic steatosis induced during HF-feeding (measured in the fed state) (11), however, the effect of sildenafil on hepatic steatosis during fasting is unknown. WT and \( Vasp^{-/-} \) mice were maintained on HF diet for 8 weeks, and for the last 2 weeks of the diet, study mice received 30 mg/kg/day oral sildenafil or vehicle. While sildenafil increased p-AMPK in WT mice following an overnight fast, this was not evident in \( Vasp^{-/-} \) mice (Figure 5C). Similarly, sildenafil reduced hepatic TG content in WT mice but not in \( Vasp^{-/-} \) mice (Figure 5D). These data suggest that the effect of sildenafil to reduce hepatic steatosis requires VASP and its downstream target, AMPK.
**Discussion**

Hepatic steatosis is a commonly observed hepatic manifestation of the metabolic syndrome in diabetes and obesity, and is thought to be the initial abnormality in NAFLD (2). NAFLD encompasses a spectrum of liver disease ranging from benign fatty liver to the more severe NASH, a condition that may progress to cirrhosis in up to 25% of patients (2).

By employing both loss of and gain of function studies, we demonstrate that VASP enhances fatty acid oxidation by activating AMPK signaling in the liver. Conversely, the absence of VASP, reduces AMPK activation and, reduces hepatic fatty acid oxidation, resulting in increased hepatic steatosis. These results suggest a novel role for VASP in attenuating the development of hepatic steatosis during obesity and furthermore may identify VASP as a potential therapeutic target.

AMPK is a serine/threonine protein kinase, which acts as a sensor of cellular energy status and regulates a wide variety of gene regulatory and metabolic pathways, including fatty acid oxidation in liver (4). Activation of AMPK occurs by phosphorylation at Thr172 catalyzed by liver kinase B1 (LKB1) in response to an increase in the AMP/ATP ratio and by calmodulin-dependent protein kinase kinase β (CaMKKβ) in response to elevated Ca\(^{2+}\) levels (4). NO has recently been reported as a
endogenous activator of AMPK through a CaMKK-dependent mechanism in endothelial cells (36). Therefore, it is reasonable to hypothesize that in hepatocytes, CaMKK signaling may link VASP to AMPK activation. We tested this hypothesis using STO-609, a pharmacological inhibitor of CaMKK and found that in the presence of STO-609, AMPK signaling (Supplemental Figure 4A) and fatty acid oxidation (Supplemental Figure 4B) is reduced. Additional studies are necessary since STO-609 may have off-target effects (37). Other possible mechanisms may involve LKB1 or protein phosphatase-2C (PP2C), a phosphatase that dephosphorylates and inactivates AMPK (4).

Since AMPK plays an important role in the regulation of gluconeogenesis and lipid metabolism, therapeutic strategies targeting AMPK signaling are promising therapies to reverse both glucose and lipid abnormalities associated with type 2 diabetes (38, 39). Our current studies suggest that targeting VASP (e.g. by the PDE5 inhibitor, sildenafil) could lead to an improvement of hepatic steatosis by activating AMPK signaling.

\( Vasp^{-/} \) mice display fasting hypoglycemia (Table 1) despite the presence of hepatic steatosis (Figure 1A) and insulin resistance at the level of Akt/IRS2 signaling (11). Hepatic steatosis and fasting hypoglycemia are also observed in \( Ppara^{-/} \) mice, a
mice model of impaired mitochondrial fatty acid oxidation (40, 41). Hypoglycemia occurring in the context of defective mitochondrial fatty acid oxidation likely due to a combination of glycogen depletion and a blunted gluconeogenic response (41). Since Ppara was regulated by VASP (Figure 1C, 2B), mechanisms of steatosis and fasting hypoglycemia in Vasp−/− mice might be similar to of Ppara−/− mice.

In Vasp−/− mice, fasting plasma TG level was reduced (Table 1) and this was associated with decreased Mtp gene expression (Figure 1C) and impaired VLDL-TG secretion (Figure 1D). Mechanistically, since transcription of Mtp is positively regulated by PPARα (29) and since VASP activates AMPK and PPARα (Figure 2B, Figure 3A), VASP may regulate Mtp thorough PPARα.

AMPK not only enhances fatty acid oxidation but also suppresses NFκ-B signaling (42). In Vasp−/− mice, reduction of fatty acid oxidation (Figure 1C) was coincident with elevation of inflammation (11). These alterations might be explained by reduced AMPK signaling (Figure 3B, C). Anti-inflammatory effect of VASP (11) might be mediated by AMPK. Indeed, restoration of AMPK by AICAR attenuated hepatic inflammation in Vasp−/− mice (Supplemental Figure 5).

Another study provides different evidence linking inflammation to AMPK, in that they show inflammatory pathway down-regulates AMPK phosphorylation and
activity (43). Since $Vasp^{-/-}$ mice demonstrated increased inflammation not only in liver (11) but also in adipose tissue (44), it is possible that reduced AMPK signaling in $Vasp^{-/-}$ mice liver could be attributed to the increased inflammation. However, alterations of AMPK signaling by VASP in vitro (Figure 3A, B) indicate a direct effect of VASP in hepatocytes. Furthermore, we demonstrated that this was CaMKK dependent (Supplemental Figure 4A, B).

NO/cGMP, regulators of VASP are implicated with mitochondrial biogenesis in adipocytes (12), which influences fatty acid oxidation capacity (13). This effect can also be exerted in the liver. NO/cGMP has been reported to enhance fatty acid oxidation in rat hepatocytes (45) and indeed, $enos^{-/-}$ mice, in which this signaling is downregulated, develop hepatic steatosis (11, 46). We consider that VASP, a downstream target, has similar roles. Since NO/cGMP/VASP signaling was downregulated during diet-induced obesity (11), reduced NO/cGMP/VASP signaling might cause impaired mitochondrial biogenesis, followed by susceptibility to develop steatosis.

VASP protein expression is found in hepatocytes, Kupffer cells, and hepatic sinusoidal endothelial cells and the cell specific contribution of VASP to the development of hepatic steatosis remains an important unanswered question. We have previously demonstrated that the absence of VASP is associated with increased
hepatocyte as well as Kupffer cell NF-κB activation during HF feeding (11). Since M1 (pro-inflammatory) activated Kupffer cells have been reported to contribute to the development of hepatic steatosis (47), additional studies are needed to clarify the role of VASP signaling in M1 activated Kupffer cells (11) and in the development of hepatic steatosis in Vasp<sup>−/−</sup> mice.

Since hepatic expression of Fatp2 was increased in Vasp<sup>−/−</sup> mice (Figure 1C), elevated incorporation of free fatty acid into the liver other than reduced fatty acid oxidation and VLDL-TG secretion might also contribute the development of steatosis. However, Fatp2 was not decreased by the overexpression of VASP (Figure 2B). Thus, whether VASP is associated with an uptake of fatty acid into the liver has not fully revealed yet.

Recently, activation of NO and cGMP signaling (34) has been reported to activate AMPK signaling in various type of cells, including endothelial cells (36) and human muscle cells (48). Our current study, which indicates that VASP enhances fatty acid oxidation by activating AMPK, is the first evidence that links VASP, a downstream molecule of cGMP, to AMPK signaling.

In summary, we demonstrated that in liver, VASP has a role to enhance fatty acid oxidation by activating AMPK. We also identified a role of VASP to promote VLDL-TG
secretion through alteration of Mtp gene expression. Improvement of hepatic steatosis in db/db mice by pharmacological enhancement of VASP signaling additionally suggests that direct activation of VASP could be a promising target for the treatment of hepatic steatosis.

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S.T. designed and performed experiments, provided data analysis, wrote the manuscript, contributed to discussion, and reviewed and edited the manuscripts. N.R-D. designed
and performed experiments. P.H. performed experiments and contributed to discussion. V.M.-S., K.O., and J.E.K. performed experiments. A.M.C., A.C., and K.E.B. designed and contributed to discussion. G.D. and A.W.C. reviewed and edited the manuscript. F.K. interpreted data and wrote, reviewed, and edited the manuscript. S.T. is the guarantor of this work, had full access to all the data, and takes full responsibility for the integrity of data and the accuracy of data analysis.

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Table 1. Metabolic parameters of WT and Vasp<sup>-/-</sup> mice fed a chow-diet

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<th>Vasp&lt;sup&gt;-/-&lt;/sup&gt;</th>
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<td>1.7±0.05</td>
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Metabolic parameters were measured at 12 weeks of age in WT or Vasp<sup>-/-</sup> mice.

*p<0.05, Data are expressed as means ± SEM (n=7). WT; wild type, BW; body weight, NEFA; nonesterified fatty acid, ALT; alanine aminotransferase
**Figure Legends**

**Figure 1. Hepatic steatosis in Vasp⁻/⁻ mice fed on a chow diet.** (A) Enzymatic measurement of hepatic triglyceride content collected after 16 hours fast (n=7). (B) RT-PCR analysis of lipolysis related genes in the epididymal adipose tissue collected after 16 hours fast (n=6) (C) RT-PCR analysis of lipid metabolism related genes in the liver collected either in fed mice or after 16 hours fast. Expression of Gapdh as shown by the ratio of CT value. (n=6) (D) Administration (ip) of Triton WR1339 (500 mg/kg), followed by measurement of plasma triglyceride level (n=5). *p<0.05. WT, wild type; CT, threshold cycle

**Figure 2. The effect of VASP on lipid metabolism in AML12 cells.** (A) AML12 cells were transduced with VASP (VASP-OE) or control (empty) vector. (B) RT-PCR analysis of lipid metabolism related genes in AML12 cells. Expression of Gapdh as shown by the ratio of CT value. (n=3) (C) Rate of [1-¹⁴C]palmitate incorporation into acid-soluble metabolites (n=4). (D) Oleic acid (either 0.1 mM or 0.4 mM) induced accumulation of triglyceride in AML12 cells. 1.3mM BSA was used as a control. (n=3) *p<0.05 CT, threshold cycle

**Figure 3. AMPK signaling is regulated by VASP in the liver.** (A) Phosphorylation of AMPK (Thr172) and ACC (Ser79) in AML12 cells with VASP overexpression. AICAR was used as a positive control. Western blot from one of three independent experiments is shown. (B) Primary hepatocytes were isolated from WT or Vasp⁻/⁻ mice and cultured. P-AMPK (Thr172) and p-ACC (Ser79) as measured by Western blot. Representative blots are shown. Rate of [1-¹⁴C]palmitate incorporation into acid-soluble metabolites (n=3). (C) p-AMPK and p-ACC in the liver collected after an overnight fast (n=6). Representative blots is shown.

**Figure 4. Involvement of AMPK signaling in the effect of VASP.** (A) AMPKα protein levels following siRNA for AMPKα1 (Prkaa1, 5nM, 48 hours) and AMPKα2 (Prkaa2, 5nM, 48 hours) in AML12 hepatocytes. (n=2) (B) RT-PCR analysis of fatty acid oxidation genes or Mtp gene in AML12 cells with siRNAs for AMPKα1 (Prkaa1, 5nM, 48 hours) and α2 (Prkaa2, 5nM, 48 hours) (n=4) (C) Oleic acid (0.1 mM) was used to treat AML12 cells for 24 hours with or without siRNAs for AMPK α1 (Prkaa1, 5nM, 48 hours) and α2 (Prkaa2, 5nM, 48 hours). (n=4) (D) Either AICAR (200 mg/kg) or PBS was injected (sc) daily for 5 days in WT and Vasp⁻/⁻ mice, followed by sacrifice.
after an overnight fast. Relative mRNA expression in the liver as measured by RT-PCR is shown (n=5). (E) Hepatic triglyceride content as measured enzymatically (n=5). (F) At the end of the 5 days treatment of AICAR protocol, Triton WR1339 (500 mg/kg) was administrated (ip) after an overnight fast, followed by a measurement of plasma triglyceride level (n=5). *p<0.05 ip, intraperitoneally; sc, subcutaneously, WT, wild type

Figure 5. Pharmacological activation of VASP ameliorates hepatic steatosis during fasting state in db/db mice and HF diet-induced obese mice. 12 week old db/db and db/+m mice fed chow diet received daily oral administration of either vehicle or the PDE-5 inhibitor sildenafil (30 mg/kg/day) for 4 weeks. For the obesity-induced hepatic steatosis study, WT and Vasp<sup>−/−</sup> mice were maintained on a HF diet for 8 weeks, and for the last 2 weeks of the diet, study mice received 30 mg/kg/day oral sildenafil or vehicle. (A) p-VASP (Ser239), p-AMPK (Thr172), and p-ACC (Ser79) as measured by Western blot in the liver samples collected after an overnight fast. Representative Western blots are shown. (B) Hepatic triglyceride content as measured enzymatically. *p<0.05 (db/+m, n=3; db/db, n=6) (C) p-AMPK (Thr172) as measured by Western blot in the liver sample collected after an overnight fast. Representative Western blots are shown. (D) Hepatic triglyceride content as measured enzymatically. *p<0.05 (n=5) WT, wild type
Figure 1

A) Liver TG (mg/g)

B) Epididymal adipose tissue

C) Liver

CT value (ratio)

fatty acid uptake

lipogenic genes

peroxisomal β-oxidation

Mitochondrial β-oxidation

VLDL secretion related gene

D) Plasma TG (mg/dl)

Figure 1
**Figure 2**

A. IB: VASP, IB: GAPDH

B. Fatty acid uptake and lipogenic genes

C. Peroxisomal β-oxidation

D. Mitochondrial β-oxidation and VLDL secretion related gene

CT value (ratio)

E. CPM/24h/μg protein

F. TG (μg/μg protein)
**Figure 3**

**A. In vitro (AML12 cells)**

- IB: p-AMPK
- IB: AMPK
- IB: p-ACC
- IB: ACC
- IB: VASP
- IB: GAPDH

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<td>VASP OE 10m</td>
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<tr>
<td>VASP OE 1h</td>
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<tr>
<td>AICAR(100 uM) (regular AML12)</td>
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</table>

**B. In vitro (primary hepatocytes)**

- IB: p-AMPK
- IB: p-ACC
- IB: AMPK
- IB: ACC
- IB: GAPDH

**CPM/24h/μg protein**

- WT
- Vasp<sup>−/−</sup>

**C. In vivo**

- IB: p-AMPK
- IB: AMPK
- IB: GAPDH

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>Vasp&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<tr>
<td>IB: GAPDH WT</td>
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<tr>
<td>IB: Vasp&lt;sup&gt;−/−&lt;/sup&gt;</td>
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<tr>
<td>IB: p-AMPK WT</td>
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<tr>
<td>IB: p-AMPK Vasp&lt;sup&gt;−/−&lt;/sup&gt;</td>
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<tr>
<td>IB: p-ACC WT</td>
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<tr>
<td>IB: p-ACC Vasp&lt;sup&gt;−/−&lt;/sup&gt;</td>
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<tr>
<td>IB: ACC WT</td>
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<tr>
<td>IB: ACC Vasp&lt;sup&gt;−/−&lt;/sup&gt;</td>
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</tbody>
</table>

**GAPDH**

- WT
- Vasp<sup>−/−</sup>

**p-AMPK/AMPK**

- WT
- Vasp<sup>−/−</sup>

**p-ACC/ACC**

- WT
- Vasp<sup>−/−</sup>
Figure 4

A. Western blot analysis showing AMPK and GAPDH expression in control (ctl) and AMPK-overexpressing (VASP-OE) conditions.

B. Bar graphs showing relative mRNA expression levels for Acox1, Ppara, Cpt1a, Pgc1a, Nrf, and Tfam in control (ctl) and AMPK-overexpressing (VASP-OE) conditions. The graphs also compare wild-type (WT) and Vasp knockout (Vasp−/−) mice.

C. Graph showing TG levels in control (ctl) and AMPK-overexpressing (VASP-OE) conditions with and without AMPK knockdown.

D. Graphs showing relative mRNA expression levels for VLDL secretion-related genes (Mtp) in control (ctl) and AMPK-overexpressing (VASP-OE) conditions with and without AMPK knockdown.

E. Bar graph showing liver TG levels in control (ctl) and AICAR-treated conditions.

F. Graph showing plasma TG levels in control (ctl) and AICAR-treated conditions.
Figure 5
Supplemental Table 1.

Metabolic parameters of WT and Vasp⁻/⁻ mice received either vehicle or AICAR

<table>
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<tr>
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<th>WT</th>
<th>AICAR</th>
<th>Vasp⁻/⁻</th>
<th>AICAR</th>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>27.7±1.6</td>
<td>27.0±1.7</td>
<td>27.8±1.0</td>
<td>26.7±1.5</td>
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<tr>
<td>Fed glucose (mg/dl)</td>
<td>131±10</td>
<td>126±9</td>
<td>133±13</td>
<td>123±12</td>
</tr>
<tr>
<td>Fast glucose (mg/dl)</td>
<td>85±2</td>
<td>81±13</td>
<td>63±8*</td>
<td>58±5</td>
</tr>
<tr>
<td>Fed insulin (mg/dl)</td>
<td>1.18±0.23</td>
<td>1.22±0.18</td>
<td>1.24±0.20</td>
<td>1.30±0.15</td>
</tr>
<tr>
<td>Fast insulin (mg/dl)</td>
<td>0.82±0.07</td>
<td>0.80±0.32</td>
<td>1.03±0.11</td>
<td>0.94±0.08</td>
</tr>
<tr>
<td>NEFA (mEq/l)</td>
<td>1.64±0.20</td>
<td>1.46±0.30</td>
<td>1.60±0.03</td>
<td>1.21±0.08</td>
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</tbody>
</table>

Either PBS (ctl) or AICAR (200mg/kg) was injected (sc) daily for 5 days in 12 weeks old WT and Vasp⁻/⁻ mice, followed by measurement of metabolic parameters. *p<0.05 vs WT ctl, #P<0.05 vs Vasp⁻/⁻ ctl. Data are expressed as means ±SEM (n=5). WT; wild type, NEFA; nonesterified fatty acid.

Supplemental Figure Legends

Supplemental Figure 1 Involvement of AMPK signaling in the effect of VASP. (A) RT-PCR analysis of fatty acid oxidation genes or Mtp gene in AML12 cells with an AMPK inhibitor, compound c (20 µM, 4 hours). (n=4) (B) Oleic acid (0.1 mM) was used to treat AML12 cells for 24 hours with or without compound c (20µM, 24 hours). (n=4)

Supplemental Figure 2 Restoration of AMPK signaling in Vasp⁻/⁻ mice by AICAR
Either AICAR (200 mg/kg) or PBS was injected (sc) daily for 5 days in WT and Vasp⁻/⁻ mice, followed by sacrifice after an overnight fast. P-AMPK (Thr172) and p-ACC (Ser79) as measured by Western blot. (n=5) *p<0.05 WT, wild type

Supplemental Figure 3 Effect of 8Br-cGMP on AMPK signaling and fatty acid oxidation. AML12 hepatocytes were stimulated with 8Br-cGMP (100µM) for 4 hours in the presence of siRNAs for AMPKα1 (Prkα1, 5nM, 48 hours) and α2 (Prkα2, 5nM, 48 hours). (A) p-AMPK (Thr172), p-ACC (Ser79), and p-VASP (Ser239) as measured by Western blot. Representative blots are shown (n=3). (B) Rate of [1-¹⁴C]palmitate incorporation into acid-soluble metabolites (n=4). (C) Relative expression of fatty acid oxidation related genes as measured by RT-PCR (n=4) *p<0.05
Supplemental Figure 4 CaMKK might be involved in the activation of AMPK by VASP in AML12 cells. (A) Phosphorylation of AMPK (Thr172) and ACC (Ser79) by VASP with or without STO-609 (10µg/ml, 4 hours). (B) STO-609 was treated for 4 hours (1µg/ml or 10µg/ml) in either control of VASP overexpressed cells. Relative mRNA expression of fatty acid oxidation related genes and Mtp genes as measured by RT-PCR. (n=3)

Supplemental Figure 5 Alterations of hepatic inflammation by AICAR in Vasp⁻/⁻ mice
12 weeks old WT and Vasp⁻/⁻ mice fed a chow diet were sacrificed after 16 hours fast. RT-PCR analysis of inflammatory genes in the liver. (n=6) *p<0.05 WT, wild type
For Peer Review Only

Supplemental Figure 1

A

<table>
<thead>
<tr>
<th>Gene</th>
<th>ctl</th>
<th>VASP-OE</th>
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<tbody>
<tr>
<td><em>Acox1</em></td>
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<tr>
<td><em>Ppara</em></td>
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B

<table>
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<tr>
<th>Compound c</th>
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<td>TG (µg/µg protein)</td>
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Supplemental Figure 2

IB: ACC
IB: AMPK
IB: p-ACC
IB: ACC

<table>
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IB: p-AMPK
IB: GAPDH
IB: AMPK
IB: p-ACC
IB: ACC

p-AMPK/AMPK
p-ACC/ACC
GAPDH

ctl     | AICAR  | ctl     | AICAR  |

WT      | Vasp^- |

* *
Supplemental Figure 3
Supplemental Figure 4


Panel B: Gene expression analysis for Peroxisomal β-oxidation (Acox1, Ppara), Mitochondrial β-oxidation (Cpt1a, Ucp2, Pgc1α, Nrf1, Tfam), and VLDL secretion related gene (Mtp) with VASP-COE treatment.