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ABOUT COVER

Associate editor of *World Journal of Hepatology*, Dr. Yong-Ping Yang is a Distinguished Professor at Peking University Health Science Center in Beijing, China. Having received his Bachelor's degree from Yanbian University in 1985, Dr. Yang undertook his postgraduate training at PLA Medical College, receiving his Master's degree in 1992. He rose to Chief Physician in the Hepatology Division of the Fifth Medical Center of the Chinese PLA General Hospital in 2003 and has held the position since. His ongoing research interests involve liver fibrosis, cirrhosis and hepatocellular carcinoma, with a particular focus on cryoablation and cryo-immunotherapy for hepatocellular carcinoma. Currently, he serves as Chairman of the Department of Liver Disease of the Chinese PLA General Hospital and as President of the Chinese Research Hospital Association for the Study of the Liver Disease. (L-Editor: Filipodia)

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ORIGINAL ARTICLE

Basic Study

Inhibition of vascular adhesion protein-1 modifies hepatic steatosis *in vitro* and *in vivo*

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Abstract

BACKGROUND

Non-alcoholic fatty liver disease (NAFLD) is associated with obesity, insulin resistance and dyslipidaemia and currently is estimated to affect up to a third of all individuals in developed countries. Current standard of care for patients varies according to disease stage, but includes lifestyle interventions common insulin sensitizers, antioxidants and lipid modifiers. However, to date specific therapies have shown little histological or fibrosis stage improvement in large clinical trials, and there is still no licensed therapy for NAFLD. Given the high prevalence, limited treatment options and significant screening costs for the general population, new treatments are urgently required.

AIM

To assess the potential for inhibition of the amine oxidase enzyme vascular adhesion protein-1 (VAP-1) to modify hepatic lipid accumulation in NAFLD.

METHODS

We have used immunochemical and qPCR analysis to document expression of VAP-1 and key functional proteins and transporters across the NAFLD spectrum. We then utilised hepatocytes in culture and human precision cut liver slices in concert with selective enzyme activity inhibitors to test the effects of activating the semicarbazide-sensitive amine oxidase activity of VAP-1 on hepatic lipid uptake and triglyceride export. A murine model of NAFLD was also used to determine the consequences of VAP-1 knockout and gene expression arrays were used to

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quantify the effects of VAP-1 activity on key lipid modifying and proinflammatory gene expression.

RESULTS

We confirmed that increasing severity of NAFLD and progression to cirrhosis was associated with a significant increase in hepatocellular VAP-1 expression. Hepatocytes *in vitro* exposed to recombinant VAP-1 and its substrate methylamine showed increased lipid accumulation as determined by quantification of Oil Red O uptake. This was recapitulated using hydrogen peroxide, and lipid accumulation was accompanied by changes in expression of the lipid transporter molecules FABP3, FATP6, insulin receptor subunits and PPARα. Human liver tissue exposed to recombinant VAP-1 or substrates for endo/exogenous VAP-1 produced less triglyceride than untreated tissue and demonstrated an increase in steatosis. This response could be inhibited by using bromoethylamine to inhibit the SSAO activity of VAP-1, and mice deficient in VAP-1/AOC3 also demonstrated reduced steatosis on high fat diet. Exposure of human liver tissue to methylamine to activate VAP-1 resulted in increased expression of FABP2 and 4, FATP3-5, caveolin-1, VLDLR, PPARGC1 and genes associated with the inflammatory response.

CONCLUSION

Our data confirm that the elevations in hepatic VAP-1 expression reported in nonalcoholic steatohepatitis can contribute to steatosis, metabolic disturbance and inflammation. This suggests that targeting the semicarbazide sensitive amine oxidase capacity of VAP-1 may represent a useful adjunct to other therapeutic strategies in NAFLD.

Key Words: Non-alcoholic fatty liver disease; Hepatocyte; Lipid; Cell biology; Vascular adhesion protein-1; Steatosis

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Core Tip: Incidence of non-alcoholic fatty liver disease (NAFLD) is dramatically increasing worldwide but to date there are no licenced therapies. The challenge remains management of the diverse pathophysiology from simple steatosis, through inflammation and fibrosis and the systemic complications of the metabolic syndrome. Vascular adhesion protein-1 (VAP-1) is an enzyme with proven contributions to systemic and hepatic glucose handling, inflammation and fibrosis. We now show an additional role in hepatic steatosis. Thus our important data suggests that targeting the semicarbazide sensitive amine oxidase capacity of VAP-1 may represent a useful adjunct to other therapeutic strategies in NAFLD.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is associated with obesity, insulin resistance and dyslipidaemia[1] and currently is estimated to affect up to a third of all individuals in developed countries[2]. Although a systemic disease, within the liver NAFLD occurs as a disease spectrum ranging from steatosis alone, to steatohepatitis (NASH) which ultimately drives the development of significant fibrosis and cirrhosis. Patients with NAFLD have high mortality and in particular are at increased risk of suffering adverse cardiovascular events[3]. The current standard of care for management of patients is stratified according to disease stage, with lifestyle interventions common in simple steatosis but more extensive clinical intervention using insulin sensitizers, antioxidants and lipid modifiers in NASH[4]. However, to date specific therapies for NASH such as

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obeticholic acid[5] and apoptosis signal regulating kinase 1 (ASK1) inhibition have shown little histological or fibrosis stage[6] improvement and there is still no licenced therapy for NASH. Transplantation is an option in some centres but as NASH will soon overtake hepatitis C infection as the major indication for liver transplantation, the demand far outstrips clinical capacity. Given the high prevalence, limited treatment options and significant cost associated with screening at risk patients, new treatments are urgently required.

VAP-1 (Semicarbazide Sensitive Amine Oxidase, SSAO, AOC3, EC 1.4.3.6) may present a novel avenue for therapeutic intervention. This molecule is a transmembrane protein and member of the copper containing amine oxidase enzyme family that is sensitive to inhibition by the urea derivative semicarbazide. It is structurally similar to other copper containing amine oxidases[7] such as lysyl oxidase (LOX). It is expressed on diverse cells including adipocytes, smooth muscle cells[8] and some endothelial cells[9], and can be secreted as a soluble protein into serum[10]. Serum concentrations of VAP-1 increase in obesity[11], diabetes[12,13] and inflammatory liver disease[14] and the molecule has promise as a serological indicator of disease severity in inflammatory liver disease[15]. Of note in patients with NASH, serum VAP-1 Levels correlate with severity of obesity and NASH, and more importantly fibrosis stage[16]. VAP-1 and other amine oxidase enzymes catalyse the deamination of amines to yield the corresponding aldehyde and hydrogen peroxide. Thus VAP-1 catalyses the oxidative deamination of both endogenous (methylamine and aminoacetone)[17] and exogenous (benzylamine)[18] amines. This enzymatic capacity is key in the context of diabetes and inflammation since the products of the reaction have been shown to alter glucose uptake[19], and administration of substrate modifies the effects of insulin[20] and activates NFκB to drive hepatic inflammation[21]. There is also evidence suggesting that serological lipid profiles[22] and atheroma risk[23] are linked to VAP-1 activity, since the insulinomimetic effects of the molecule alter lipid metabolism and storage[24] and prime adipocyte differentiation and lipolysis[25]. Since transgenic mice overexpressing VAP-1/SSAO show increased BMI and abdominal fat pad weight if exposed to methylamine[26] it is likely that VAP-1 contributes to the storage and distribution of lipids in NAFLD. In support of this recent studies from our group have demonstrated that wild type mice given an anti-VAP-1 therapeutic antibody, show reduced steatosis on MCD diet[16]. However, to date the mechanisms underlying this response have not been characterised, particularly in a human context. Therefore, in the current investigation we aimed to assess the potential for VAP-1 inhibition to modify hepatic lipid accumulation.

MATERIALS AND METHODS

Human and murine tissue samples

All human tissue used was collected at the Liver and Hepatobiliary Unit, Queen Elizabeth Hospital, Birmingham, with prior written informed patient consent and local research ethics committee approval (06/Q702/61). Normal and steatotic donor tissue was surplus to requirement for transplantation, whilst NASH and ALD tissue was collected from end-stage fibrotic explanted livers upon transplantation. Tissue was immediately snap frozen and stored at -80 °C or formalin-fixed and paraffin-embedded. For functional assays and generation of tissue slices, tissue samples of approximately 30 g were cut from the periphery of freshly collected livers and immediately placed into (DMEM) prior to use.

All mice were maintained and housed under conventional conditions in the Biomedical Services Unit at the University of Birmingham, United Kingdom. All animal experiments were performed under a Home Office project license in accordance with United Kingdom legislation and welfare guidelines, and studies were approved by the local ethical review board. Male 8-10 wk old WT (Charles Rivers Laboratories Margate, United Kingdom) or *VAP-1*$^{-/-}$ mice (*AOC3* constitutive KO, Taconic, Denmark) were fed a high fat diet (HFD, Special Diets Services, Essex, United Kingdom) for 12 wk. Mice were sacrificed and the liver was removed and immediately snap frozen and stored at -80 °C for subsequent analysis.

Immunohistochemistry and histological analysis

Haematoxylin and eosin staining was performed on Formalin fixed, paraffin embedded sections from human or mouse liver using standard protocols. To quantify lipid content in hepatocytes, fresh frozen tissue sections were incubated in 60% isopropanol for 5 min followed by Oil Red O reagent for 15 min at room temperature.

This was tipped off and 60% isopropanol was added for another 5 min. Slides were washed twice with water and finally mounted using aqueous mountant (Thermoscientific, Shandon). Sections were imaged using brightfield microscopy and % staining area was determined using morphometric analysis *via* Image J software version 1.42 (NIH), using 5 non-overlapping fields selected at random from each mouse (at × 20 magnification). 7 mm sections of formalin-fixed or snap frozen human or murine liver tissue were stained with haematoxylin and eosin or Van Geison's stain[27] according to standard protocols. For analysis of VAP-1 expression fixed tissue sections were stained using standard indirect immunohistochemical methods as described previously[16].

Generation of precision-cut liver slices

To generate precision-cut liver slices (PCLS), 8 mm tissue cores from fresh tissue samples were aseptically obtained and placed in DMEM (Invitrogen, United Kingdom) at 4 °C prior to slicing[28]. A Krumdieck tissue slicer (Alabama Research and Development, United States) was set up aseptically in a class II microflow tissue safety hood according to the manufacturers instructions. Tissue cores were placed into the Krumdieck tissue slicer assembly and aseptic 240 μm thick PCLS were cut with a blade cycle speed ranging from 20-70/min depending on the type of tissue used (fatty or normal). PCLS were then immediately transferred to tissue culture media consisting of Williams E media (Sigma, United Kingdom) supplemented with 2% FCS (Invitrogen, United Kingdom), 0.1 μmol/L dexamethasone (Sigma, United Kingdom), and 0.5 μmol/L insulin (Novo-Nordisk) unless otherwise noted for specific assays. PCLS were cultured for up to 48 h *ex vivo* in static culture at 37 °C in 5% CO_2 in a humidified atmosphere.

Maintenance of Huh7.5 cells

The human hepatoma-derived hepatocyte cell line Huh7.5 was also used in this study. The cells were seeded in to a T75 cm^2 flask (Corning, United Kingdom) cultured in complete Dulbecco's modified eagle medium (DMEM, GIBCO®, Invitrogen) containing 2 mmol/L L-Glutamine, 100 U/mL Penicillin and 100 μg/mL Streptomycin (Sigma, Dorset, United Kingdom). The medium was also supplemented with 10% foetal calf serum (FCS, Invitrogen, Paisley, United Kingdom) and 1 mL of non-essential amino acids (GIBCO®, Invitrogen). The cells were maintained in a 5% CO_2 humidified atmosphere at 37 °C.

Assessment of lipid uptake in PCLS or cultured cells after VAP-1 treatment

In order to study the effects of rVAP-1 activity on lipid uptake in an intact organ culture system, PCLS were cultured in 24 well plates in Williams E media (Sigma, United Kingdom) supplemented with 2% FCS (Invitrogen, United Kingdom) and 0.1 μmol/L dexamethasone (Sigma, United Kingdom). Insulin was removed from the culture media unless specifically being studied. Similarly Huh7.5 cells subcultured into 24 well plates were used to quantify effects of VAP-1 activity on lipid uptake and retention. PCLS or cells were then treated with SSAO substrates (methylamine 200 μm, benzylamine 200 μm), recombinant VAP-1 (rVAP-1 500 ng/mL Biotie Therapeutics, Finland), insulin (0.10 IU), hydrogen peroxide (10 μm) or specific enzyme activity inhibitors (VAP-1 activity inhibitor 2-bromoethylamine hydrobromide (BEA) 400 μm, or MOA inhibitor clorgyline 200 μm, MAOB inhibitor pargyline 200 μm, or the lysyl oxidase inhibitor β-aminopropionitrile BAPN, 250 μm) alone or in combination for 18 h. Media alone was used as the control condition. This was followed by a 6-h incubation with 0.25 μm palmitic acid. When using cultured Huh7.5 cells, duplicate plates were treated identically and fixed and stained with Hoechst dye upon termination of experiment for signal normalisation after treatment.

PCLS or Huh7.5 cells were then stained with Oil Red O to permit lipid quantification. Here cells or PCLS were fixed briefly in 60% isopropanol for 5 min followed by a 45 min incubation with Oil Red O solution. After a brief rinse in isopropanol and water, the Oil Red O reagent was solubilized out of the treated cells in order for spectrophotometric quantification. Here 300 μL of isopropanol was incubated in each well on a plate shaker for 5 min. 100 μL of solubilised solution from each well or isopropanol control was added to triplicate wells in a 96 well falcon plate, and the plate was read at an absorbance of 500-520 nm. For PCLS signal was expressed per 500 mg of tissue. For cell enumeration, data from Huh7.5 treated with Oil red O and Hoechst dye was manipulated to express the amount of Oil red O per 100000 cells.

Assessment of triglyceride secretion from PCLS

To quantify triglyceride secreted into the culture supernatant of PCLS under different conditions we used a chromogenic assay (Cayman Chemical Company: Colorimetric TG assay CAT10010303) according to manufacturer's instructions.

RNA extraction from human liver tissue

Human liver tissue samples, roughly 2 cm/2 cm, were preserved in RNA later and stored at -80 °C. To isolate total RNA, blocks were removed from -80 °C storage and placed on ice, approximately 30 mg of tissue was excised. RNA was also extracted from treated PCLS and here the PCLS were submerged in buffer RLT + β-mercaptoethanol immediately after treatment. Total RNA was isolated from 30 mg liver tissue using RNeasy kit (Qiagen). Tissue was dissociated in RLT lysis buffer, placed in gentleMACS™ M Tubes (Miltenyi Biotech) and homogenized by gentleMACS ™Dissociator using program RNA_01.01. RNA concentration and purity were measured using a Nanophotometer™ (IMPLEN).

Quantitative real-time PCR

To investigate the relative expression of the major lipid transporters in primary cells and human liver tissue the Fluidigm® 96.96 Dynamic Array™ was used. Taqman fluorogenic 5′ nuclease assays using gene-specific 5′FAM labeled probes were used in this array. All RNA samples were diluted to 125 ng/μL and approximately 1 μL of total RNA was transcribed to cDNA using the superscript TM III first strand synthesis supermix kit (Invitrogen) according to the manufacturer's instructions. All cDNA samples were then preamplified with the TaqMan PreAmp Master Mix (Applied Biosystems) according to manufacturers instructions. The Preamp thermocycling parameters were as follows: 95 °C for 10 min, 14 cycles at 95 °C for 15 s followed by 60 °C for 4 min. The PreAmplified template was diluted (at least 1:5 dilution) with 20 μL of 1 × Tris-EDTA buffer 100 × (Sigma) and stored at -20 °C until further use.

Fluidigm® 96.96 Dynamic Array™ Integrated Fluidic Circuit chip preparation

The 96.96 syringes containing 150 μL of control line fluid were gently inserted into the dynamic array. The plate was then primed for 20 min on the IFC loader (136 × script), before 5 μL of each sample and probe were dispensed on to the 96.96 dynamic array on respective inlets on the plate. The plate was loaded on the IFC for 1 h and 30 min on the load mix (136 ×) script. The plate was then run in the fluidigm for two hours and 10 min. All conditions were run in triplicate for each tissue or cell sample (Supplementary Table 1).

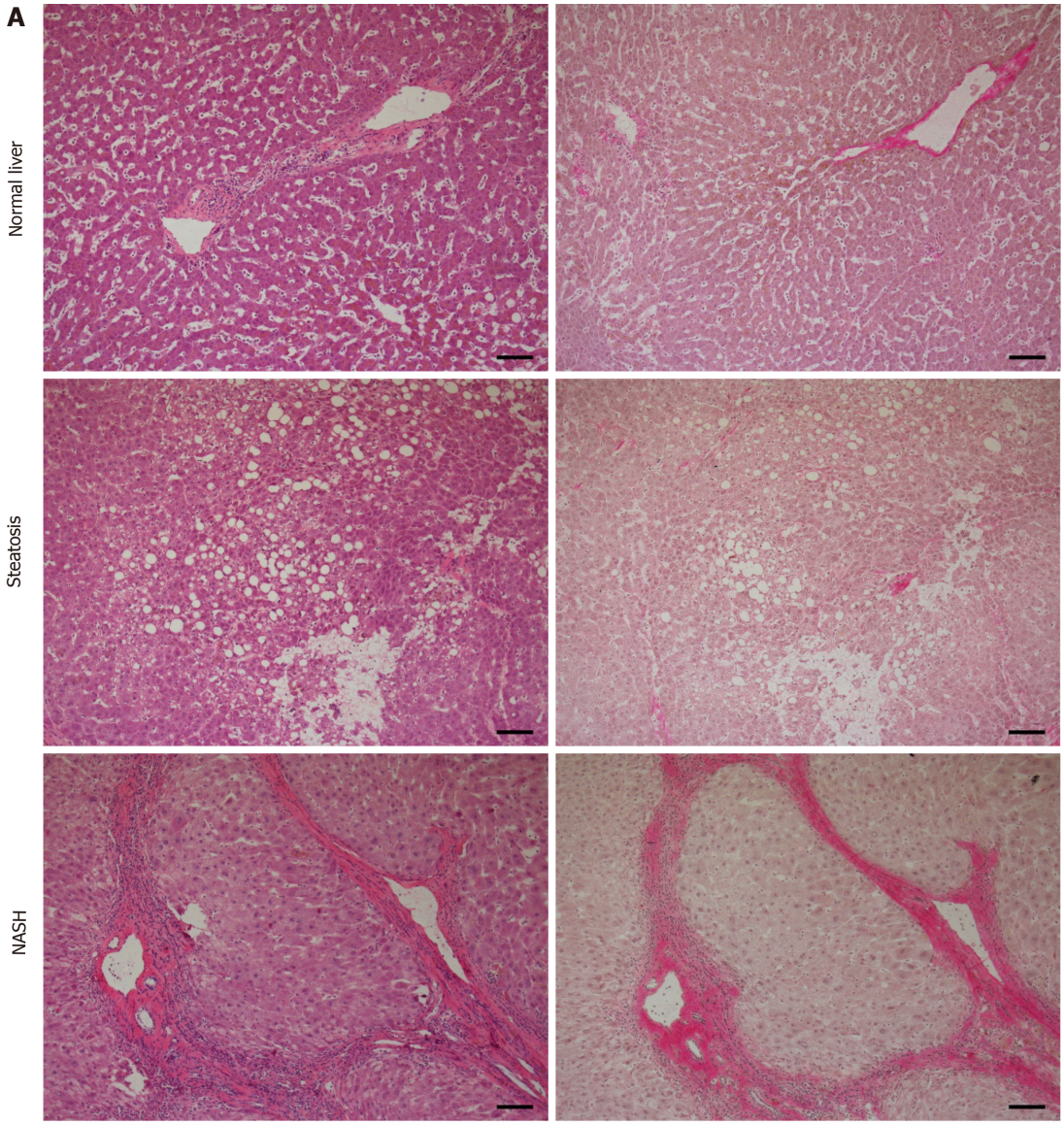
Statistical analysis

Independent student's *t* tests were used to compare means of two samples unless otherwise stated. Analysis was performed using Graphpad Prizm. For gene array data results are expressed as mean of five replicate samples per tissue ± SEM run on triplicate arrays and normalized to pooled endogenous controls (B-actin and GAPDH). Data was log transformed using 2-delta CT. The relative expression values were used to calculate fold changes within each matched tissue sample. Thus for each patient/liver, the mean of the replicates were calculated and expressed as fold change *vs* the control condition.

RESULTS

Expression of VAP-1 increases in chronic liver disease

Several studies have previously demonstrated that VAP-1 is expressed on hepatic cells[29,30] and contributes to inflammation and fibrogenesis[16] in the context of disease. We wished to confirm its presence in our tissue samples and thus selected livers across the spectrum of disease from normal tissue, through steatosis to end stage cirrhosis in NASH. Figure 1A shows example of the typical histological appearance of selected livers within these categories. Fatty donor material demonstrated predominantly macrovesicular steatosis with little evidence of inflammation or fibrosis (Figure 1A). In contrast material from end-stage NASH cirrhosis contained significant lobular and scar associated inflammation. Van Geison's staining confirmed the presence of significant bridging fibrosis and presence of regenerative nodules in the cirrhotic samples as expected. Histochemical staining with antibody directed against VAP-1 (Figure 1B) confirmed staining was localised predominantly to perivascular structures


A
Normal liver
Steatosis
NASH
B
Normal liver
NASH

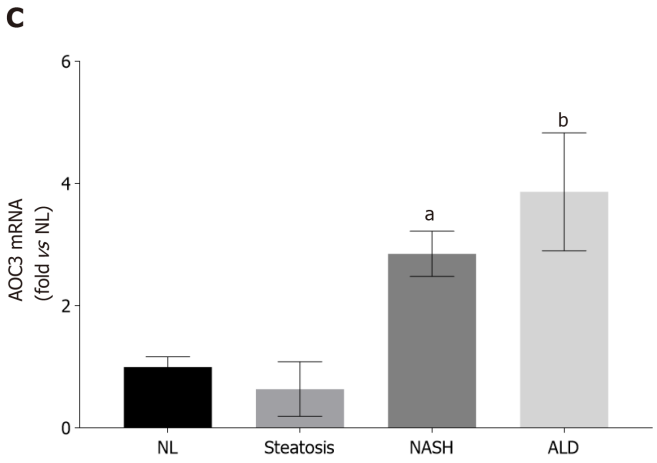


Figure 1 Hepatocellular expression of vascular adhesion protein-1 increases in nonalcoholic steatohepatitis. A: Representative brightfield images of sections from indicated disease types stained with Hematoxylin and Eosin (left panels) and Van Geison's Stain (right panels). Original magnification 10 ×, images representative of multiple fields of view from *n* = 3 Livers of each disease type. Scale bar is 100 μmol/L; B Immunohistochemical staining for vascular adhesion protein-1 (VAP-1) in representative acetone fixed frozen sections from normal and nonalcoholic steatohepatitis (NASH) livers. Isotype matched control antibody was negative (not shown). Fields were captured at 10 × original magnification with inset pictures captured at 40 × original magnification; C: Analysis of VAP-1 (AOC3) expression by quantitative qPCR analysis. mRNA expression of AOC3 in whole liver RNA from normal, steatotic, NASH, and alcohol-related cirrhosis (ALD) livers using fluidigm qPCR array®, run on triplicate arrays. Results are expressed as the mean fold change in gene expression normalized to pooled endogenous controls β-actin and GAPDH relative to normal livers defined as 1 ± SEM with means from five normal l, four steatotic, three NASH, and four ALD livers. [a]*P* < 0.05 or [b]*P* < 0.01 using a one way ANOVA with Bonferroni correction. NASH: Nonalcoholic steatohepatitis; ALD: Alcohol-related cirrhosis.

in normal liver and that expression increased dramatically in fibrotic tissue and was localised within fibrotic tissue and sinusoidal areas. QPCR analysis of AOC3 gene expression confirmed these findings (Figure 1C) with VAP-1 mRNA increasing significantly in the context of NASH. This was similar to the picture seen in alcohol-related cirrhosis (ALD, Figure 1C).

Uptake of fatty acids by hepatocytes is increased in the context of VAP-1 activity

Next we used HuH7.5 cells to determine whether the amine oxidase activity of VAP-1 impacts upon hepatocyte lipid handling. Figure 2 shows that exposure of Huh7.5 cells to Oleic acid for 18 h, leads to accumulation of lipid within the cells that can be extracted and quantified or visualised using Oil red O (Figure 2A). Co-incubation of cells with recombinant Vap-1 and its substrate methylamine, increased uptake whilst methylamine alone had no effect. Importantly addition of exogenous H_2O_2 the recreate the generation *via* the SSAO activity of VAP-1 recapitulated the response (Figure 2B). We also used qPCR arrays to determine the consequences of oleic acid uptake on gene expression by Huh7.5 cells. Figure 2C shows that we observed notable changes in expression of genes linked to lipid transport and partitioning (FABP3, FATP6), and lipid metabolism (PPARA and PPARg) after treatment. To test if the same response would occur in primary hepatocytes we utilised precision cut liver tissue. Slices of approximately 250um thickness were generated from donor liver tissue, and reproducibility of cutting and viability in culture were confirmed (Supplementary Figure 1). PCLS exposed to OA for 24 h maintained morphological integrity and accumulated lipid within hepatocytes (Supplementary Figure 2B). As there was a gradual decline in viability over time, all experiments were performed on PCLS cultured for a maximum of 24 h. Figure 3A shows quantification of triglyceride content in supernatant from treated slices and reveals that exposure of liver tissue to methylamine, VAP-1 or H_2O_2 led to a reduction in triglyceride secretion (Figure 3A) Exposure of cultured normal human liver tissue to VAP-1 also induced a modest but significant lipid accumulation (Figure 3B). In agreement with our data with HuH7.5 cells, exposure to substrate for the SSAO activity of VAP-1 in the form of methylamine also increased lipid accumulation in tissue. However, addition of exogenous recombinant VAP-1 and methylamine resulted in the most dramatic increase. Bromoethylamine (VAP-1 inhibitor) reduced accumulation to control levels, whilst inhibitors of other monoamine oxidases A and B (MAOA and MAOB) did not reduce the uptake seen in the presence of VAP-1 plus substrate. We did note however that use of the lysyl oxidase inhibitor β-aminopropionitrile also reduced lipid accumulation.

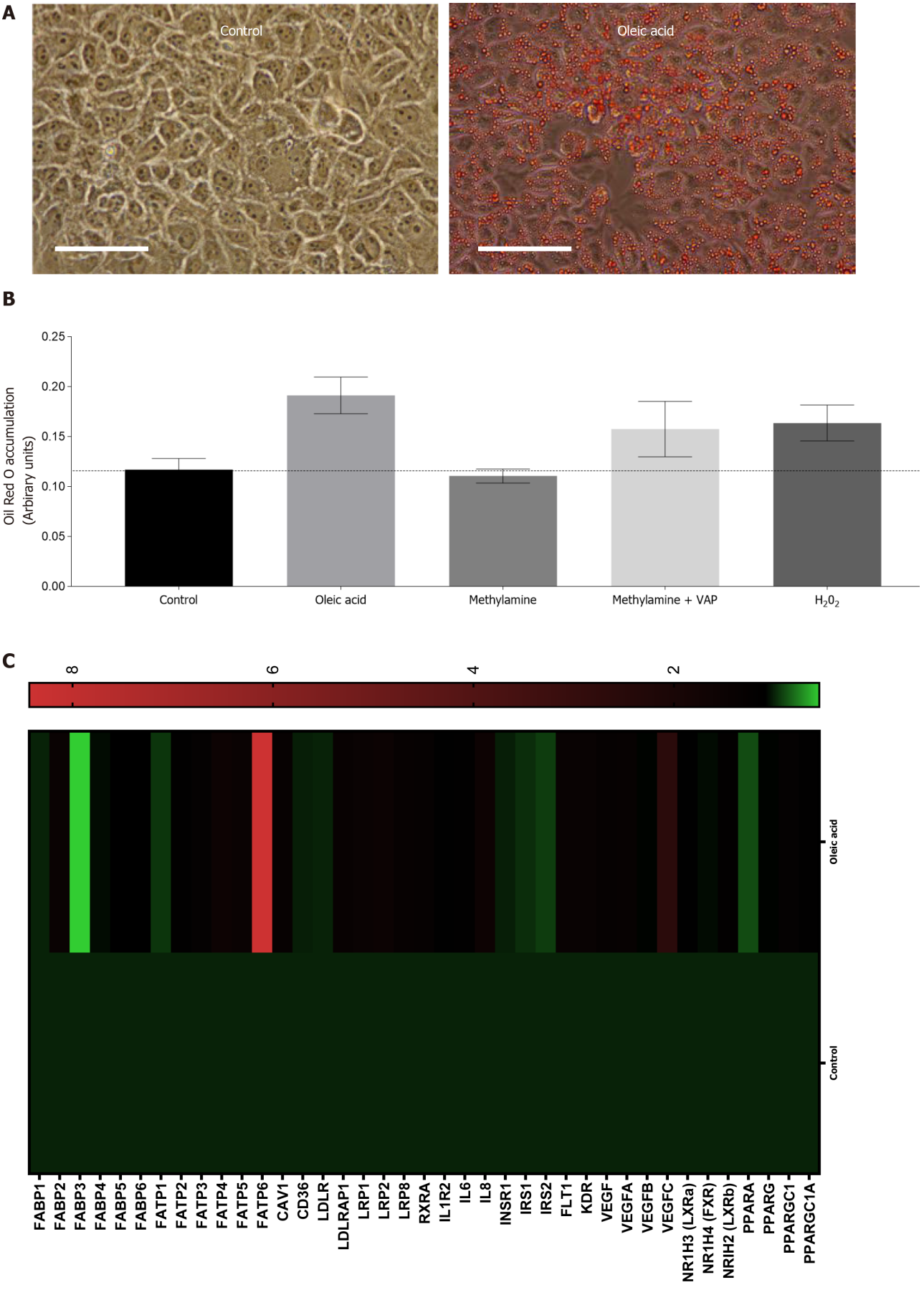


Figure 2 Exposure of Huh7.5 cells to products of vascular adhesion protein-1 enzyme activity leads to lipid accumulation and gene expression changes. A: Representative phase contrast images of confluent control Huh7.5 (left) or cells pretreated with 250 μm Oleic Acid for 6 h. All wells were

fixed and stained with Oil Red O, and images were captured at 40 × original magnification (representative of n = 3 samples per condition). Bar is 50 mm; B: Quantification of oleic acid accumulation after vascular adhesion protein-1 (VAP-1) stimulation. Huh7.5 were pretreated with either methylamine (200 μm) alone, or in combination with recombinant VAP-1 (500 ng/mL) or H_2O_2 (10 μmol/L), for approximately 18 h. This was followed by incubation for 6 h with 250 μm OA. Cells were fixed and stained with Oil Red O and solubilized. Signal is expressed in arbitrary units. Data are mean ± SEM of triplicate experiments; C: Analysis of mRNA expression in HuH7.5 cells after exposure to oleic acid by quantitative qPCR analysis. mRNA expression for indicated genes was assessed using fluidigm qPCR array® according to manufacturer's instructions. Data is expressed as fold changes in relative gene expression compared to pooled housekeeping genes in control (untreated cells). Data are representative of triplicate conditions run on triplicate gene array plates.

Mice deficient in VAP-1 show reduced hepatic steatosis on high fat diet.

To confirm our observation that VAP-1 contributes to hepatic lipid accumulation in a more physiological context we utilised mice deficient in SSAO activity and exposed them to a high fat diet for 12 wk. Figure 3C shows that whilst wild type livers demonstrated extensive macrovesicular hepatic steatosis, this was significantly reduced in SSAO knockout animals. Thus Oil Red O staining quantification showed a significant decrease in knockout animals after 12 wk on diet.

SSAO activity alters expression of hepatic lipid transporters

Finally, in order to determine the possible molecular mechanism of our response we assessed changes in lipid transporter and key metabolic response gene expression following exposure of liver tissue to SSAO substrate. We documented the baseline expression of transporter molecule RNA in normal, steatotic and NASH cirrhotic livers (Supplementary Figure 2). Simple steatosis was associated with modest changes in members of the FABP, and FATP families. In contrast the development of NASH or alcohol related cirrhosis was associated with more profound increases in transporter expression along with changes in proinflammatory mediators and signalling molecules (Supplementary Figure 2). We next used precision cut liver slices used to assess the impact of SSAO activity on transporter molecule gene expression and noted that there were indeed selective changes in expression. Figure 4 shows that in general activation of the endogenous enzymatic capacity of VAP-1within tissue resulted in a modest but significant upregulation of lipid transporter molecules with the notable examples of FABP2, and 4 in addition to FATP4 and 5. We also report increased expression of caveolin 1, VLDLR, IRS1, VEGFc and PPARGC1 (Figure 4 and Supplementary Tables 2 and 3).

DISCUSSION

Recent evidence has implicated the semicarbazide sensitive amine oxidase VAP-1 as a potential therapeutic target in metabolic[23] and liver disease[16] and indicate that levels of soluble VAP-1 in serum[15,31-33] have value as a prognostic marker. We have previously demonstrated that VAP-1 function supports key pathophysiological processes in the progression from NAFLD to NASH through its contribution to glucose homeostasis[34] hepatic inflammation[18,29,30] and fibrosis[16]. In this study we suggest that VAP-1 expression in the human liver is not significantly changed by steatosis, which is similar to the reported similarity in SSAO activity in adipose tissue in obese *vs* lean individuals[35]. However we note a profound increase in hepatic expression of VAP-1 in NASH and other cirrhotic disease in agreement with previous evidence[16,19,33]. We have also previously demonstrated that induction of hepatic steatohepatitis in murine models, leads to increased hepatic VAP-1 expression[16]. There are well described roles of VAP-1 in supporting leukocyte[30,36] and particularly monocyte[23,29] recruitment into tissue, and studies suggesting that inhibition of hepatic monocyte recruitment improves steatohepatitis and fibrosis[37]. Thus targeting VAP-1 may have effects on multiple contributing pathways in the pathogenesis of NAFLD.

One of the hallmarks of NAFLD is the presence of hepatic steatosis which is driven by increased abundance of free fatty acids from diet and adipocytes[38] and as a consequence of hepatic de novo lipogenesis[39]. In particular accumulation of the saturated free fatty acid palmitic acid (PA) is associated with disease progression[40] and hepatocyte lipotoxicity, which are a precursor to development of inflammation and fibrosis in NASH. We saw evidence of steatosis in Huh7.5 cells after exposure to OA (Figure 2), but no significant effect of exposure to methylamine, a substrate for VAP-1. Whilst this amine has been widely demonstrated to drive lipid accumulation and differentiation in adipocytes[41,42], the lack of response here likely reflects the absence of VAP-1 expression in this cell line. However, the combination of

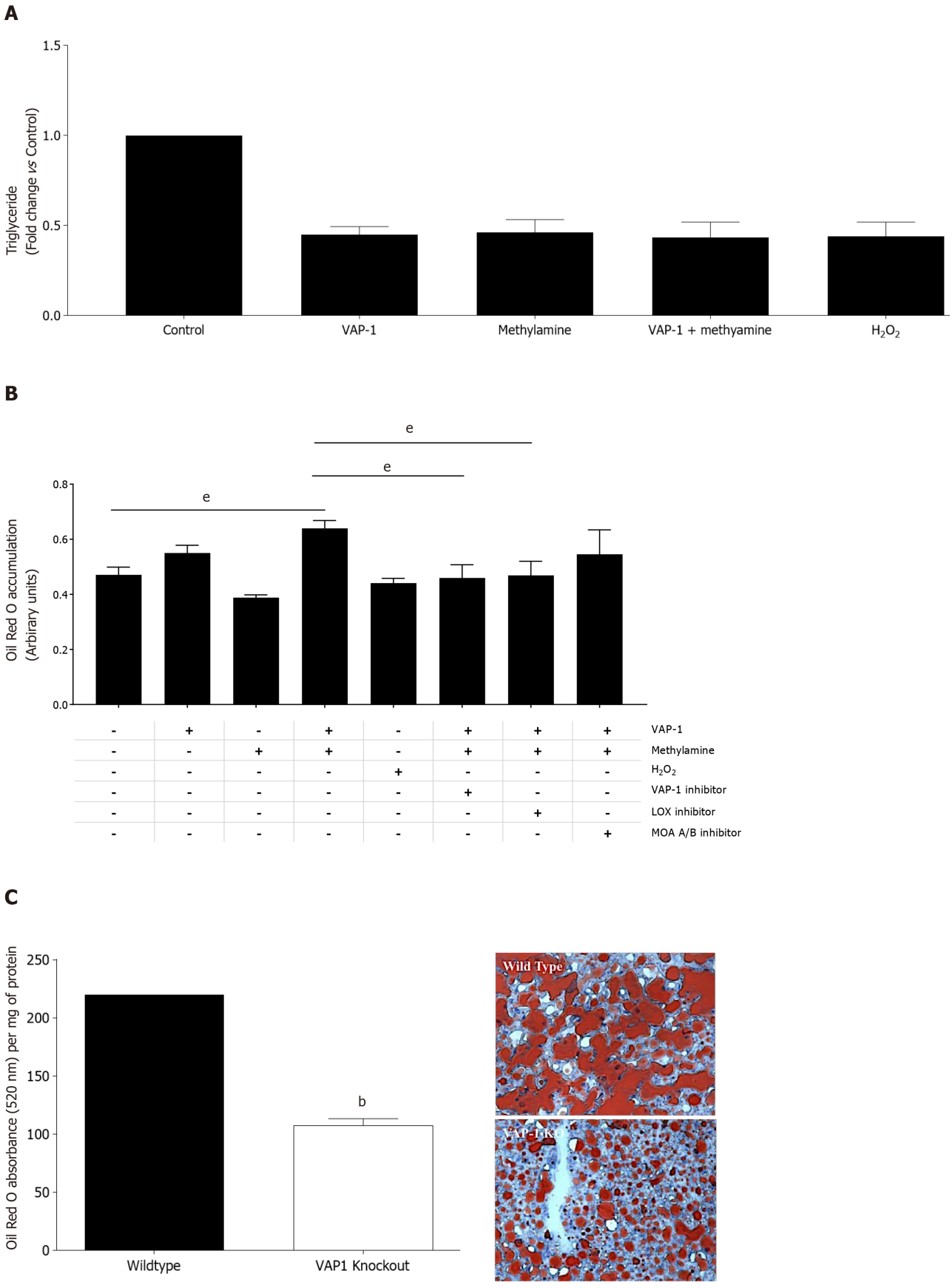


Figure 3 Activation of vascular adhesion protein-1 enzyme activity results in reduced triglyceride export and increased steatosis in human liver tissue and VAP-1/AOC3 knockout protects against high fat diet induced steatosis in mice. A: PCLS were pretreated with either, methylamine 200 μm, vascular adhesion protein-1 (VAP-1) 500 ng, H_2O_2 10 μmol/L, or a combination of methylamine + VAP-1, benzylamine+VAP-1 for approximately 18 h and then 6 h with 250 μm fatty acid. Supernatants were collected after treatments and triglyceride secretion was quantified using a commercial assay (Cayman

Chemical Company) according to manufacturer's instructions. Data are triplicate samples from n = 2 normal livers ± SEM. P < 0.01 for all using a one way ANOVA; B: Lipid uptake in PCLS from normal liver tissue pretreated with either methylamine 200 μm, and rVAP1 500 ng/mL alone or in combination, or, H_2O_2 10 μmol/L. Some slices were exposed to the combination of methylamine and VAP-1 plus selective enzyme inhibitors: Bromoethylamine (VAP-1 inhibitor 400 μm), β-aminopropionitrile (lysyl oxidase inhibitor, BAPN 250 μm) or the Monoamine oxidase A and B inhibitors Clorgylline and Pargylline (both at 200 μm). After approximately 18 h incubation, slices were exposed to 250 μm oleic acid for 6 h. PCLS were fixed and stained with Oil RedO, which was solubilized and signal normalized to per 500 mg of tissue. Data are mean of triplicate samples from n = 2 normal livers ± SEM. Significance expressed as ^{e}P < 0.001 in one way ANOVA with Tukeys correction for multiple comparisons; C: Left - Accumulation of lipid in WT and VAP-1 KO mice fed on a high fat diet for 12 wk. 7um cryosections from WT and VAP-1 KO mouse livers were stained with ORO, which was then solubilized and signal expressed relative to protein concentration for each group of mice, Data are mean ± SEM of three mice per group. Significance expressed as ^{b}P < 0.01 one way ANOVA. Right – representative brightfield microscopy images of Oil red O stained cryosections from WT and VAP-1 KO mice.

recombinant VAP-1 and methylamine induced an accumulation of lipid in these cells. This is likely linked to peroxide generation as a consequence of amine oxidase activity, since addition of exogenous hydrogen peroxide recapitulated the response (Figure 2) and the effect was reduced in the presence of bromoethylamine but not MAOA or B inhibitor (clorgylline and pargyline). Hydrogen peroxide is a known adipocyte lipolysis inhibitor[43], and insulin mimic which increases hepatic glucose uptake[19]. Thus enhanced lipid accumulation within our cells in culture may reflect increased free fatty acid and glucose uptake and reduced lipolysis and export. In support of this concept we performed additional experiments using precision cut liver tissue slices. Here an endogenous supply of both VAP-1 and physiological amines would be present. Once again the administration of oleic acid induced a modest hepatocyte-specific lipid accumulation, but we also confirmed that methylamine induced lipid accumulation within the tissue, most likely due to activation of endogenous VAP-1. Similar results have been reported *in vivo* where benzylamine increases adipose tissue fat deposition in diabetic rats[44], and transgenic mice overexpressing VAP-1/SSAO supplemented with methylamine have increased BMI and abdominal fat pad weight[26] however this is the first observation in human liver tissue. Interestingly addition of exogenous VAP-1 caused a profound steatosis, suggesting the presence of exogenous substrates within tissue such as tyramine, histamine or dopamine[45]. When both VAP-1 + MA were added, the stimulatory effect was less marked which may suggest preferential or competitive use of methylamine or benzylamine over endogenous substrates. This also suggests the endogenous substrates may have multiple or more potent effects. In agreement Salmi *et al*[46], have shown that addition of BA reduces VAP-1-dependent lymphocyte binding to endothelial cells. Thus they suggested BA was a competitor for an endogenous substrate for VAP-1[46]. Regardless of substrate specificity, the observation that BEA specifically inhibited the MA/BA + VAP-1 effect, and that H_2O_2 recapitulates the response confirms that VAP-1/SSAO alters lipid accumulation in PCLS. We also observed reduced triglyceride export from treated slices in the context of VAP-1 activation which suggests that net accumulation of lipid is linked to both increased FFA uptake and reduced triglyceride export.

Our studies with mice deficient in VAP-1 suggested that this protected the animals from hepatic steatosis induced by high-fat diet exposure. To explain this, and our reductions in TG export and increased steatosis when SSAO activity was primed, we performed PCR arrays on cultured cells and liver tissue slices to quantify changes in expression of key lipid transporters and proinflammatory molecules. This also shed light on mechanisms which underpin the characteristic insulin resistance and altered sugar handling that are also a feature of metabolic syndrome and have previously been attributed at least in part to VAP-1 activity[8,47,48]. Our baseline analysis of liver tissue across the spectrum of NAFLD (Supplementary Figure 2) confirmed increasing dysregulation of insulin responses, PPAR activity and lipid transport and metabolism[49] as disease progresses, in keeping with altered fatty acid partitioning and binding, and lipolysis. For example FATP6 is known to play a role in the uptake of long chain fatty acids such as oleic and palmitic acid[50] and as such our increased expression in oleic acid exposed hepatocytes, or methylamine treated PCLS makes sense. Similarly, CD36 increases on hepatocytes during diet induced obesity in rodent models, and correlates with extent of hepatic triglyceride storage and secretion[51]. We observed increased RNA expression in liver samples from patients with steatosis in NASH and ALD. In conditions of lipid excess, this transmembrane scavenger receptor can operate to transport long chain fatty acids into the cell for transition into lipid droplets or use as an energy source. Mice with CD36-deficient hepatocytes are protected from high fat diet induced hepatic steatosis and have improved insulin sensitivity[52]. Thus our altered CD36 expression in diseased liver tissue may reflect a compensatory response to nutrient excess.

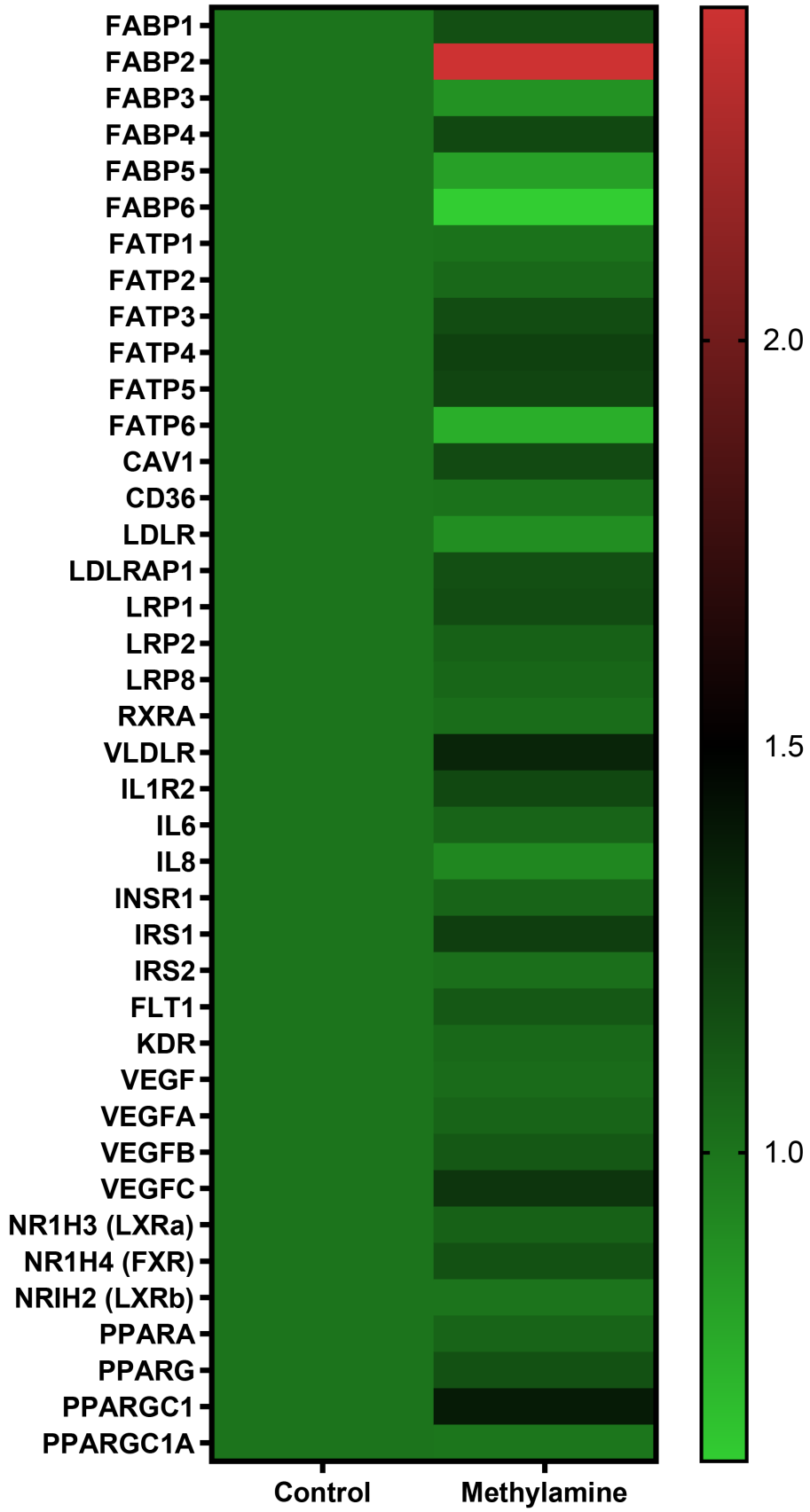


Figure 4 Exposure of precision-cut liver slices from human donor liver tissue cells to substrate for vascular adhesion protein-1 enzyme activity leads to gene expression changes. Precision-cut liver slices were treated with methylamine 200 μm for approximately 4.5 h. RNA was extracted and mRNA expression was carried out using a fluidigm qPCR array® run on triplicate arrays. Results are expressed as the mean fold change in gene expression normalized to pooled endogenous controls β-actin and GAPDH relative to untreated control livers. Data are indicative of triplicate arrays prepared from 2 donor livers.

The dramatic upregulation of FABP2 on steatotic tissue slices after even a short exposure to methylamine is in agreement with studies suggesting that FABP2 upregulation on cell lines is associated with reduced lipoprotein export. FABP4 was also increased in both treated cells and slices and was particularly elevated in NASH[49] and ALD liver tissue. FABP4 is shed from adipose tissue and plasma FABP4 Levels are considered an early indicator in the development of the metabolic syndrome[53]. Given the reported regulation of adipocyte FABP4 expression by PPARα[54], and the acquisition of an adipocyte-like phenotype by hepatocytes during steatotic liver injury this may also relate to PPARα activation[55] following VAP-1 engagement. Thus it is possible that similar regulatory mechanisms govern expression of FABP4 in hepatocytes. Increasing clinical evidence for targeting hepatic fat metabolism *via* PPAR blockade[56] is in keeping with our elevated expression in diseased tissue and after priming of VAP-1 activity *in vitro*.

We also noted early changes in expression of mRNA for Caveolin-1, an integral membrane protein found in caveoli that has been linked to formation and function of these intracellular structures[57]. In particular the molecule has been linked to insulin signalling and translocation of GLUT4 to the cell membrane[19], and is increased during adipogenesis[58]. Our reported increases in treated PCLS and diseased liver fit with the ability of hydrogen peroxide generated as a consequence of the enzyme activity of VAP-1 to prime uptake of glucose as a fuel for de novo lipogenesis. Our reported alterations in insulin receptor subunit expression are also suggestive of altered insulin-dependent responses in NAFLD and fit with the changes in insulin receptor expression when tissue slices are exposed to methylamine. Importantly the changes in gene expression reported in our tissue slice studies, occurred after a relatively short period of *in vitro* VAP-1 activation (4.5 h). Thus it is perhaps not surprising that we see only small but significant magnitude changes in expression of genes involved in the early stages of nutrient handling such as FABP2, VLDLR, caveolin and Insulin receptor subunits at this timepoint. We also note that it would be important to utilize specific VAP-1 inhibitors such as semicarbazide or bromoethylamine to confirm that the gene expression changes we report when cells and tissue slices are exposed to methylamine do indeed relate to its specific metabolism by VAP-1.

The contribution of other amine oxidase enzymes was tested through addition of BAPN, Clorgylline or Pargylline in combination with MA/BA + VAP-1. These inhibitors did not lead to inhibition of lipid accumulation, and if anything caused an increase. We observed the same effect in Huh7.5 exposed to methylamine (Figure 2) suggesting substrate, and possibly cell-specific effects of inhibitors. Interestingly when clorgylline and pargyline were added in combination with exogenouse VAP-1 we observed an increase in lipid accumulation compared to OA alone. This may suggest that monoamine oxidase blockade leads to upregulation of VAP-1 activity, or these inhibitors may be causing allosteric effects in VAP-1 thus increasing enzyme activity and lipid accumulation. Of note, presence of the LOX inhibitor BAPN did alter liver lipid accumulation when VAP-1 and methylamine were also present. Since administration of BAPN does not reduce weight gain in atherogenic rat models[59] it is unlikely that LOX has a significant role in systemic lipid handling. There are also reports suggesting that this agent is not specific for LOX and may also have a moderate inhibitory effect on VAP-1 in some cells[41,60]. Thus our response may reflect SSAO inhibition by BAPN. However oxidation of lysine by LOX leads to collagen and elastin crosslinking and ECM remodelling, and thus increased expression in fibrotic NASH livers is in keeping with previous reports of increases on hepatic stellate cells and myofibroblasts in disease[61], and antifibrotic benefit of lysyl oxidase blockade[62].

CONCLUSION

Thus, in conclusion we have used human and murine model systems to demonstrate that metabolic features of NAFLD, linked to altered glucose and insulin responses, steatosis and lipid uptake and altered triglyceride export are all influenced by the amine oxidase activity of VAP-1. These findings are summarized in Figure 5. In light of previous evidence showing that VAP-1 also plays a role in M2 macrophage infiltration[63] and IL-1b function in steatosis, has roles in atheroma development[23] and influences hepatic inflammation and fibrogenesis[16] we would argue that the increased pharmaceutical interest in amine oxidase inhibitors is well placed[64].

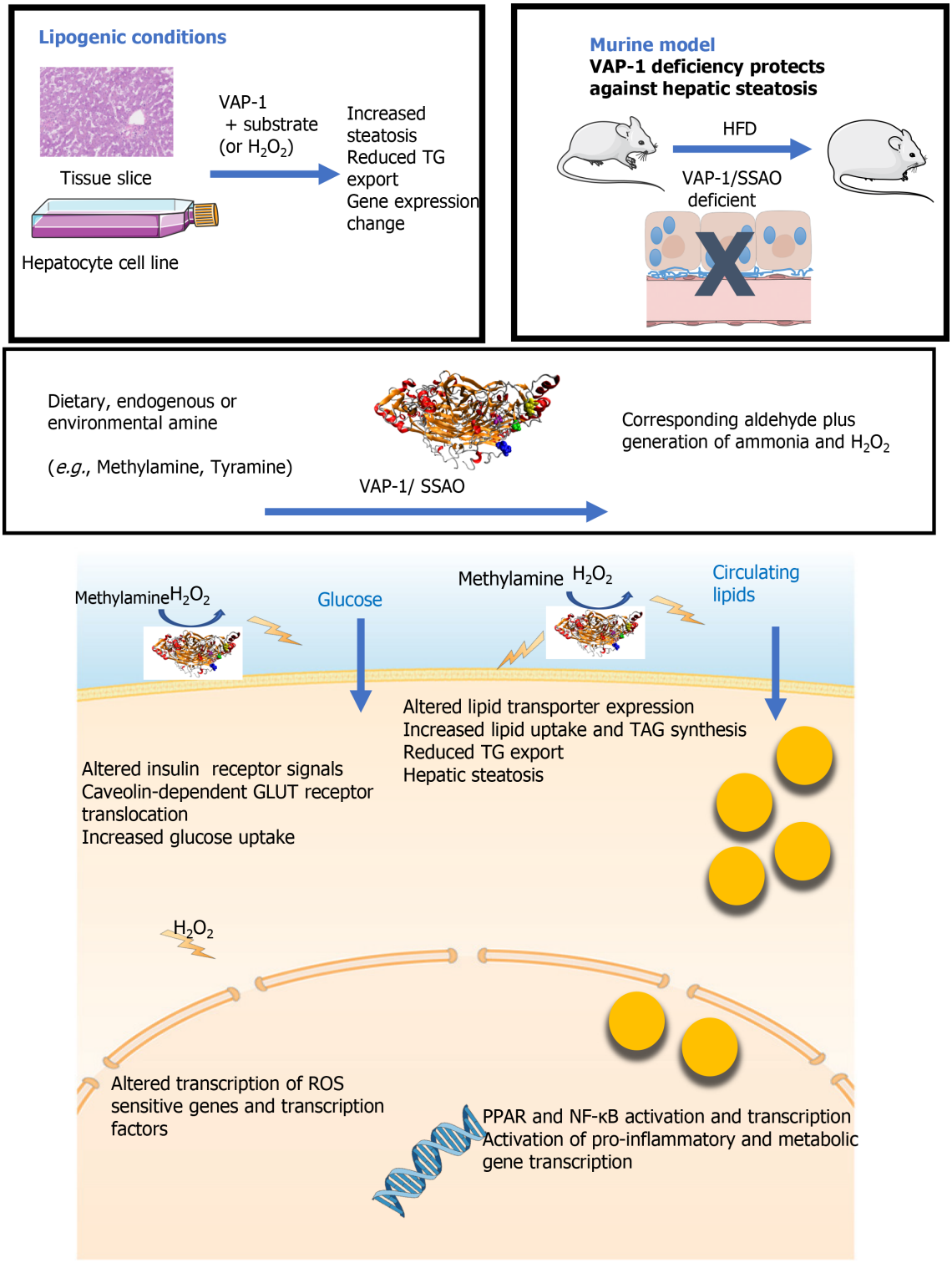


Figure 5 Graphical summary the impact of vascular adhesion protein-1 on the hepatic pathogenesis of non-alcoholic fatty liver disease. Exposure of human liver tissue or hepatocytes in culture to vascular adhesion protein-1 (VAP-1) in the presence of endogenous or exogenous (methylamine) substrate led to a reduction in TG export and increased steatosis. In tissue this was accompanied by changes in metabolic gene expression. VAP-1 deficient mice are protected against hepatic steatosis when fed a high fat diet. These findings can be explained by the enzymatic capacity of VAP-1 to reduce amine substrates to the corresponding aldehyde accompanied by generation of potent signaling molecules such as hydrogen peroxide. Addition of hydrogen peroxide to our culture systems recapitulated the effects of VAP-1 activation. Our previous studies suggest that increased inflammation, steatosis and fibrosis in the context of non-alcoholic fatty liver disease in part relate to the ability of VAP-1 to support leukocyte recruitment across endothelial cells, to prime hepatic glucose uptake and to activate hepatic stellate cells. We now show additional effects on transcription of key lipid transporter molecules and transcription factors. The upregulation of FABP4, FABP2, FATP3-5 and LRP1 along with the VLDLR which alter uptake and intracellular targeting of lipid molecules, Transport of fatty acids to the nucleus by receptors such as FABP2 will also activate nuclear receptors such as PPARs and NF-κB, hence influencing gene transcription. VAP-1: Vascular adhesion protein-1; HFD: High fat diet.

ARTICLE HIGHLIGHTS

Research background

Current standard of care for non-alcoholic fatty liver disease (NAFLD) patients varies according to disease stage, but includes lifestyle interventions common insulin sensitizers, antioxidants and lipid modifiers. However, to date specific therapies for have shown little histological or fibrosis stage improvement in large clinical trials and there is still no licensed therapy for NAFLD. Given the high prevalence, limited treatment options and significant screening costs for the general population, new treatments are urgently required.

Research motivation

Vascular adhesion protein-1 (VAP-1) is an enzyme with proven contributions to systemic and hepatic glucose handling, inflammation and fibrosis. We now show an additional role in hepatic steatosis.

Research objectives

In the current investigation, we aimed to assess the potential for inhibition of the amine oxidase enzyme VAP-1 to modify hepatic lipid accumulation in NAFLD.

Research methods

We have used a combination of human cell cultures, a murine model and human precision cut liver slices to understand the contribution of the semicarbazide sensitive amine oxidase enzyme VAP-1 to lipid handling in NAFLD. This molecule is of increasing therapeutic interest due to its ability to regulate hepatic inflammation and fibrosis.

Research results

VAP-1 increases lipid accumulation and reduces triglyceride export by hepatocytes. This is linked to alterations in expression of key lipid transporters including FABP1, 2 and 4, FATP2-5 and LRP1 and key regulators such as PPARα. In agreement, VAP-1 deficient mice are protected against steatosis on high fat diet.

Research conclusions

We suggest the multifaceted effects of VAP-1 within the liver in NAFLD make it an interesting target for pharmacological intervention.

Research perspectives

We would argue that the increased pharmaceutical interest in amine oxidase inhibitors is well placed.

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