Lipid-induced Signaling Causes Release of Inflammatory Extracellular Vesicles from Hepatocytes

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Abstract

BACKGROUND & AIMS—Hepatocyte cellular dysfunction and death induced by lipids, and macrophage-associated inflammation are characteristics of nonalcoholic steatohepatitis (NASH). The fatty acid palmitate can activate death receptor 5 (DR5) on hepatocytes, leading to their death, but little is known about how this process contributes to macrophage-associated inflammation. We investigated whether lipid-induced DR5 signaling results in release of extracellular vesicles (EV) from hepatocytes, and whether these can induce an inflammatory macrophage phenotype.

METHODS—Primary mouse and human hepatocytes and Huh7 cells were incubated with palmitate, its metabolite lysophosphatidylcholine, or diluent (control). The released EV were isolated, characterized, quantified, and applied to macrophages. C57BL/6 mice were placed on chow or a diet high in fat, fructose, and cholesterol to induce NASH. Some mice were also given the ROCK1 inhibitor fasudil; 2 weeks later, serum EVs were isolated and characterized by immunoblot and nanoparticle-tracking analyses. Livers were collected and analyzed by histology, immunohistochemistry, and quantitative PCR.

RESULTS—Incubation of primary hepatocytes and Huh7 cells with palmitate or lysophosphatidylcholine increased their release of EV, compared with control cells. This release was reduced by inactivating mediators of the DR5 signaling pathway or ROCK1 inhibition. Hepatocyte-derived EV contained TRAIL and induced expression of interleukin-1, beta (Il1b) and Il6 mRNAs in mouse bone marrow-derived macrophages. Activation of macrophages required...
DR5 and RIP1. Administration of the ROCK1 inhibitor fasudil to mice with NASH reduced serum levels of EV; this reduction was associated with decreased liver injury, inflammation, and fibrosis.

**CONCLUSIONS**—Lipids, which stimulate DR5, induce release of hepatocyte EV, which activate an inflammatory phenotype in macrophages. Strategies to inhibit ROCK1-dependent release of EV by hepatocytes might be developed for treatment of patients with NASH.

**Keywords**

exosomes; microvesicles; lipotoxic; cell death

**INTRODUCTION**

Nonalcoholic steatohepatitis (NASH), characterized by hepatic lipotoxicity, inflammation and varying stages of fibrosis, has become a significant public health concern, confounded by the absence of effective pharmacotherapy. A better understanding of the molecular and cellular mechanisms underlying NASH development and progression may facilitate identification of novel therapeutic strategies.

NASH is characterized pathologically by hepatocellular lipoapoptosis (i.e., apoptosis induced by toxic lipid mediators) and inflammatory cell infiltration represented, in part, by activated macrophages.1 We have recently reported that death receptor 5 (DR5, also known as TNFRSF10B) signaling pathways play a key role in mediating hepatocyte lipoapoptosis and macrophage activation *in vitro* and *in vivo*.2, 3 For example, saturated free fatty acids (FFA), which are elevated in NASH and mediate lipoapoptosis,1 can activate the DR5 proapoptotic signaling cascade via a ligand-independent manner in cultured hepatocytes.2 In a mouse model of NASH, DR5 genetic inactivation is quite salutary and represses hepatocyte lipoapoptosis, macrophage-associated inflammation and fibrosis.3 A decrease in hepatocyte lipotoxicity by DR5 genetic deletion would be anticipated to secondarily attenuate liver inflammation by macrophages. However, macrophage DR5 deficiency also represses their activation in lipotoxic-relevant *in vitro* paradigms in a cell-autonomous manner, suggesting DR5 initiates proinflammatory signals in macrophages. Collectively, these observations implicate a dual role for DR5 signaling in NASH: i) a hepatocytic injury process; and ii) a proinflammatory signaling cascade in macrophages. However, it remains enigmatic how the hepatocyte injury promotes DR5-mediated macrophage activation.

Extracellular vesicles (EV), such as exosomes and microvesicles, have recently been recognized as critical mediators of cell-to-cell communication in health and disease states.4 EV are membrane-defined nanometer-sized vesicles released by cells into the extracellular milieu in a highly regulated manner. Conventionally, exosomes originate from intracellular multivesicular bodies while microvesicles bud directly from the plasma membrane.4 Following their release, EV interact with target cells, in which they may trigger a myriad of responses. The missing link between hepatocyte injury and development of inflammation led us to propose that proapoptotic lipotoxic signaling by DR5 may induce release of proinflammatory EV from hepatocytes, which, in turn, activate macrophages by a DR5-dependent process. We hypothesized that a likely mechanism mediating this intercellular...
communication would be release of TRAIL-bearing EV from hepatocytes which stimulate proinflammatory cascades in macrophages.

**METHODS**

Please see supplemental material for detailed description of all experimental procedures.

**RESULTS**

**Lipotoxicity induces release of EV from hepatocytes**

The FFA palmitate induces hepatocyte lipoapoptosis via its intracellular metabolite lysophosphatidyl choline (LPC), which also accumulates in the liver of NAFLD patients proportionally to disease severity. To explore the effect of lipotoxicity on EV release, we first established treatment conditions which does not induce cell death in primary hepatocytes and Huh7 cells to avoid collection of apoptotic bodies. A 4-hour treatment with 20 μM LPC did not induce apoptosis in these cells (Supplementary Fig. 1B–C) and thus was utilized for our experiments. Upon the LPC treatment, released EV were isolated from the cell culture media and quantified by nanoparticle tracking analysis (NTA). Over a 4-hour incubation period, LPC induced a ~3-fold increase in release of EV in mouse hepatocytes (Fig. 1A). According to NTA, the size distribution of hepatocyte-derived EV was 40–300 nm with a mode size of 85 nm; these size characteristics were confirmed by electron microscopy (Fig. 1C). Treatment with LPC slightly increased the mean size of the EV (123 nm, Fig. 1B). Immunoblot analysis indicated that isolated EV contained established exosomal and microvesicular markers such as Alix, TSG101, and ARF6 (Fig. 1D). LPC had virtually the same effect on EV release in primary rat and human hepatocytes (Fig. 1E). Interestingly, the human hepatoma cell line Huh7 dramatically responded to LPC treatment (a ~400-fold increase in EV release, Fig. 1E and Supplementary Fig. 1D–E). Because LPC is an active metabolite of palmitate, we next assessed whether the parent compound also affects EV release. Primary mouse, rat and human hepatocytes and Huh7 cells were treated with the palmitate (C16:0) and the non-toxic monounsaturated oleate (C18:1), as a control. Indeed, palmitate had a similar effect to LPC in primary hepatocytes and Huh7 cells. Of note, the non-toxic oleate did not induce significant EV release compared to vehicle-treated controls (Fig. 1F). Collectively, these data confirm that toxic lipids induce EV release from human, rat and mouse hepatocytes.

**Lipotoxicity-induced EV release is DR5, caspase and ROCK1-dependent**

Lipotoxicity in hepatocytes *in vivo* and *in vitro* is, in part, mediated via ligand-independent DR5 signaling and caspase activation. We examined the potential role of DR5 proapoptotic signaling cascade in EV release by employing hepatocytes isolated from mice genetically deficient in DR5, caspase 8 (hepatocyte-specific) and caspase 3. While in wild-type (WT) mouse hepatocytes LPC induced at least a 3-fold increase in EV release, in DR5−/−, caspase 8−/− and caspase 3−/− hepatocytes LPC treatment failed to induce EV release above that observed in vehicle-treated controls (Fig. 2A). Ligand-independent DR5 activation can also be induced by endoplasmic reticulum stress via CHOP (CCAAT/enhancer-binding protein homologous protein) activity. Indeed, hepatocytes isolated from...
CHOP−/− mice also failed to display an increase in EV release following LPC treatment (Fig. 2A). In line with these observations, genetic targeting of caspase 8 by shRNA or pharmacological inhibition with a pan-caspase inhibitor zVAD-fmk also decreased LPC-induced EV release from Huh7 cells (Fig. 2B). During proapoptotic stimuli, active caspase 3 cleaves and activates Rho-associated kinase 1 (ROCK1), which promotes plasma membrane blebbing and perhaps shedding of microvesicles.6, 7 Therefore, we posited that ROCK1 is involved in EV release during hepatocyte lipotoxicity. Although LPC treatment did not induce significant apoptosis in Huh7 cells at 4 h of incubation (Supplementary Fig. 1C), it did result in generation of cleaved ROCK1, an active form of this kinase (Fig. 2C). Both ROCK1 shRNA-mediated knockdown and pharmacologic inhibition by fasudil were associated with a significant decrease in EV release in response to LPC (Fig. 2C).

Interestingly, EV isolated from vehicle and LPC-treated Huh7 cells had a distinct protein composition. EV from vehicle-treated cells were enriched in typical exosomal proteins such as LAMP-1, Alix, TSG101, and CD634. On the other hand, proteins such as beta-actin and ARF-6, a protein required for vesicle budding from plasma membrane5, were more abundant in EV from LPC-treated cells. Quite strikingly, plasma membrane asialoglycoprotein receptor (ASGPR) 1, which is selectively expressed by hepatocytes, was very abundant in lipotoxic vesicles, but was absent in EV from vehicle-treated cells (Fig. 2D). Most released exosomes (by current definition) appear to originate from a Rab27-dependent event where Rab27b is mediating docking of multivesicular bodies to the plasma membrane.8 To assess whether lipotoxicity-induced EV represent exosomes, we silenced Rab27b using siRNA approach as described previously.9 Reducing mRNA expression of Rab27b by 90% did not significantly affect LPC-induced EV release from Huh7 cells (Fig. 2E). Besides genetic manipulation, we examined effects of pharmacological inhibition of exosome release. It is well established that GW4869, an inhibitor of neutral sphingomyelinase, blocks exosomes biogenesis and effectively diminishes exosome release at a concentration range 1–10 μM.10 In our experiments, however, pre-treatment with GW4869 (10 μM, 20 h) had no effect on the number of released EV from LPC-treated Huh7 cells (Fig. 2F). Taken together, lipotoxicity-induced EV release by hepatocytes appears to be mediated via a DR5 proapoptotic signaling pathway which involves downstream caspase activation, with proteolytic activation of ROCK1. Moreover, lipotoxicity-induced EV likely originate from the plasma membrane and, therefore, represent microvesicles.

Lipotoxic hepatocyte-derived EV activate macrophages in a DR5-dependent manner

To ascertain if hepatocyte lipotoxicity may elicit macrophage inflammatory responses via EV, mouse bone marrow-derived macrophages (BMDM) were incubated with purified EV released from cultured primary mouse hepatocytes. Besides LPC-induced hepatocyte lipotoxicity, hepatocytes isolated from mice fed the FFC diet (high saturated fat, high fructose and high cholesterol) were utilized to simulate chronic lipotoxicity. Macrophage activation was assessed by induction of proinflammatory cytokines interleukin (IL)-1β and IL-6 and amount of secreted IL-6 by ELISA. Purified EV (10^10/mL) from LPC-treated hepatocytes or FFC diet hepatocytes significantly increased IL-1β and IL-6 mRNA expression and secretion. This effect was not observed when employing EV released from hepatocytes isolated from chow-fed mice or inert latex beads of the same size and concentration (Fig. 3A). Lipotoxic hepatocyte-derived EV increased gene expression of
cytokines that are characteristic of classical activation (M1-like phenotype), but not alternative macrophage activation (M2-like, Supplementary Fig. 2A), suggesting that lipotoxic EV promote a proinflammatory responses. To demonstrate that an EV-unrelated fraction was not responsible for macrophage activation, we destroyed EV by boiling and observed that this abolished their stimulatory activity (Supplementary Fig. 2B). We then examined whether phagocytosis is required for macrophage activation by EV. Cytochalasin D, a pharmacological inhibitor of phagocytosis, efficiently inhibited phagocytosis of fluorescently labeled EV, but had no effect on macrophage activation (Supplementary Fig. 2C–D). These data suggest that a protein component of EV and EV-macrophage interaction at the level of plasma membrane is likely responsible for proinflammatory macrophage activation.

We have previously reported that the FFC diet induces hepatocyte mRNA expression of the DR5 ligand (TRAIL) and that DR5 deficiency suppresses macrophage-associated inflammation in a murine model of NASH. Thus, besides expression of TRAIL by hepatocytes (Supplementary Fig. 2D), we also examined hepatocyte-derived EV by immunoblot analysis and identified TRAIL associated with the purified EV (Fig. 3B). A monoclonal antibody against TRAIL detected a ~65 kDa band in EV preparations as well as in Jurkat cells, a positive control for TRAIL protein. The higher molecular size of TRAIL on EV has been previously described and may represent homotrimeric TRAIL aggregates. In parallel, the presence of TRAIL on hepatocyte-derived EV was also confirmed by immunogold labeling and electron microscopy (Fig. 3C). In accordance with these observations, the EV-induced upregulation of IL-1β and IL-6 and increased IL-6 secretion was blunted in DR5−/− macrophages, suggesting that DR5 signaling also plays a role in macrophage activation (Fig. 3D). In addition, the effect of circulating vesicles from chow and FFC diet-fed mice on macrophage activation was examined. Serum EV from FFC diet-fed mice induced a several fold increase in the proinflammatory cytokines expression and secretion in BMDM, while those from chow-fed mice had no effect compared to control (Fig. 3E). To assess human disease relevance of this phenomenon, serum EV were isolated from patients with NASH. Circulating EV from NASH patients significantly induced expression and secretion of IL-1β, while EV from healthy subjects lacked these effects (Fig. 3F). Together, these data suggest that hepatocyte-derived TRAIL-bearing EV are capable of activating macrophages in a DR5-dependent manner, likely by presenting the cognate ligand to the acceptor macrophages and that circulating EV in NASH have proinflammatory properties.

**Mechanism of TRAIL-mediated macrophage activation**

Inflammatory signaling by TRAIL, as opposed to proapoptotic signaling, remains incompletely understood. To gain greater insight regarding TRAIL signaling in macrophages, we employed recombinant human TRAIL, primary human macrophages and human monocytic cell line THP-1, which can be differentiated into macrophage-like cells. Treatment with TRAIL induced IL-1β mRNA levels in both THP-1 macrophages and primary human macrophages (Fig. 4A). In non-apoptotic signaling by TRAIL, RIP1, a kinase that interacts with the death domains of adaptor proteins FADD (Fas-associated death domain) and TRADD (TNFRSF1A-associated via death domain), is recruited to a secondary
cytoplasmic complex which activates several non-apoptotic signaling cascades. Therefore, we further interrogated the signaling complexes responsible for TRAIL-initiated proinflammatory signaling by performing immunoprecipitation studies. TRAIL treatment in THP-1 macrophages induced formation of a complex formed by FADD, RIP1, TRADD, cIAP-1 (inhibitor of apoptosis protein-1), cFLIP\L (long splice variant of cellular caspase-8 (FLICE)-like inhibitory protein), TRAF6 (TNF receptor associated factor 6), and TRAF-6 downstream kinase IKK\β (inhibitor of IκB kinase-β) in the absence of DR5 and RIP3 (Fig. 4B). The absence of DR5 and RIP3 in the complex suggested that TRAIL induced a secondary signaling complex as opposed to a proapoptotic and/or necroptotic signaling complex, respectively. Unexpectedly, caspase 8 in this complex was detected in all the time points following TRAIL-treatment (including time 0) precluding an assessment of participation of this protein in the signaling cascade. To further confirm involvement of a FADD/RIP1 signaling complex in macrophage activation, we utilized RIP1\−/− THP-1 cells, in which the gene encoding RIP1 was disrupted using CRISPR/Cas9 technology. Wild-type, but not RIP1\−/−, THP-1 cells acquired an activated phenotype upon incubation with LPC-treated Huh7-derived EV or recombinant human TRAIL, as demonstrated by upregulation and increased secretion of IL-1β (Fig. 4C–D). Lipotoxic Huh7-derived EV also induced a several fold increase of IL-1β in BMDM (not shown). Proinflammatory signaling often converges on nuclear factor-κB (NF-κB) to induce transcriptional regulation of inflammatory cytokines such as IL-1β. Indeed, treatment of THP-1 macrophages with lipotoxic EV or TRAIL resulted in NF-κB pathway activation as evidenced by increased nuclear translocation of p65, an NF-κB component responsible for its transcriptional activity (Fig. 4E). This, however, was not observed in RIP1\−/− THP-1 macrophages. These data are consistent with a recent report demonstrating that TRAIL-induced NF-κB activation in cells expressing cFLIP is dependent on RIP1. To corroborate RIP1 complex involvement in activation of primary macrophages, we also utilized a highly selective inhibitor of RIP1, necrostatin-1. Pre-treatment with necrostatin-1 prevented mouse BMDM activation induced by TRAIL-bearing EV, and, again, this was associated with decreased nuclear translocation of p65 (Fig. 4F, Supplementary Fig. 2F). Collectively, the data are consistent with non-canonical TRAIL signaling in macrophages resulting in an activated proinflammatory phenotype. The proinflammatory signaling cascade appears to be RIP1-dependent as it is attenuated by necrostatin-1 or RIP1 deletion.

**ROCK1 inhibition improves NASH in vivo**

Next, we employed a murine model of NASH to assess the potential role of hepatocyte-derived EV in NASH-associated liver injury, inflammation and fibrosis. To modulate EV release in vivo, we utilized a ROCK1 inhibitor, fasudil, which effectively blocked lipotoxicity-induced EV release in our in vitro experiments (Fig. 2D). Mice were fed the FFC diet for 6 months followed by a 2-week administration of fasudil (50 mg/kg p.o. q.d.). After completion of the study, circulating EV were isolated from serum and quantified by NTA. The FFC diet induced more than a 2-fold increase in serum EV concentration compared to chow diet, which was almost completely abolished by fasudil treatment (Fig. 5A, Supplementary Fig. 3A–B). To detect hepatocyte-originated EV among the circulating EV, we assessed the abundance of the hepatocyte-specific protein CYP2E1 in the serum EV protein lysates. Immunoblot analysis of pooled samples demonstrated that the abundance of
CYP2E1 was higher in circulating EV isolated from FFC diet-fed mice compared to chow diet (Fig. 5B). In line with the fasudil effect in vitro, CYP2E1 abundance in serum EV was significantly decreased in FFC-fed mice treated with fasudil (Fig. 5B). As previously reported by us, the FFC diet caused hepatocellular injury as demonstrated by elevated serum alanine aminotransferase (ALT). Of note, fasudil treatment markedly decreased the FFC diet-induced increase in serum ALT (Fig. 5C). We next assessed typical morphologic features of NASH such as steatosis and macrophage-associated inflammation. Fasudil did not change body weight, liver weight or accumulation of neutral triglyceride caused by the FFC diet (Supplementary Fig. 3C–F), but had a positive effect on macrophage-mediated hepatic inflammation. In evidence of this, macrophage accumulation and activation within the liver were assessed using immunohistochemistry for Mac-2, a surface marker of phagocytically active macrophages, and hepatic IL-1β transcript levels, respectively. The FFC diet increased the number of Mac-2-positive cells within the liver parenchyma, which was markedly suppressed by the fasudil treatment (Fig. 5D). In accordance, fasudil therapy decreased FFC diet-induced levels of IL-1β mRNA in the liver. Finally, we examined the effect of fasudil treatment on FFC diet-induced liver fibrosis. Sirius red staining demonstrated the presence of chicken wire-like perisinusoidal fibrosis in the liver of FFC diet-fed mice, which was significantly attenuated by fasudil (Fig. 5E). Similarly, the FFC diet induced hepatic α-smooth muscle actin (αSMA) mRNA, a surrogate for fibroblast activation, which was again diminished by fasudil therapy. Taken together, these observations indicate that ROCK1 inhibition may be salutary in NASH by reducing the release of proinflammatory EV from hepatocytes.

**DISCUSSION**

EV are membrane-defined, cellular-derived nanoparticles released into the extracellular milieu where they function as mediators of intercellular communication. EV are released continuously under basal conditions, although cellular stress may further increase the magnitude of released vesicles. Indeed, we observed a significant induction of EV release from hepatocytes upon treatment with toxic lipids such as palmitate and its active metabolite, LPC. These results are consistent with the recent report by Povero et al. indicating that treatment with palmitate increases release of EV from HepG2 cells and rat hepatocytes. Our study extends the prior observations by elucidating, in part, the cellular mechanism driving lipotoxicity-induced EV release and the subcellular origin of these vesicles. In the present study, we report that toxic lipid-initiated EV release is mediated by a DR5 proapoptotic signaling, consisting of a CHOP → DR5 → caspase 8 → caspase 3 signaling cascade activating ROCK1. Of these molecules, only caspase 3 has been previously implicated in EV release in platelets and hepatocytes. Since these molecular mediators significantly promote the release of proinflammatory hepatocyte EV, they all represent potential targets for NASH therapy. In particular, the caspase-dependence of EV release has therapeutic implications for NASH. For example, pan-caspase inhibitors are salutary in preclinical models of NASH. The therapeutic benefits of caspase inhibition have been attributed to a reduction in hepatocyte cell death, however, the current study suggests that caspase inhibitors may also be anti-inflammatory by attenuating release of proinflammatory EV from lipotoxic hepatocytes. We have also shed light on the subcellular
origin of these EV. In general, EV may be derived from exosomes originating from multivesicular bodies within the cell or from microvesicles shed from the plasma membrane. Our inhibition studies utilizing genetic and pharmacological approaches to target ROCK1, a kinase required for membrane blebbing, suggest that the majority of lipotoxicity-induced EV originate from plasma membrane. On the contrary, inhibition of exosome biogenesis and release by knockdown of Rab27b did not have any effect on lipotoxicity-induced EV release. Thus, according to the current nomenclature, the lipotoxicity-induced EV can likely be classified as microvesicles.

Our current data strongly suggest that lipotoxic hepatocytes release proinflammatory signals in the form of EV that can induce macrophage activation. Lipotoxic hepatocyte-derived EV, but not normal hepatocyte EV, induced expression and release of proinflammatory cytokines, such as IL-1β and IL-6, in macrophages. We have previously reported that apoptotic bodies shed by dying hepatocytes induce macrophage proinflammatory activation. In the present study, we observed that a distinct population of EV of plasma membrane origin and a diameter of ~100 nm are similarly capable of activating macrophages. To our knowledge, this is the first report to directly link hepatocyte lipotoxic injury to a macrophage proinflammatory response without presence of overt hepatocyte cell death. These observations support the evolving concept that proapoptotic signaling and/or apoptosis is not an immunologically silent process, especially when initiated by death receptors. Indeed, others have also stressed this concept demonstrating that Fas ligand proapoptotic signaling cascades mediate release of CCL2 (chemokine (C-C motif) ligand 2, also known as MCP-1) from hepatocytes. Whether CCL2 is present on EV or not will require further studies.

In the current study, we observed that hepatocyte-derived EV contain TRAIL as part of their cargo and activate macrophages, in part, in a DR5-dependent manner. TRAIL has been extensively studied as a ‘death ligand’ and initiator of apoptotic signaling cascade via its cognate receptor DR5. However, several recent reports suggest that TRAIL can promote completely different cellular processes including survival, proliferation, migration and metastasis. Cytokine and chemokine production has been described upon Fas receptor stimulation, however the proinflammatory effects of TRAIL signaling remain largely unexplored. Interestingly, the proinflammatory properties of TRAIL should not be unexpected given similarities between its signaling cascades and those of TNFs. Herein, we have demonstrated that stimulation of TRAIL receptor with recombinant TRAIL or TRAIL-bearing EV activates macrophages towards a proinflammatory phenotype via NF-κB signaling pathway. Upon TRAIL treatment, a secondary cytosolic signaling complex is formed, which consists of kinase RIP1, among others. The proinflammatory activation was mediated by RIP1, providing another piece of evidence that RIP1 represents a novel therapeutic target for inflammatory disorders. At the time of resubmitting this manuscript, Gao et al. reported that TRAIL activates macrophages via an NF-κB pathway consistent with our results. Taken together, DR5 signaling appears to play an important role in NASH pathogenesis: i) by promoting ligand-independent proapoptotic signaling in hepatocytes resulting in lipotoxicity-induced hepatocyte proinflammatory EV release and
eventual hepatocyte apoptosis; and ii) by mediating proinflammatory signaling in macrophages via non-canonical TRAIL-DR5 signaling pathway.

Besides TRAIL, there are likely other activators of proinflammatory signaling within lipotoxic EV. Indeed, our early protein mass spectrometry analysis revealed more than 2000 proteins on the isolated EV, which however did not include any classical proinflammatory cytokines, such as interleukins or interferon. We do not know yet the strength of other potential macrophage activators, hierarchal relationship and relative kinetics between EV-derived activators, which all remain to be elucidated. Also crosstalk between DR5 and other receptors involved in macrophage activation is plausible. Crosstalk between DR5 and other activators may be quite likely given the burgeoning information regarding plasma membrane crosstalk processes.

To extend our studies of EV release to an in vivo context, a clinically relevant ROCK1 inhibitor fasudil was employed in a nutrient-excess model of NASH. This model includes a diet high in saturated fats, cholesterol and the addition of fructose and glucose in the drinking water, and was developed to replicate a western diet. The model recapitulates several features of human NASH including neutral lipid accumulation, the presence of ballooned hepatocytes, an accumulation of inflammatory cells, and chicken wire-like perisinusoidal fibrosis. Interestingly, two weeks of fasudil therapy attenuated all the injurious features of NASH in this model, which correlated with a decrease in both total and hepatocyte-derived circulating EV. These results highlight the role of EV in the pathogenesis of NASH. In addition to ROCK1, fasudil is also able to inhibit ROCK2 with a similar potency and both ROCK isoforms have also been implicated in macrophage polarization and functions. ROCK1 deficiency has been shown to promote macrophage migration and recruitment and in response to various chemoattractants in vitro and in vivo. On the other hand, selective ROCK2 inhibition suppresses M2-like phenotype and accentuates M1-like polarization in macrophages. Our in vivo study with fasudil demonstrated decreased macrophage infiltration and decreased M1-like activation in the liver, which is in contrast to ROCK1 or ROCK2 inhibition in macrophages when compared to the above mentioned studies. This suggests that fasudil treatment likely does not influence the macrophage phenotype observed in our model. Finally, because fasudil is approved in Japan for the treatment of cerebral vasospasm and is also currently in clinical trials for some other indications, it could potentially be repurposed for the treatment of human NASH.

In summary, we identified hepatocyte-derived EV as a potential link between hepatocyte lipotoxicity and macrophage-mediated inflammation in NASH. Proinflammatory EV are an attractive target for developing novel therapeutic

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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**Abbreviations**

- **αSMA**: alpha smooth muscle actin
- **ALT**: alanine aminotransferase
- **ARF6**: ADP-ribosylation factor 6
- **ASGPR1**: asialoglycoprotein receptor
- **BMDM**: bone marrow-derived macrophage
- **CCL2**: chemokine (C-C motif) ligand 2
- **cFLIP<sub>L</sub>**: long splice variant of cellular caspase-8 (FLICE)-like inhibitory protein
- **cIAP-1**: inhibitor of apoptosis protein-1
- **CHOP**: CCAAT/enhancer-binding protein homologous protein
- **DR5**: death receptor 5
- **EV**: extracellular vesicle
- **FADD**: Fas-associated via death domain
- **FFA**: free fatty acid
- **FFC**: high fat, high fructose and high cholesterol
- **IKKβ**: inhibitor of IκB kinase-β
- **IL**: interleukin
- **LPC**: lysophosphatidylcholine
- **NASH**: nonalcoholic steatohepatitis
- **NF-κB**: nuclear factor kappa-light-chain-enhancer of activated B cells
- **OA**: oleic acid
- **PA**: palmitic acid
- **RIP**: receptor-interacting protein kinase
- **ROCK1**: rho-associated, coiled-coil-containing protein kinase 1
- **TNF**: tumor necrosis factor
- **TRADD**: TNFRSF1A-associated via death domain
- **TRAF6**: TNF receptor associated factor 6
- **TRAIL**: tumor necrosis factor-related apoptosis-inducing ligand
- **TSG101**: tumor susceptibility gene 101
WT wild-type

References


Figure 1. Lipotoxicity induces release of EV from hepatocytes

Primary hepatocytes or Huh7 cells were treated with lysophosphatidylcholine (LPC, 20 μM for 4 h), palmitate (PA, 400 μM for 16 h or 800 μM for 4 h), oleate (OA, 400 μM for 16 h or 800 μM for 4 h) or vehicle (Veh). EV were isolated by differential ultracentrifugation, except for primary human hepatocytes where commercially available kit was employed. (A) Representative image of NTA demonstrating concentration and size distribution of mouse hepatocyte EV. (B) Size of mouse hepatocyte EV. (C) Transmission electron photomicrographs of mouse hepatocyte EV. (D) Immunoblot for EV markers in mouse hepatocyte EV. (E) Primary mouse, rat and human hepatocytes, and Huh7 cells were treated with LPC and EV release was quantified using NTA. (F) Primary mouse and rat hepatocytes were treated with OA and PA (400 μM), primary human hepatocytes and Huh7 cells were treated with OA and PA (800 μM) and EV release was quantified using NTA. Bar graphs represent mean ± SEM. ***P < .001, **P < .01, *P < .05 compared to vehicle.
Figure 2. Lipotoxicity-induced EV release is mediated by DR5 proapoptotic signaling and ROCK1 activity
Primary mouse hepatocytes and Huh7 cells were treated with lysophosphatidylcholine (LPC, 20 μM for 4 h). Isolated EV were quantified by NTA. (A) LPC-induced EV release in wild-type hepatocyte and hepatocytes genetically deficient in DR5, caspase 8, caspase 3 and CHOP. (B) LPC-induced EV release from Huh7 cells where caspase 8 was silenced using shRNA and from Huh7 cells which were pre-treated with a pan-caspase inhibitor zVAD-fmk (20 μM, 1 h). (C) Immunoblot for active ROCK1 in Huh7 and LPC-induced EV release from Huh7 cells where ROCK1 was silenced using shRNA and from Huh7 cells which were pre-treated with fasudil (100 μM, 1 h). (D) Immunoblot for EV markers in Huh7-derived EV. (E) LPC-induced EV release from Huh7 cells where Rab27b was silenced using siRNA. (F) LPC-induced EV release from Huh7 cells which were pre-treated with GW4869 (10 μM, 20 h). Bar graphs represent mean ± SEM. ***P < .001, **P < .01, *P < .05 compared to controls.
Figure 3. Lipotoxic hepatocyte-derived EV activate macrophages and contain TRAIL

(A) EV were obtained from cultured hepatocytes isolated from chow and FFC diet-fed mice, and from LPC-treated hepatocytes (20 μM, 4 h). Mouse bone marrow-derived macrophages (BMDM) were incubated with inert beads and EV at concentration of 10^10/mL for 8 h and 24 h for mRNA and protein analysis by qPCR and ELISA, respectively. (B) Immunoblot of LPC-treated mouse hepatocyte EV with Jurkat cell lysate as a positive control. (C) Representative transmission electron photomicrographs of LPC-treated mouse hepatocyte EV which were immunogold-labeled with an anti-TRAIL antibody (scale bar 100 nm). (D) BMDM obtained from wild-type and DR5^-/- mice were incubated with LPC-treated mouse hepatocyte EV at concentration of 10^10/mL for 8 h (mRNA) and 24 h for mRNA and protein analysis by qPCR and ELISA, respectively. (E) BMDM were incubated with serum EV isolated from chow and FFC-diet fed mice. (F) THP-1 cells were incubated with EV isolated
from serum of healthy subjects and patients with NASH. Bar graphs represent mean ± SEM.

***$P < .001$, **$P < .01$, *$P < .05$ compared to controls or as indicated.
Figure 4. Macrophage activation occurs via non-canonical TRAIL signaling
(A) THP-1 macrophages and primary human macrophages were treated with TRAIL (200 ng/mL, 6 h). (B) THP-1 macrophages were treated with TRAIL (50 ng/mL, 0–4 h). Cell lysates were subjected to immunoprecipitation of FADD. (C–D) Wild-type and RIP1<sup>−/−</sup> THP-1 cells (with CRISPR/Cas9-mediated RIP1 deletion) were incubated with TRAIL (50 ng/mL) or EV (10<sup>10</sup>/mL) derived from LPC-treated Huh7 cells for 2 h and 24 h for mRNA and protein analysis by qPCR and ELISA, respectively. (E) Wild-type or RIP1<sup>−/−</sup> THP-1 cells were incubated with TRAIL (50 ng/mL) or EV (10<sup>10</sup>/mL) released from LPC-treated Huh7 cells for 1 h. Nuclear translocation of p65 was examined by immunofluorescence. (F) Mouse BMDM were pre-treated with necrostatin-1 (25 μM, 1 h) followed by incubation
with LPC-treated mouse hepatocyte EV (10^{10}/mL) for 8 h and 1 h for qPCR and p65 immunofluorescence analysis, respectively. Bar graphs represent mean ± SEM. ***P < .001, **P < .01, *P < .05 compared to controls or as indicated.
Figure 5. ROCK1 inhibition improves NASH in a murine nutrient-excess model
C57BL/6 mice were placed on chow or FFC diet for 6 months and then received fasudil (50 mg/kg p.o. q.d.) or vehicle for 2 weeks. (A) EV were isolated from serum and quantified by NTA. (B) Immunoblot analysis of pooled serum EV samples. (C) Serum ALT activity. (D) Immunohistochemistry for macrophage marker Mac-2. Representative photomicrographs taken with a 20 × objective are shown (scale bar 200 μm). Morphometric analysis of Mac-2-positive area and liver expression of IL-1β. (E) Sirius red staining on liver tissue sections (20 × objective, scale bar 200 μm). Morphometric analysis of Sirius red staining and liver expression of αSMA. Bar graphs represent mean ± SEM. ***P < .001, **P < .01, *P < .05.
Lipotoxicity during NASH activates DR5 proapoptotic signaling cascade activating ROCK1, which promotes release of TRAIL-bearing EV from hepatocytes. EV activates macrophages via a non-canonical, non-apoptotic DR5 signaling pathway in a RIP1-dependent manner. A ROCK1 inhibitor, fasudil, prevents lipotoxicity-induced EV release from hepatocytes and thus attenuates macrophages activation.