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Interferon regulatory factor 1-Rab27a regulated extracellular vesicles promote liver ischemia/reperfusion injury

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Abstract

The role and regulators of extracellular vesicles (EV) secretion in hepatic ischemia/reperfusion (IR) injury have not been defined. Rab27a is a GTPase known to control EVs release. Interferon regulatory factor 1 (IRF-1) is a transcription factor that plays an important role in liver IR and regulates certain GTPases. However, the relationships among IRF-1, Rab27a, and EVs secretion are largely unknown. Here, we show induction of IRF-1 and Rab27a both in vitro in hypoxic hepatocytes and in vivo in warm IR and orthotopic liver transplantation livers. Interferon γ stimulation, IRF-1 transduction, or IR promoted Rab27a expression and EVs secretion. Meanwhile, silencing of IRF-1 decreased Rab27a expression and EVs secretion. Rab27a silencing decreased EVs secretion and liver IR injury. Ten putative IRF-1 binding motifs in the 1,692 base pairs Rab27a promoter region were identified. Chromatin immunoprecipitation and electrophoretic mobility shift assay verified five functional IRF-1 binding motifs, which were confirmed by Rab27a promoter luciferase assay. IR-induced EVs contained higher oxidized phospholipids (OxPL). OxPLs on EVs surface activated neutrophil through toll like receptor 4 (TLR-4) pathway. OxPL-neutralizing E06 antibody blocked the effect of EVs and decreased liver IR injury. These findings provide a novel mechanism by which IRF-1 regulates Rab27a transcription and EVs secretion, leading to OxPL activation of neutrophils and subsequent hepatic IR injury.

Keywords

transcription factor; promoter; oxidized phospholipid; neutrophil; toll like receptor 4

Introduction

Extracellular vesicles (EV) are phospholipid bilayer particles that are generated by a conserved biological process. Secreted by most cell types and ubiquitously present in body fluids, EVs exert profound biological effects (1–3). Their complicated effects result from their intricate composition of lipids, proteins, RNAs, and DNAs (4). However, the regulatory mechanisms of EVs secretion, especially under different pathophysiologic situations, remain largely unknown. Ischemia/reperfusion (IR) injury is a major clinical pathophysiologic concern in surgical resection, organ transplantation, myocardial infarction, and stroke (5, 6). The effects of EVs during IR injury are mainly unknown.

Previously, we have shown that interferon regulatory factor 1 (IRF-1) is a transcription factor that plays an important role in hepatic IR injury (7). IRF-1 has been shown to regulate GTPase expression (8) and secretion of melanosome, a type of EV (9). Interestingly, many GTPases participate in vesicular trafficking and EVs secretion. In particular, Rab27a, a small GTPase, is known to play an important role in EVs secretion (10). Therefore, we hypothesize that IRF-1 may regulate EVs secretion through Rab27a in hepatic IR.

We further sought to examine the mechanisms by which EVs regulated by IRF-1 and Rab27a affect IR injury. Oxidative stress is a major pathophysiologic mechanism during IR injury. Oxygen radicals generated during oxidative stress can cause phospholipid oxidation. Phospholipids, an integral component of cell membranes, are also present on the EVs. Oxidized phospholipids (OxPL) are found in dying cells (11) and IR-damaged tissues (12–14). Of note, oxidized membranes of EVs are known to contain OxPLs. The surface OxPLs of EVs are secreted by parent cells undergoing oxidative stress and can activate toll-like receptor 4 (TLR-4) (15). There is evidence that TLR-4 ligands are increased during IR injury and that TLR-4 activation can promote IR injury (16, 17). TLR-4 on neutrophils, a major immune cell population in IR injury, can be activated during IR (18). We hypothesize that IRF-1-Rab27a regulated EVs can promote IR injury by activating neutrophils through EV OxPLs and neutrophil TLR-4 pathway.

Materials and Methods

Ethics

Human hepatocytes (hHC) and mouse hepatocytes (mHC) isolation were performed using protocols approved by University of Pittsburgh Institutional Review Board. The University of Pittsburgh Institutional Animal Care and Use Committee approved all murine experiments.

Mice

Male C57BL/6J wild type (WT) and IRF-1 knock out (KO) (B6.129S2-Irf1^{tm1Mak/J}, IRF-1^{-/-}) mice were purchased from Jackson Laboratory. TLR-4 global KO and TLR-4^{loxP/-};Lyz-cre (Lyz-TLR4 KO) mice from B6 background were provided by Dr. Timothy R. Billiar. All animals were housed or bred in the specific pathogen-free animal facility at the University of Pittsburgh School of Medicine and had access to food and water ad libitum.

Cells and Reagents

Huh-7, Hep G2 human hepatoma cell lines and Hepa 1–6 mouse hepatoma cell line were purchased from American Type Culture Collection (ATCC; Rockville, MD, USA). Human IRF-1 expression plasmid and backbone plasmid were ordered from Origene (Rockville, MD). β -galactosidase expression (β -Gal) plasmid was purchased from Promega (Madison, WI). Mouse IRF-1 expression adenovirus (Ad-mIRF-1) and β -galactosidase expression adenovirus (Ad-LacZ), mice Ad-Rab27a shRNA, adenovirus scramble shRNA (Ad-SCR) and control adenovirus backbone (Ad-CTL), targeting both human and mice Ad-IRF-1 shRNA were ordered from Vector Biolabs (Malvern, PA). Antibodies against IRF-1 (SC-640, Santa Cruz), CD63 (SC-15363, Santa Cruz), calnexin (SC-6465, Santa Cruz), CD81 (160523-006, System Biosciences), HSP70 (ab2728, Abcam), Rab27a (ab55667, Abcam), β -actin (8H10D10, CST), Lamin A/C (4C11, CST) were used for Western Blot. TopFluor™ labeled E06 antibody (Avanti Polar Lipids), which targets oxidized phospholipid, was used to detect oxidized phospholipid on EVs by flow cytometry.

Other Methods

Please see supporting information for details about liver warm IR model, hepatocytes hypoxia and re-oxygenation, liver damage evaluation, serum neutrophil elastase activity test, histology and immunofluorescence staining, EV isolation and quantification, EV labeling with PKH26, adenovirus infection in vitro and in vivo, qPCR, Rab27a activity assay using GTP-Rab27a pull-down and detection method, chromatin immunoprecipitation (ChIP), electrophoretic mobility shift assay (EMSA), luciferase assay and plasmid subclone, detection OxPLs on EV surface by flow cytometer, neutrophil isolation and co-culture with EVs, cold IR model (mouse orthotopic liver transplantation, OLTx), serum cytokine detection, and statistical analysis.

Results

Rab27a is induced during liver IR. Rab27a silencing during IR decreases liver damage and serum EVs (sEV) concentration

During warm IR in mice, hepatic Rab27a mRNA and protein were induced in a time-dependent manner (supporting Fig. S1A, and Fig. 1A, 1B). As expected, liver damage peaked at 6 hours after warm IR (Fig. 1C). Silencing of hepatic Rab27a expression with Ad-Rab27a shRNA reduced liver injury as measured by serum alanine aminotransferase (ALT) levels, while Ad-SCR and Ad-CTL had no effect (Fig. 1C). Likewise, hepatic necrosis after IR was significantly decreased by Rab27a silencing as shown by liver H&E staining (Fig. 1D and supporting Fig. S1C). Western blot for Rab27a confirmed silencing of Rab27a protein with decreased basal and IR-induced Rab27a expression in liver after Ad-Rab27a shRNA pre-treatment for 48 hours (supporting Fig. S2A, S2B). To detect Rab27a activity, we used Slp homology domain (SHD) GST fusion protein to pull down GTP conjugated Rab27a as an assay for Rab27a activity. The active form of Rab27a, GTP conjugated Rab27a, was also increased during liver IR (supporting Fig. S1D and S1E). The GTP bound active form of Rab27a corresponds well with total Rab27a levels including Rab27a silencing or IRF-1 KO group (data not show) after different time points of reperfusion. These data

confirm that Rab27a activity is increased during IR along with increased Rab27a transcription.

Next, we isolated sEVs from sham and IR-treated mice with serum EV isolation kit. sEVs concentration increased in a time-dependent manner 6–12 hours after warm IR as quantified with CD81 exosome ELISA kit (Fig. 1E), and corresponded to the Rab27a protein expression and its activity shown in Fig. 1A, supporting Fig. S1D and S1E. Silencing of Rab27a expression in vivo with Ad-Rab27a shRNA decreased sEVs 6–24 hours after IR (Fig. 1F). Bigger sEVs pellets were observed 6 hours after IR (supporting Fig. S2C), and sEVs were visually confirmed by transmission electron microscopy (TEM) (supporting Fig. S2D). By western blot analysis, sEVs lysates contained markers CD63, CD81, and HSP70, but not endoplasmic reticulum marker calnexin (supporting Fig. S2E).

IRF-1 KO mice have decreased Rab27a induction, sEVs secretion, and liver damage after IR

As we have previously shown, hepatic IRF-1 expression was rapidly induced 2 hours after warm IR (Fig. 2A, supporting Fig. S3A). IRF-1 KO mice had significantly decreased liver injury shown by serum ALT (Fig. 2B) and histology (Fig. 2C & 2D) compared to WT mice 6 hours after IR. Rab27a protein expression was induced 6 and 12 hours after IR in WT mice, but not in IRF-1 KO mice (Fig. 2E, supporting Fig. S3B). Concentrations of sEVs were significantly decreased at 6 and 12 hours after IR in the IRF-1 KO mice compared to WT (Fig. 2F). These data show that hepatic Rab27a is not effectively induced by IR injury when IRF-1 is deficient.

Hypoxia/re-oxygenation promotes mHC and hHC EV secretion in an IRF-1 and Rab27a dependent manner

In order to confirm the relationships among IRF-1, Rab27a, and EV secretion, we isolated mHC and hHC and exposed them to hypoxia/re-oxygenation to simulate IR injury (19). Rab27a was markedly induced 6 hours after re-oxygenation in both mHC (Fig. 3A and supporting Fig. S3C) and hHC (Fig. 3B and supporting Fig. S3C). To examine the effects of hypoxia/re-oxygenation on EV secretion from cultured HCs, supernatants were collected 24 hours after re-oxygenation and EVs were isolated by ultracentrifugation. Hypoxia/re-oxygenation significantly induced EV secretion in mHC (Fig. 3C). This effect was abrogated when Rab27a was silenced with Ad-Rab27a shRNA infection (48 hours pretreatment) (Fig. 3C).

Next, to determine the effect of IRF-1 on hepatocyte EV (hepEV) secretion, mHCs were isolated from IRF-1 KO mice. Unlike WT mHCs, these cells did not show increased EV secretion after re-oxygenation (Fig. 3D). Increased EV secretion was also observed in hHC (Fig. 3E) during hypoxia/re-oxygenation. Silencing of hHC IRF-1 with Ad-IRF-1 shRNA decreased Rab27a protein expression (supporting Fig. S4A and S4B) and EV secretion in response to hypoxia/re-oxygenation (Fig. 3E). EVs were examined and confirmed by TEM and western blot for EV markers (supporting Fig. S4C and S4D). These data can confirm that Rab27a induction during IR is not coming from immune cell infiltration.

Induction of IRF-1 by interferon γ (IFN γ) promotes Rab27a transcription, translation and subsequent EV secretion

To confirm the link between IRF-1 and Rab27a, we added IFN γ to induce endogenous IRF-1 in primary hHC and the human hepatoma cell line Huh-7. IFN γ induced IRF-1 and Rab27a mRNA expression 6 hours after stimulation in a dose-dependent manner (supporting Fig. S5A). IRF-1 and Rab27a protein levels were also induced by IFN γ in a dose-dependent manner 12 hours after stimulation (Fig. 4A and 4B). Likewise, both IRF-1 and Rab27a mRNA and protein were induced by IFN γ in the Huh-7 cell line (Fig. 4C, 4D, supporting Fig. S5B, S5C). Consistent with the Rab27a induction, EV secretion was also increased in hHC (supporting Fig. S5D) and Huh-7 (supporting Fig. S5E) after IFN γ stimulation. Fluorescence microscopy also showed that IFN γ increased IRF-1 and Rab27a expression after 12 hours treatment (supporting Fig S6). Since cytoplasmic multiple vesicular bodies (MVB) store the EVs before releasing, we examined the effect of IFN γ by confocal microscopy on MVBs and showed that IFN γ induced MVBs in hHC and Huh-7 cells (supporting Fig. S7A, S7B).

Overexpression of IRF-1 induces Rab27a expression and EV secretion

Next, to determine if IRF-1 over-expression (in the absence of exogenous IFN γ) also increased Rab27a expression and EV secretion, the murine hepatoma cell line Hepa1-6 was infected with Ad-mIRF-1. IRF-1 protein expression was confirmed at 24–48 hours after Ad-mIRF1 infection (Fig. 4E and 4F). This was associated with increased Rab27a protein expression (Fig. 4E and 4F) and increased EV secretion (supporting Fig S5F) in Hepa1-6 cells. Thus, both endogenous and exogenous IRF-1 induces Rab27a expression and EV secretion.

IRF-1 promotes Rab27a transcription through DNA binding motifs in Rab27a promoter region

As IRF-1 is a transcription factor, we hypothesized that IRF-1 could bind directly to the Rab27a promoter region and promote Rab27a transcription. The bioinformatics software PROMO was used to predict IRF-1 binding motifs. Ten possible motifs were found within a 1.6 kilo base (kb) pairs area of the human Rab27a promoter region (supporting Fig S8). We designed 5 PCR primers around this DNA region (supporting Table S1) and performed ChIP assay. IRF-1 Ab pulled down a protein-DNA complex from ChIP primer 3 set (Fig. 5A). Immune precipitation was not observed with the other primers (data not shown). Positive ChIP results were identified in the primer 3 region which spans from –647 to –337 base pairs of the human Rab27a promoter and contains 5 IRF-1 binding motifs as illustrated in Fig. 5B. We confirmed the binding ability of the 5 motifs with EMSA (supporting Fig. S9). These findings indicate that IRF-1 binds to the Rab27a promoter region.

Next, luciferase assays were done using the –1.4 kb pairs Rab27a promoter reporter plasmid. Stimulation with IFN γ for 6 hours increased Rab27a promoter activity in a dose-dependent manner (Fig. 5C). Transfection efficiency was normalized to β -galactosidase. Co-transfection of IRF-1 expression plasmid also increased Rab27a promoter activity in a dose-dependent manner (Fig. 5C).

To verify that the active IRF-1 DNA binding motifs were in the primer 3 region spanning from -647 to -337 base pairs in the human Rab27a promoter, we generated Rab27a promoter deletional constructs using restriction endonucleases (Fig. 5D). The full length -1.4 kb pairs Rab27a promoter and the truncated -1.1 kb pairs Rab27a promoter showed significantly increased luciferase activity in response to IFN γ stimulation or IRF-1 over expression. In contrast, deletion of the five IRF-1 DNA motifs from -647 to -337 base pairs abrogated most of the promoter inducibility (Fig. 5E & 5F). These data further confirm that IRF-1 binds the Rab27a promoter region in vivo.

EVs from hepatic IR or hypoxic HC carry higher surface OxPLs that activate TLR-4 pathway in neutrophils

OxPLs increase during inflammation and IR injury (14) and promote inflammation (20). We used the well-characterized E06 Ab to detect OxPLs on the surface of EVs attached to beads conjugated with anti-CD81 antibody with flow cytometry (21). We found increased amounts of OxPLs on the surface of sEVs from IR mice compared to sham (Fig. 6A & 6B), as well as increased amounts of OxPLs on EVs secreted by hypoxic hepatocytes (hypo-hepEV) in comparison to normoxic hepatocytes secreted EVs (norm-hepEV) (Fig. 6C & 6D). In contrast, the IRF-1 and Rab27a induction by IFN γ did not increase the OxPLs on EVs (Fig. S10A and S10B).

Since OxPLs are known to activate the TLR-4 pathway, we assessed the effect of EVs on the neutrophil TLR-4 pathway using WT and TLR-4 KO bone marrow derived neutrophils (BMDNs). First, we showed by ImageStream that NF- κ B p65 was translocated to the cell nucleus when the BMDNs were co-cultured with hypo-hepEVs (supporting Fig. S11A). In contrast, p65 remained in the cytoplasm when the TLR-4 KO BMDNs were co-cultured with hypo-hepEVs (supporting Fig. S11B). To quantify the p65 nuclear translocation, a similarity score was generated where a higher score indicated greater nuclear translocation. Hypo-hepEVs co-cultured with WT (but not TLR-4 KO) BMDNs had greater similarity scores compared to norm-hepEVs or PBS (supporting Fig. S11C, S11D and S11E). Neutralization of the OxPLs with E06 Ab abrogated the p65 nuclear translocation (supporting S11E), which indicates that the BMDN NF- κ B nuclear translocation depends on EV OxPLs.

Next, we tested BMDN activation by measuring supernatant IL-6 from the co-cultures. We observed that hypo-hepEVs promoted BMDN IL-6 production when co-cultured with WT BMDNs but not when co-cultured with TLR-4 KO BMDNs (Fig. 6E), suggesting that BMDN activation is dependent on TLR-4 pathway. Neutralization of the OxPLs from hypo-hepEVs with E06 Ab abrogated the increased IL-6 production (Fig. 6F). Thus, the OxPLs from hypo-hepEVs activate BMDN NF- κ B nuclear translocation and IL-6 production in a TLR-4 dependent manner.

Hypo-hepEVs reverse the protective effect of Rab27a silencing in vivo in a TLR-4 dependent manner

To confirm that OxPLs cause liver damage during IR, we injected WT IR mice with E06 Ab at time of reperfusion and found decreased IR damage as measured by ALT and necrotic area (Fig 7A, supporting Fig S12A, and S12D). Next, to determine if hypo-hepEVs promote

liver injury in the absence of IR injury, we injected WT mice with hypo-hepEVs and found no significant effect (data not shown). Hypo-hepEVs could reverse the protection effect of Rab27a silencing during IR by in vivo delivery. The increased liver damage was mediated at least in part by OxPLs because the E06 Ab ameliorated the injury (Fig. 7B, supporting Fig S12B, and S12E). Additionally, Lyz-TLR-4 KO mice, in which TLR-4 is knocked out specifically in myeloid cells (neutrophils, macrophages, et. al.), were treated with hypo-hepEVs, there was no significant increase in liver damage after IR, suggesting that the hypo-hepEVs signal through TLR-4 pathway (Fig. 7C, supporting Fig S12C and S12F). Serum cytokine levels of IL-6 and TNF- α decreased by E06 Ab, further confirming that OxPLs promote liver damage (Fig. 7D). Likewise, in the setting of Rab27a silencing in vivo, IL6 and TNF- α concentrations in sera increased by injection of hypo-hepEVs and was reversed by OxPLs neutralization with E06 Ab (Fig. 7E).

Since the in vitro data showed that IR released EVs activate neutrophils through TLR-4 pathway, we examined neutrophil infiltration and activation in vivo. The immunofluorescence staining showed that livers from IR-treated IRF-1 KO, Rab27a silencing, and Lyz-TLR-4 KO mice had less Ly6G positive neutrophil infiltration after 6 hours of reperfusion (supporting Fig. S13A, Fig. 7F) as compared to WT counterparts. Neutrophil elastase activity, a neutrophil activation marker, was also decreased in IRF-1 KO, Rab27a silencing, and Lyz-TLR-4 KO mice liver after 6 hours of reperfusion (Fig. 7F). The hypo-hepEVs could reverse the diminished neutrophil infiltration and elastase activity after Rab27a silencing as compared with the norm-hepEVs injection group. Furthermore, E06 Ab abrogated the effects (Supporting Fig. S13B and Fig. 7F). Hence, the in vitro and in vivo data showed that IR released EVs can activate neutrophil TLR-4 pathway through their surface OxPLs.

Rab27a induction during liver transplant IR is mediated in part by IRF-1 signaling

Expression of hepatic Rab27a has not been examined in the liver transplant setting. We performed murine B6 isograft OLTx after 20 hours cold storage in UW solution, and observed that hepatic Rab27a was induced in a time-dependent manner (Fig. 8A). Since our data implicated IRF-1 in regulating Rab27a expression, we used IRF-1 KO mice as donors of liver isografts. We found that Rab27a induction after OLTx with IRF-1 KO donor grafts was markedly decreased in comparison to WT donor grafts (Fig. 8B). Hepatic IRF-1 protein was induced in vivo in a time-dependent manner after OLTx (Fig. 8C) as before (7). In comparison to WT grafts, IRF-1 KO grafts led to significantly decreased OLTx IR injury as measured by serum ALT (Fig. 8D). To confirm a functional role for Rab27a in OLTx IR injury, we silenced the liver graft Rab27a with Ad-Rab27a shRNA intravenous injection and transplanted the grafts 48 hours later. ALT levels were significantly decreased in recipients of Ad-Rab27a shRNA treated grafts in comparison to Ad-SCR (Fig. 8E).

Next, we measured the effect of OLTx IR on sEV levels and found that sEVs doubled in WT mice 6 hours after OLTx (Fig. 8F). IRF-1 KO isografts did not exhibit this increase in sEVs, suggesting that sEV secreting induction is IRF-1 dependent. Silencing Rab27a in the donor grafts abrogated the increase in sEVs levels after OLTx (Fig. 8F). Likewise, apoptosis measured by TUNEL staining was decreased after OLTx in IRF-1 KO or Rab27a silenced

liver grafts (supporting Fig. S14C, S14D). Serum IL-6 and TNF- α levels were consistent with these results (supporting Fig. S14E and S14F). Taken together, these data suggest that Rab27a plays an IRF-1 dependent role in OLTx IR injury.

Discussion

The major and novel findings of this study are: (i) hepatic IR injury in vivo or hypoxia/re-oxygenation of hepatocytes in vitro induced Rab27a expression and subsequent EV secretion in an IRF-1 dependent manner, (ii) Rab27a activity also increased during liver warm IR and correlated well with the increase of Rab27a transcription and translation; (iii) IRF-1 induced Rab27a transcription by binding to five specific IRF-1 binding motifs in the Rab27a promoter, (iv) IRF-1 knockout or IRF-1 silencing decreased Rab27a expression and EV secretion, (v) EV surface OxPLs generated during redox stress activate neutrophils in a TLR-4 dependent manner to exacerbate liver damage. Taken together, these findings demonstrate that oxidative stress during hepatic IR injury induces IRF-1 that regulates Rab27a transcription to control EV secretion. Secreted EVs contribute to hepatic IR through OxPL activation of neutrophil TLR-4 pathway.

Based on the current literature, the term EV includes exosome, microvesicle, and apoptotic cell-derived EV, each EV with different biogenesis, size and molecular composition (22). Exosomes are small EVs that originate within the endocytic compartment of living cells. They are isolated from culture supernatants or bodily fluids by ultra-centrifugation (100,000g). However, it has been shown that the population of small EVs includes vesicles of exosomal and non-exosomal origin (23). Thus, to avoid confusion with the terminology of EV and be consistent throughout the manuscript, we have used the term “extracellular vesicle (EV)” instead of “exosome”.

There are abundant literatures showing that EVs play important roles in cell communication (24, 25) and can be used as bio-markers (26). The mechanisms, by which cells regulate EV secretion, especially after physiological or pathological stress, remain largely unknown. IR injury is inevitable during organ transplantation, stroke, or myocardial infarction. IR (27) and hypoxia (28) have been shown to enhance EV secretion, but the mechanisms remain unknown. It has been demonstrated that Rab27a plays an important role in EV secretion (10); however the regulators of Rab27a mediated EV secretion are unknown. This study is the first to show that IRF-1 regulates Rab27a mediated EV secretion during IR injury. Although we found that IFN γ -treated Huh-7 cells increased their content of MVBs, the ultimate mechanisms by which the IRF-1-Rab27a axis increases generation and secretion of EVs will require more detailed analysis in future studies.

Transcriptional regulation allows cells to respond to a variety of intracellular and extracellular stresses. Previously, we showed that hepatic IR activates the transcription factor IRF-1 (7). Here, we demonstrate that IRF-1 induces Rab27a transcription and translation, which promotes EV secretion. We also showed that the activity of Rab27a (determined by the concentration of GTP bound active form of Rab27a) correlated well with total Rab27a levels. These data are consistent with the notion that the main regulation of Rab27a is at the transcriptional level during IR.

IRF-1, as well as other IRF family members, promotes transcription of target genes by binding the IRF element (IRF-E) or interferon stimulating response element (ISRE) in target gene promoter regions (29). ISREs in the Rab27a promoter region were first predicted by bioinformatics and then confirmed by ChIP and EMSA. Similar to our findings that IRF-1 regulates EV secretion, others have shown that IRF-1 regulates the secretion of melanosomes, another type of EV (9). Furthermore, IRF family members regulate the expression of other GTPases (30–32), which play important roles in cellular vesicle transport (33). Our research demonstrates the important role of interferon pathway and IRF family members in the release of EVs.

Our data show that EV secretion is increased in both warm and cold liver IR. Silencing of Rab27a led to decrease EV secretion and subsequent alleviation of liver damage. Though it has been demonstrated that adenovirus concentrates mainly in the liver after intravenous injection, we cannot rule out the effects of extra hepatic Rab27a silencing, particularly in neutrophils, where Rab27a is known to be active (34). Western blot showed that Rab27a expression is not significantly changed in platelets or kidney tissue. There was minimal decrease in lung tissue. This further confirms the protective effects of Ad-Rab27a shRNA during liver IR comes from the liver Rab27a silencing. It also means that the protection effect should come from the inhibition of liver EVs secretion. In addition, our liver transplant experiments with Rab27a silencing in the liver grafts resulted in decreased sEV concentration and post-OLTx IR injury, which confirms the importance of hepatic Rab27a-EV pathway during transplantation related IR. Because the recipients were Ad-Rab27a shRNA free. Though the mechanisms of cold and warm IR are not identical, these data further confirm that inhibiting liver EV secretion through Rab27a silencing has a protective effect in IR injury.

A myriad of components mediate the effects of EVs. There is limited evidence that EVs secreted by hepatocytes promote liver repair and regeneration after IR (27). In our study, we found that EVs secreted after hepatic IR promote damage through surface OxPLs. IR injury, a form of oxidative stress, results in formation of oxygen radical species. These free radicals can oxidize unsaturated fatty acids in phospholipids and generate OxPLs (35). OxPLs are found not only in IR related diseases (14, 35), but also in dying cells, and acute lung injury (20). Previous in vitro studies have demonstrated that EVs contain surface OxPLs (15, 36). To our knowledge, this is the first in vivo study that showed inflamed tissue releases OxPLs through EVs. Accordingly, treatment of mice with the E06 Ab, which blocks the effects of OxPLs, decreased IR damage and blocked neutrophil activation by hypo-hepEVs.

TLR-4 ligands play a pivotal role in IR injury (37). Our data show that EVs express membrane OxPLs and act as TLR-4 ligands. In particular, phospholipids are major components of eukaryotic bio-membranes (38). Oxidized 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphorylcholine (POVPC), one type of OxPL, has been found on EVs (36). Oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (oxPAPC), another OxPL, has been shown to exacerbate acute lung injury through a TLR-4 pathway (20). In concordance with our work, another in vitro study found that EVs secreted during oxidative stress contain OxPLs, which can activate the TLR-4 pathway (15). EVs isolated from body fluids are also able to activate immune cells through TLR-4 pathway (39).

The specific role of OxPLs in the TLR-4 pathway remains controversial. Some studies show that OxPLs can activate TLR-4 pathway (20), while others refute that finding (40) or suggest that OxPLs inhibit LPS-mediated TLR-4 activation (41). Our data clearly show that OxPLs activate neutrophil TLR-4. We propose that TLR-4 activation is dependent on the cell type, and TLR-4 activation by OxPLs is less efficacious than by LPS, a theory verified by Oskolkova, et al (42). This may explain why hypo-hepEVs, unlike LPS, could not significantly cause naïve mice liver damage. Since other cells such as macrophages and hepatocytes also express TLR-4, we cannot rule out TLR-4 activation by OxPLs positive EVs on other cell types. In any case, our work confirms that IR induced EVs promote liver damage and that blockade of this mechanism may have potential as a therapeutic target.

In conclusion, our research shows that EVs induced by liver IR are regulated by the IRF-1-Rab27a axis, and that the EVs promote IR injury through neutrophil TLR-4 pathway. Rab27a and IR generated EVs represent new targets to ameliorate liver damage during hepatic IR injury and other IR related diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviation

IRF-1	interferon regulatory factor 1
IR	ischemia/reperfusion
OLTx	orthotopic liver transplantation
OxPL	oxidized phospholipid
TLR-4	toll like receptor 4
MVB	multiple vesicular body
hHC	human hepatocyte
mHC	mouse hepatocyte
ChIP	chromatin immunoprecipitation
EMSA	Electrophoretic Mobility Shift Assay
EV	extracellular vesicle
ALT	alanine aminotransferase

Ad	adenovirus
shRNA	short hairpin RNA
TEM	transmission electron microscopy
IFNγ	interferon γ
TLR-4 flox	TLR-4 ^{loxP/-}
Lyz-TLR4 KO	TLR-4 ^{loxP/-} -Lyz-cre
hepEV	hepatocyte secreted extracellular vesicle
Norm-hepEV	normoxic hepatocyte secreted extracellular vesicle
Hypo-hepEV	hypoxic hepatocyte secreted extracellular vesicle
BMDN	bone marrow derived neutrophil
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling

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Figure 2. IRF-1 KO decreases Rab27a induction, sEV secretion, and liver damage after IR
 (A) Western blot of IRF-1 expression in ischemic liver lobes of WT mice after IR; (B) ALT levels in WT and IRF-1 KO mice after IR. (C) H&E staining of ischemic liver lobes of WT and IRF-1 KO mice after IR with 6 hours reperfusion; (D) Extent of necrotic area in H&E stained ischemic liver lobes of WT or IRF-1 KO mice. (E) Western blot of Rab27a in WT and IRF-1 KO liver lobes after IR. (F) sEVs concentrations in WT and IRF-1 KO mice as measured by CD81 exosome ELISA kit. $n = 6$ in each group. Bar = 200 μm . * $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$. Abbreviation: sEV, serum extracellular vesicle.

Figure 3. IRF-1 and Rab27a promote hepatocyte EV secretion during hypoxia/re-oxygenation in vitro

Western blot of Rab27a protein in mHC (A) and hHC (B) following hypoxia/re-oxygenation in vitro; (C) EV secretion by 48 hours Ad-Rab27a shRNA or Ad-SCR pretreated mHC during 24 hours re-oxygenation after hypoxia; (D) EV secretion by WT or IRF-1 KO mHC during 24 hours re-oxygenation after hypoxia; (E) EV secretion by 48 hours Ad-IRF-1 shRNA or Ad-SCR pretreated hHC during 24 hours re-oxygenation after hypoxia. Hepatocyte-derived EVs were isolated by ultracentrifugation. After lysed with lysis buffer, EV protein concentrations were detected with BCA method. $n = 6$ for each group. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Abbreviations: mHC, mouse hepatocyte; hHC, human hepatocyte; Ad-Rab27a shRNA, adenovirus encoding Rab27a short hairpin RNA; Ad-SCR, adenovirus encoding scramble short hairpin RNA; Ad-CTL, control adenovirus.

Figure 4. IRF-1 and Rab27a are induced by human IFN γ and Ad-mIRF-1

(A) Western blot analysis of IRF-1 and Rab27a expression in hHC after 12 hours IFN γ treatment. (B) Quantification of IRF-1 and Rab27a proteins in hHC after 12 hours IFN γ treatment. (C) Western blot analysis of IRF-1 and Rab27a expression in Huh-7 after 12 hours IFN γ treatment. (D) Quantification of IRF-1 and Rab27a proteins in Huh-7 after 12 hours IFN γ treatment. (E) Western blots of IRF-1 and Rab27a expression in Hepa 1–6 cells after Ad-mIRF-1 infection. (F) Quantification of IRF-1 and Rab27a proteins in Hepa 1–6 cells after Ad-mIRF-1 infection. $n = 6$. Abbreviations: hHC, human hepatocyte; Ad-LacZ, β -galactosidase expression adenovirus; Ad-mIRF-1, mouse interferon regulatory factor 1 expression adenovirus.

Figure 5. IRF-1 mediates Rab27a up-regulation through specific binding to Rab27a promoter
 (A) ChIP results from the third primer in hHCs and Huh-7 cells. (B) Schematic representation of IRF-1 binding sites in the Rab27a promoter region as predicted by PROMO bioinformatics software and single endonuclease target site within Rab27a promoter driven luciferase vector; (C) Rab27a driven luciferase activity as normalized to β -galactosidase activity. (D) Deletional human Rab27a promoter constructs schema. (E) Deletional human Rab27a promoter activities after IFN γ treatment. (F) Deletional human Rab27a promoter activities after IRF-1 overexpression. Experiments were performed after 48 hours of IRF-1 expression plasmid co-transfection or IFN γ 6 hours treatment. $n = 6$ in each group. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. Abbreviations: hHC, human hepatocyte; ChIP, Chromatin immunoprecipitation; TSS, transcription starting site; β -Gal, β -galactosidase.

Figure 6. OxPLs are present on the EV surface

Flow cytometry analysis (A, C) and MFI (B, D) of E06 Ab positive mouse sEVs (A, B) and hepEVs (C, D) after hypoxia and re-oxygenation in vitro. (E) IL-6 levels in culture supernatant of hypo-hepEVs or norm-hepEVs treated WT or TLR-4 KO neutrophils for 6 hours. (F) IL-6 levels in culture supernatant of hypo-hepEVs and E06 Ab treated WT neutrophils for 6 hours. $n = 6$ for each groups. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Abbreviations: sEV, serum extracellular vesicle; OxPL, oxidized phospholipid; MFI, mean fluorescence intensity; hepEV, hepatocyte secreted extracellular vesicle; TLR-4, toll like receptor 4; Hypo-hepEV, EV secreted by hypoxia and re-oxygenation hepatocyte; Norm-hepEV, EV secreted by normoxic hepatocyte.

Figure 7. Hypo-hepEVs reversed the protection effects of Rab27a silencing. Liver neutrophil numbers quantified by immunofluorescence staining and their activation measured by serum NE (A) ALTs after E06 Ab portal vein injection in WT IR mice. (B) ALTs after hypo-hepEVs portal vein injection in Rab27a silenced mice undergoing hepatic IR. (C) ALTs after hypo-hepEVs portal vein injection in Lyz-TLR-4 KO mice after liver IR. (D) Serum concentrations of IL-6 and TNF- α after E06 Ab portal vein injection in WT mice undergoing IR. (E) Serum concentrations of IL-6 and TNF- α after hypo-hepEVs injection in Rab27a silenced mice. (F) Liver Ly6G positive neutrophils quantification and serum NE activity measurement 6 hours after reperfusion. $n = 6$ in each group. * $p < 0.05$; ** $p < 0.01$;

*** $p < 0.0001$. Abbreviations: Hypo-hepEV, EV secreted by hypoxia and re-oxygenation hepatocyte; Norm-hepEV, EV secreted by normoxic hepatocyte; Ad-Rab27a shRNA, adenovirus encoding Rab27a short hairpin RNA; Ad-SCR, adenovirus encoding scramble short hairpin RNA; Ad-CTL, control adenovirus; TLR-4, toll like receptor 4; TLR-4 flox, TLR-4^{loxP/loxP} mice; Lyz-TLR4 KO, TLR-4^{loxP/-}; Lyz-cre mice. HPF, high power field (40×); NE, neutrophil elastase.

Figure 8. Liver cold IR induces IRF-1, Rab27a and sEV secretion

(A) Western blot of Rab27a protein in transplanted WT livers and its relative band intensity quantification. (B) Western blot of Rab27a protein in transplanted IRF-1 KO livers and its quantification. (C) Western blot of IRF-1 protein in transplanted WT livers and its quantification. (D) ALTs of recipients with WT and IRF-1 KO donors 6 hours after transplantation. (E) ALT of recipients with Rab27a silenced or control donors 6 hours after transplantation. (F) sEV concentrations of recipients with Rab27a silenced or control donors 6 hours after transplantation. $n = 6$ in each group. Donor livers were preserved in 4 °C UW solution for 20 hours. * $p < 0.05$; **** $p < 0.0001$. Abbreviations: Ad-Rab27a shRNA, adenovirus encoding Rab27a short hairpin RNA; Ad-SCR, adenovirus encoding scramble short hairpin RNA; Ad-CTL, control adenovirus; sEV, serum extracellular vesicle.