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A Novel Resolvin-Based Strategy for Limiting Acetaminophen Hepatotoxicity

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OBJECTIVES: Acetaminophen (APAP)-induced hepatotoxicity is a major cause of morbidity and mortality. The current pharmacologic treatment for APAP hepatotoxicity, N-acetyl cysteine (NAC), targets the initial metabolite-driven injury but does not directly affect the host inflammatory response. Because of this, NAC is less effective if given at later stages in the disease course. Resolvins, a novel group of lipid mediators shown to attenuate host inflammation, may be a therapeutic intervention for APAP hepatotoxicity.

METHODS: The temporal patterns of liver injury and neutrophil activation were investigated in a murine model of APAP hepatotoxicity. In addition, the effect of neutrophil depletion and resolvin administration on the severity of liver injury induced by APAP was studied. In vitro studies to investigate the mechanism of resolvin effect on hepatocyte injury and neutrophil adhesion were performed.

RESULTS: We demonstrate that hepatic neutrophil activation occurs secondary to the initial liver injury induced directly by APAP. We also show that neutrophil depletion attenuates APAP-induced liver injury, and administration of resolvins hours after APAP challenge not only attenuates liver injury, but also extends the therapeutic window eightfold compared to NAC. Mechanistic in vitro analysis highlights resolvins' ability to inhibit neutrophil attachment to endothelial cells in the presence of the reactive metabolite of APAP.

CONCLUSIONS: This study highlights the ability of resolvins to protect against APAP-induced liver injury and extend the therapeutic window compared to NAC. Although the mechanism for resolvin-mediated hepatoprotection is likely multifactorial, inhibition of neutrophil infiltration and activation appears to play an important role.

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INTRODUCTION

Acetaminophen (APAP) and APAP-containing products are the most commonly used antipyretic-analgesic medications worldwide. Although APAP is safe when taken at therapeutic doses in the majority of patients, overdoses of APAP can lead to significant morbidity and mortality. In fact, APAP-induced hepatotoxicity accounts for 50% of acute liver failure cases, and is the leading reason for liver transplantation for acute liver failure in the United States.1–3 In addition, the risk of developing APAP hepatotoxicity is further increased in the large cohort of patients with preexisting chronic liver disease.4

The pathogenesis of APAP-induced hepatotoxicity begins with its metabolism by perivenular hepatocytes, leading to the generation of reactive metabolites such as N-acetyl-p-benzoquinone-imine (NAPQI) that directly trigger oxidative stress, mitochondrial damage, and hepatocellular injury.5–7 Growing evidence suggests that as this injury propagates throughout the hepatic lobule, an exuberant host inflammatory response is activated, resulting in hepatic neutrophil infiltration and significant collateral damage.8–13 Without timely treatment, many patients develop fulminant hepatic failure and multiorgan dysfunction.

N-acetyl cysteine (NAC) is the only FDA-approved pharmacologic therapy for APAP hepatotoxicity.1,14 However, it suffers from a limited rescue window (the time between APAP ingestion and initiation of therapy), as it targets only the initial reactive metabolite-driven injury that occurs at the earliest stage of disease pathogenesis.14,15 Unfortunately, many patients are asymptomatic during this stage and do not present for treatment. As such, there is a need to better understand the complete pathogenesis of APAP hepatotoxicity with a particular emphasis on the later stages, in order to develop novel therapies that extend the rescue window to beyond that of NAC.

Immune cells play a dynamic role in activating, maintaining, and resolving inflammation at the site of tissue injury. Kupffer cells, natural killer cells, and neutrophils have all been implicated in APAP hepatotoxicity by releasing various...

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inflammatory mediators including cytokines, chemokines, and reactive oxygen species. Although neutrophil recruitment into the liver and peripheral activation has been demonstrated in APAP hepatotoxicity, neutrophil contribution to the progression and severity of injury is controversial. Whereas some data show that neutrophils establish a host inflammatory response that amplifies overall liver injury, other data suggest that their activation may be a critical event for injury resolution following APAP overdose.

Recently endogenous lipid mediators derived from omega-3 polyunsaturated fatty acids have been shown to control important events during inflammation, such as neutrophil migration, adhesion, activation, and clearance. In particular, docosahexaenoic acid-derived lipid mediators, known as resolvins, regulate critical cellular events in the resolution of inflammation. Resolvins are synthesized by neutrophils during the resolution phase of inflammation, and serve to block the secretion of interleukin-1 beta and tumor necrosis factor-alpha, as well as stimulate nitric oxide production, thereby reducing neutrophil adhesion to the endothelium and inhibiting neutrophil infiltration into the tissue. Resolvins have also been shown to potently and specifically inhibit neutrophil chemotaxis by directly acting on circulating neutrophils. Such resolvins have proven protective in murine models of inflammatory bowel disease, colitis, sepsis, asthmatic airway inflammation, conjunctivitis, myocardial ischemia-reperfusion injury, and burn injury.

In this study, we tested whether inhibition of neutrophil recruitment attenuates APAP-induced hepatotoxicity. Specifically, we hypothesized that administration of a resolin compound, 7S, 16R, 17S-trihydroxy-4Z, 8E, 10Z, 12E, 14E, 19Z-docosahexaenoic acid (RvD2), after APAP overdose would lessen liver injury by reducing the secondary, neutrophil-predominant sterile inflammatory phase seen with APAP hepatic injury.

METHODS

Animals. C57BL/6 mice were purchased from Jackson Laboratory. Male mice between the ages of 6–8 weeks were used for all experiments. All animal protocols were approved by Massachusetts General Hospital Subcommittee on Research Animal Care. For survival experiments, animals were killed when they became moribund according to the criteria of lack of response to stimuli or lack of righting reflex. Ten animals per group were used for each experiment.

Cell culture. Primary rat hepatocytes were isolated and maintained as previously described. Briefly, hepatocytes were isolated from female Lewis rats by a two-step collagenase perfusion technique. Isolation yields ranged from 200 to 300 million hepatocytes/rat with viabilities ranging from 85 to 95%, and purity was >95%. The culture medium for hepatocytes consisted of Williams E supplemented with 20 ng/ml epidermal growth factor (EGF), 14 ng/ml glucagon, 0.5 U/ml insulin, 7.5 μg/ml hydrocortisone, 100 U/ml penicillin, and 100 μg/ml streptomycin. Isolated hepatocytes were stimulated in suspension (2 million cells/ml) with NAPQI (Cayman Chemical, Ann Arbor, MI) at various concentrations (100 μM, 250 μM, 500 μM, and 1,000 μM) for 4 h, in the presence or absence of RvD2 (Cayman Chemical, Ann Arbor, MI) at 10 μM and 100 μM. Hepatocellular damage was assayed by centrifuging the cells and measuring alanine aminotransferase (ALT) in the supernatant using the Infinity ALT (GPT) Liquid Stable Reagent (Thermo Scientific, Middletown, VA). Cell cytotoxicity was assayed by measuring lactate dehydrogenase in the supernatant using a lactate dehydrogenase cytotoxicity assay kit (Cayman Chemical, Ann Arbor, MI). As a positive control, Triton X-100 was used to lyse hepatocytes (Sigma-Aldrich, St Louis, MO).

Human microvascular endothelial cells (HMVECs) (Lanza, United States) were cultured as previously described using MCDB 131 media (Caisson Laboratories, North Logan, UT). Experiments were performed using standard phenol red free Dulbecco’s modified Eagle’s media (Thermo Fisher, Wal- tham, MA, GIBCO) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and l-glutamine (200 mM). Polymorphonuclear granulocytes (PMNs) were isolated from human peripheral blood by gradient separation using Lymphocyte-poly (Cedarlane Labs, Burlington, NC) according to the manufacturer’s protocol. To assay adhesion between PMNs and HMVECs in vitro, PMNs were co-cultured for 1 h with HMVECs in 12-well plates followed by three washing steps. Experimental pretreatment of HMVECs included NAPQI at 250 μM for 4 h. Experimental pretreatment of PMNs included RvD2 at 1 μM for 1 h. For image analysis, HMVEC nuclei were labeled with Hoechst 33342, PMNs with Calcein-AM stain (Life Technologies, Grand Island, NY). Images were acquired using an inverted microscope equipped with an incubation chamber (Axiovert, Zeiss, Oberkochen, Germany) and analyzed using ImageJ 1.43 u. To account for partial detachment of NAPQI-injured HMVECs, the number of adherent PMNs was normalized to the density of HMVECs. Washing steps effectively eliminated nonspecific PMN adherence to HMVEC-free areas.

APAP-induced hepatotoxicity. APAP (Sigma, St Louis, MO) solution was made fresh for each experiment in 0.9% saline at 20 mg/ml and heated until dissolved. APAP was dosed at 400 mg/kg, and injected intraperitoneally after 15 h of starvation. Animals were euthanized by ketamine/xylazine overdose at the manufacturer’s protocol. To assay adhesion between PMNs and HMVECs in vitro, PMNs were co-cultured for 1 h with HMVECs in 12-well plates followed by three washing steps. Experimental pretreatment of PMNs included RvD2 at 1 μM for 1 h. For image analysis, HMVEC nuclei were labeled with Hoechst 33342, PMNs with Calcein-AM stain (Life Technologies, Grand Island, NY). Images were acquired using an inverted microscope equipped with an incubation chamber (Axiovert, Zeiss, Oberkochen, Germany) and analyzed using ImageJ 1.43 u. To account for partial detachment of NAPQI-injured HMVECs, the number of adherent PMNs was normalized to the density of HMVECs. Washing steps effectively eliminated nonspecific PMN adherence to HMVEC-free areas.

Myeloperoxidase assessment. Freshly excised livers were snap-frozen and then crushed in liquid nitrogen. A MPO assay was performed on these samples. Briefly, pulverized liver tissue was added to a non-denaturing lysis buffer (TRIZMA, Sigma, 10% glycerol, and 0.2% Tween), and samples were exposed to three cycles of freeze-thawing, and then centrifuged to collect the lysate. The MPO reaction was...
carried out by first mixing 20 μl of liver lysate with 20 μl assay buffer (0.167 mg/ml o-dianisidine-HCL, 50 mM Na₂HPO₄, pH 5.4) in a 96-well plate, and then adding 200 μl of development solution (0.1% H₂O₂, 50 mM Na₂HPO₄, pH 5.4). Absorbance was measured at 450 nm every 15 s. MPO activity is expressed as the change in absorbance per minute per milligram of liver lysate protein (ΔmOD/min/mg protein). Immunohistochemical staining for MPO was also performed from excised liver tissue.

**In vivo neutrophil depletion.** Neutrophils were depleted using an antibody-mediated strategy using a method previously described. Briefly, mice were injected intravenously with 2 mg/kg of Anti-Gr1 monoclonal antibody (Beckman Coulter, Brea, CA) followed by injection of 1 mg/kg 20 h later. Vehicle-control mice received rat IgG2b. APAP was injected intraperitoneally 4 h after the second dose of anti-Gr1 neutrophil depleting antibody. Animals were killed by ketamine/xylazine injection at various time points for collection of serum and liver tissue for MPO activity assay and histology.

**Resolvin D2 treatment.** Resolvin D2 (Cayman Chemical, Ann Arbor, MI) was reconstituted in 0.9% saline. It was given intravenously via tail vein (25 μg/kg) at various time points after APAP administration. This supraphysiologic dose of RVD2 was chosen to ensure optimal neutrophil interaction in this proof-of-concept study, and was based on previous investigations with RVD2.

**N-acetylcysteine treatment.** NAC (Sigma) was made fresh for each experiment in 0.9% saline at 20 mg/ml. NAC
Mice were treated with resolvin D2 (RvD2) at various city. Resolvin therapy attenuates APAP-induced hepatotoxicity (Figure 2b).

Furthermore, mice treated with anti-GR1 before a lethal dose of APAP exhibited no mortality when followed for 10 days after APAP administration. We observed a significant reduction in hepatic MPO activity in RvD2-treated mice as compared to vehicle-treated animals (Figure 3c). To confirm that RvD2 treatment resulted in diminished hepatic neutrophil infiltration, MPO activity from livers of RvD2 and vehicle-treated mice was measured. We observed significant survival benefit as compared to vehicle-treated mice (Figure 3b). In concordance with the serologic and histological data, we also found that treatment with RvD2 offered significant survival benefit as compared to vehicle-treated mice (Figure 3c). To confirm that RvD2 treatment resulted in diminished hepatic neutrophil infiltration, MPO activity from livers of RvD2 and vehicle-treated mice was measured. We observed a significant reduction in hepatic MPO activity in RvD2-treated mice as compared to vehicle-treated animals (Figure 3d).

RvD2 extends the APAP-treatment rescue window compared to NAC. We next sought to compare the ability of RvD2 to rescue from APAP hepatotoxicity with that of NAC, the "gold-standard" therapy for APAP overdose. We found that NAC therapy rescued mice from APAP-induced liver injury if given within 90 min of APAP administration. However, NAC therapy given 3 h following APAP had no effect on liver injury (Figure 4a). This finding likely relates to the fact that

RESULTS

Temporal evolution of APAP-induced hepatotoxicity. In order to define the temporal pattern of APAP hepatotoxicity, mice were treated with a sub-lethal dose of APAP (400 mg/kg) and examined for liver injury at multiple time points following treatment. We found evidence of significant liver injury as early as 3 h after APAP administration. ALT, a serum marker for hepatocellular injury, was increased at hour 3 compared to hour 0 (3100 IU/l vs. 30 IU/l, ***P<0.001). Evidence for ongoing liver injury in the form of rising ALT persisted until termination of the experiment at hour 24 (Figure 1a). In conjunction with ALT, histological evaluation of liver injury revealed progressively worsening inflammation, hemorrhage, and hepatocyte necrosis through the course of the experiment (Figure 1b). Taken together, these results show continuing liver injury up to 24 h after APAP administration.

The temporal pattern of APAP-induced hepatic neutrophil infiltration was then evaluated using hepatic MPO activity as a surrogate for neutrophil activation. We observed an increase in neutrophil activation at 3 h following APAP administration. However, we observed a much sharper rise in hepatic neutrophil infiltration 12 h following APAP administration (Figure 1b, c).

Neutrophil depletion attenuates APAP-induced liver injury. Mice were pretreated with an antibody directed against granulocyte-differentiation antigen-1 (anti-GR1) at 20 and 4 h before APAP treatment. Anti-GR1 monoclonal antibodies have previously been shown to specifically deplete both peripheral and liver neutrophils in mice, with efficiency rates of >95%. Mice pretreated with anti-GR1 antibody before APAP-administered attenuated hepatocellular injury, based on ALT values, compared to vehicle-treated mice at all time points throughout the study. These results are consistent with previously published data. Notably, the difference in liver injury between anti-GR1-treated and vehicle-treated mice was most significant at later time points (12 and 24 h) after APAP administration (Figure 2a), suggesting that neutrophils play a more prominent role in ongoing liver injury and inflammation as opposed to the initial injury. Furthermore, mice treated with anti-GR1 antibody before a lethal dose of APAP exhibited no mortality when followed for 10 days after APAP administration, in contrast to vehicle-treated mice receiving APAP, which experienced significant mortality (Figure 2b).

Resolvin therapy attenuates APAP-induced hepatotoxicity. Mice were treated with resolvin D2 (RvD2) at various time points following APAP administration and examined for markers of liver injury. RvD2, an endogenous lipid mediator with anti-inflammatory properties, has been previously shown to inhibit neutrophil trafficking to sites of inflammation. We observed that mice treated with RvD2 9 h following APAP administration exhibited less severe hepatic injury. Specifically, ALT levels in RvD2-treated mice were significantly reduced at all time points compared to vehicle-treated mice (Figure 3a). Furthermore, the severity of hepatocyte necrosis, inflammation, and hemorrhage was reduced in RvD2-treated mice both at 12 and 24 h post APAP overdose (Figure 3b). In concordance with the serologic and histological data, we also found that treatment with RvD2 offered significant survival benefit as compared to vehicle-treated mice (Figure 3c). To confirm that RvD2 treatment resulted in diminished hepatic neutrophil infiltration, MPO activity from livers of RvD2 and vehicle-treated mice was measured. We observed a significant reduction in hepatic MPO activity in RvD2-treated mice as compared to vehicle-treated animals (Figure 3d).

Figure 2 Neutrophil depletion attenuates acetaminophen (APAP)-induced hepatotoxicity. Neutrophils were depleted using an antibody-mediated strategy. Wild-type (WT) mice were intravenously injected with 2 mg/kg of Anti-Gr1 antibody and then with 1 mg/kg 20 h later. Vehicle-treated mice were injected with control rat IgG2b. APAP treatment began 4 h after the second dose of anti-Gr1 neutrophil depleting antibody. (a) Time course of serum alanine aminotransferase (ALT) levels from neutrophil-depleted WT mice or vehicle-treated WT mice (N=10 mice/group) after APAP treatment (intraperitoneally (IP), 400 mg/kg). (b) Kaplan–Meier survival curve for neutrophil-depleted WT mice or vehicle-treated WT mice (N=10 mice/group) after a single lethal dose of APAP (750 mg/kg). **P<0.005; ***P<0.001.
NAC primarily targets reactive metabolite-induced injury that occurs within the first hour or so of APAP overdose. These results are also consistent with clinical data showing that the efficacy of NAC decreases in humans when treatment is delayed following APAP ingestion. Conversely, treatment with RvD2 up to 12 h following APAP led to significant attenuation of liver injury (Figure 4b).

RvD2 does not protect against NAPQI-induced hepatotoxicity but does inhibit neutrophil activation in vitro. To investigate the mechanistic aspects of our in vivo observations, we studied the effect of RvD2 on NAPQI-induced hepatocyte injury in vitro. Specifically, rat primary hepatocytes were cultured with NAPQI in the presence or absence of RvD2 and examined for hepatocyte injury. Our in vivo data suggest neutrophils likely do not contribute to initial injury induced by APAP; supporting the notion that direct toxicity from NAPQI drives initial injury. Therefore, we hypothesized that RvD2 would not affect NAPQI-related hepatocyte injury. Accordingly, we found that the treatment with escalating doses of RvD2 offered no protection against hepatocyte injury and death induced by NAPQI (Figure 5a).

Multiple inflammatory mediators activate neutrophils and enhance their interaction with endothelial cells, thereby promoting neutrophil infiltration into active sites of tissue injury. Previous work has demonstrated the ability of RvD2 to inhibit neutrophil migration to sites of injury. We showed that co-culturing of human neutrophils and endothelial cells in the absence of NAPQI leads to minimal neutrophil adhesion. Conversely, the addition of NAPQI strongly increases neutrophil adhesion to endothelial cells. Notably, NAPQI-induced neutrophil adhesion to endothelial cells is significantly attenuated by RvD2 treatment (Figure 5b).

**DISCUSSION**

In this study we demonstrate that hepatic neutrophil infiltration occurs secondary to APAP-induced liver injury, and that neutrophil depletion attenuates liver injury. Furthermore, we show that administration of RvD2 after APAP overdose attenuates liver injury and extends the therapeutic rescue window as compared to the gold standard of treatment, NAC. Mechanistic in vitro analysis highlights RvD2’s ability to inhibit neutrophil attachment to endothelial cells in the presence of NAPQI, the reactive metabolite of APAP, suggesting RvD2 may prevent neutrophil migration into the liver following APAP injury.

Although inflammation is host protective against a variety of insults and pathogens, excessive inflammation can be damaging. For example, sepsis results from an overly exuberant and persistent inflammatory response to a
pathogen, resulting in multisystem organ dysfunction. Furthermore, the inability to dampen inflammation may contribute to the development of certain chronic inflammatory diseases, such as inflammatory bowel disease and rheumatoid arthritis. Accordingly, the ability to modulate the resolution phase of the inflammatory response may prove critical in the treatment of these disorders. Endogenous lipid mediators, termed resolvins, have been shown to actively contribute to the inflammatory response induced by APAP, and, in fact, may provide definitive evidence for the role of neutrophils in APAP-induced liver injury. This exciting finding deserves further investigation as a potential treatment strategy, either as an alternative to or in combination with NAC, for the treatment of APAP overdose in patients.

The contribution of neutrophils to the pathogenesis of APAP-induced hepatotoxicity has been the subject of debate. Consistent with our data, previous reports have documented attenuation of APAP liver injury in neutrophil-depleted mice, suggesting an essential role for neutrophils in the injury process. In addition, recent data show neutrophils contribute to the sterile inflammatory response seen in APAP-induced liver injury in a TLR-9-dependent manner. However, other reports suggest that neutrophils are not critical to the inflammatory response induced by APAP, and, in fact, may contribute to liver regeneration and recovery following the APAP insult. Although our study does not provide definitive evidence for the role of neutrophils in APAP liver injury, it provides experimental support for their importance.

Despite these exciting findings, certain limitations need to be noted. As this was a proof-of-concept study, we did not test various doses of RvD2 to ascertain the lowest dose needed to obtain protection. It is unclear if the supraphysiological dose used in this study would be tolerated clinically; therefore, the use of RvD2 as a therapeutic target in humans with APAP overdose remains uncertain. Furthermore, it must be noted that in this study we did not investigate the effects of RvD2 administration systemically and on immune cells other than neutrophils, such as macrophages and natural killer cells.
Further work is needed to comprehensively define the effect of resolvins on the multiple inflammatory pathways involved in the APAP liver injury.

In summary, we highlight the ability of RvD2 to protect against APAP-induced liver injury, and compared to NAC therapy, significantly extend the therapeutic rescue window by eightfold following APAP overdose. Although the mechanism for RvD2-induced hepatoprotection is likely multifactorial, inhibition of neutrophil migration and activation plays an important role. Further study into the potential use of RvD2 in the treatment of APAP-induced hepatotoxicity is thus warranted.

CONFLICT OF INTEREST

Guarantor of the article: Martin L. Yarmush, MD, PhD.
Specific author contributions: Suraj J. Patel and Jay Luther designed the concept, performed experiments, and wrote manuscript. Stefan Bohr, Arvin Iracheta-Vellve, Matthew Li, and Kevin R. King performed experiments. Raymond T. Chung and Martin L. Yarmush provided final approval.

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Potential competing interests: None.
Study Highlights

**WHAT IS CURRENT KNOWLEDGE**
- Acetaminophen (APAP) overdose can induce severe liver injury and failure.
- APAP-induced hepatotoxicity accounts for 50% of acute liver failed cases, and is the leading reason for liver transplantation for acute liver failure (ALF) in the United States.
- N-acetyl cysteine (NAC), the only FDA-approved therapy for APAP overdose, has limited therapeutic window, limiting its clinical efficacy.

**WHAT IS NEW HERE**
- Resolvis can rescue from APAP-induced hepatotoxicity and improve morbidity and mortality in an animal model as compared to current gold standard therapy with NAC.
- Resolvis also extend the therapeutic window eightfold compared to NAC, which is of critical importance given the clinical need for therapies that provide protection when given long after APAP ingestion.
- Resolvis act by dampening the secondary host inflammatory response generated by APAP instead of acting on the toxic metabolite.
- Resolvis block neutrophil entry and activation in hepatic tissue, thereby limiting inflammation.

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