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RESVERATROL PREVENTS PROTEIN NITRATION AND RELEASE OF ENDONUCLEASES FROM MITOCHONDRIA DURING ACETAMINOPHEN HEPATOTOXICITY

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Abstract

Overdose of acetaminophen (APAP) is a common cause of acute liver injury and liver failure. The mechanism involves formation of a reactive metabolite, protein binding, oxidative stress and activation of c-Jun N-terminal kinase (JNK), mitochondrial dysfunction, and nuclear DNA fragmentation caused by endonucleases released from damaged mitochondria. Previous work has shown that the natural product resveratrol (RSV) can protect against APAP hepatotoxicity in mice through prevention of lipid peroxidation and anti-inflammatory effects. However, these earlier studies did not take into consideration several fundamental aspects of the pathophysiology. To address this, we treated C57Bl/6 mice with 300 mg/kg APAP followed by 50 mg/kg RSV 1.5h later. Our results confirmed that RSV reduced liver injury after APAP overdose in mice. Importantly, RSV did not inhibit reactive metabolite formation and protein bindings, nor did it reduce activation of JNK. However, RSV decreased protein nitration after APAP treatment, possibly through direct scavenging of peroxynitrite. Interestingly, RSV also inhibited release of apoptosis-inducing factor and endonuclease G from mitochondria independent of Bax pore formation and prevented the downstream nuclear DNA fragmentation. Our data show that RSV protects against APAP hepatotoxicity both through antioxidant effects and by preventing mitochondrial release of endonucleases and nuclear DNA damage.

Graphical Abstracts

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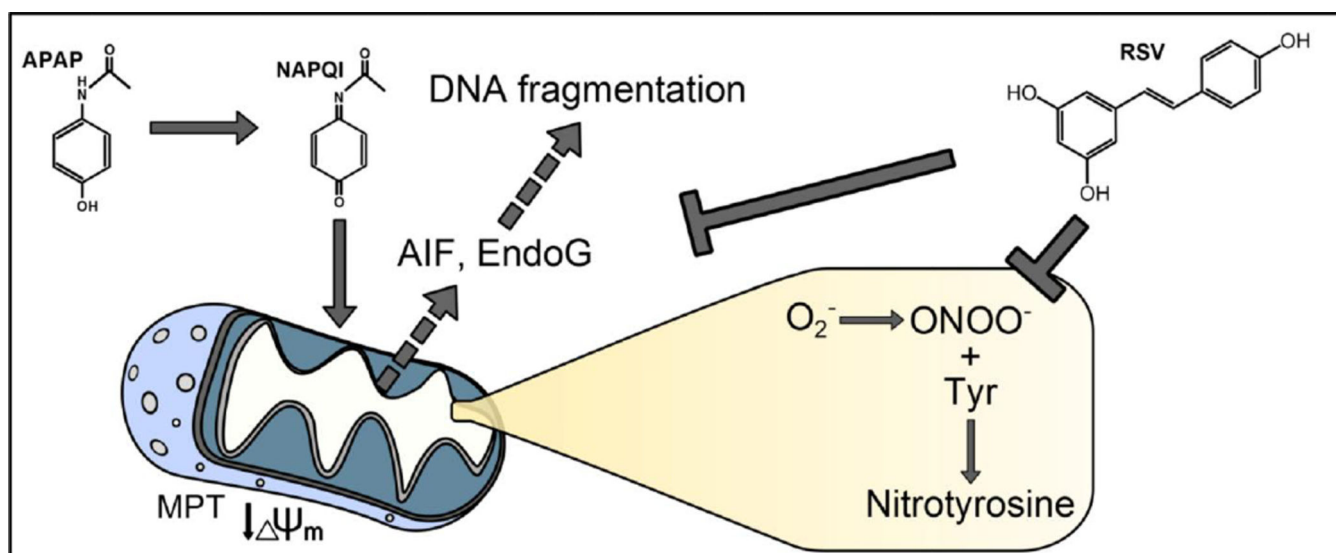
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CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.



Keywords

Acetaminophen; resveratrol; drug hepatotoxicity; oxidant stress; DNA fragmentation; mitochondrial dysfunction

1. INTRODUCTION

Acetaminophen (APAP) is an analgesic and antipyretic drug that is widely used in Western countries. While it is safe at therapeutic doses, overdose can cause acute liver injury and liver failure (Budnitz et al., 2011; Larson et al., 2005). The toxicity of APAP is initiated by the formation of a reactive metabolite, NAPQI, which can deplete glutathione and bind to cellular proteins, especially proteins in mitochondria (Tirmenstein et al., 1989). The mitochondrial protein binding disturbs the normal respiration of the mitochondria (Meyers et al., 1988) and enhances the formation of reactive oxygen species (ROS) (Jaeschke, 1990) and peroxynitrite (Hinson et al., 1998; Cover et al., 2005). The resulting oxidative stress activates the c-jun N-terminal kinases (JNK) 1/2 (Gunawan et al., 2006; Hendersen et al., 1997; Saito et al., 2010a). JNK activation seems to involve several upstream kinases, including apoptosis signal regulating kinase 1 (Nakagawa et al., 2008), glycogen synthase kinase 3 β (Shinohara et al., 2010), mixed lineage kinase 3 (Sharma et al., 2012), and possibly the receptor interacting protein kinase 3 (Ramachandran et al., 2013). Activated JNK then translocates to the mitochondria and eventually triggers the opening of the mitochondrial membrane permeability transition (MPT) pore (Hanawa et al., 2008; Kon et al., 2004; Masubuchi et al., 2005). This results in mitochondrial dysfunction and release of mitochondrial intermembrane proteins. In particular, apoptosis-inducing factor (AIF) and endonuclease G (EndoG) are released and translocate to the nucleus where they cause nuclear DNA fragmentation (Bajt et al., 2006, 2011). The extensive mitochondrial dysfunction and nuclear DNA damage leads to necrotic cell death (Gujral et al., 2002). Importantly, emerging evidence suggests that these mechanisms are also relevant for the

pathophysiology of APAP-induced liver injury in humans (McGill et al., 2012a; 2014; Xie et al., 2014).

The use of natural products in the prevention and treatment of liver diseases has gained considerable popularity (Zhang et al., 2013; Nair et al., 2014; Jaeschke et al., 2011; 2013). Among these products, resveratrol (RSV), a natural polyphenolic compound primarily found in the skin of grapes, is particularly well-studied (Bishayee et al., 2010). It has been shown to protect against liver damage caused by hepatic ischemia-reperfusion (Hassan-Khabbar et al., 2008; 2010; Plin et al., 2005), ethanol toxicity (Ajmo et al., 2008; Min et al., 2008), high fat diet (Ahn et al., 2008) and hepatotoxic agents such as APAP (Masubuchi et al., 2009; Sener et al., 2006) and carbon tetrachloride (Rivera et al., 2008). The mechanisms that have been proposed to explain this protection include antioxidant effects, up-regulation of anti-oxidant enzymes, modulation of inflammatory responses, and induction of mitochondrial biogenesis (Lagouge et al., 2006; Baur et al., 2006; Sener et al., 2006; Masubuchi et al., 2009; Bishayee et al., 2010). The first group to examine the effect of RSV in APAP-induced liver injury concluded that it protected by acting as an antioxidant, preventing lipid peroxidation (LPO) and possibly neutrophil infiltration (Sener et al., 2006). A more recent study suggested that RSV protected by lowering expression of the pro-inflammatory cytokine TNF- α (Masubuchi et al., 2009). However, these mechanisms are in conflict with other data. For example, it has been clearly demonstrated that the low level of LPO caused by APAP is not critical for the liver injury (Knight et al., 2003). Furthermore, there is considerable evidence that the sterile inflammatory response after APAP does not contribute to the liver injury phase in mice or humans (Cover et al., 2006; Williams et al., 2010a; 2010b; 2011; 2014; Xie et al., 2013), although it may be needed for later recovery and regeneration (Jaeschke et al., 2012; Holt et al., 2008). In addition, neither of these studies examined the effect of RSV on metabolic activation of APAP (i.e. NAPQI formation), which is an essential prerequisite for mechanistic investigation of any intervention in this model (Jaeschke et al., 2011; 2013). Finally, several critical events in the initiation and progression of APAP hepatotoxicity, including JNK activation and mitochondrial translocation, mitochondrial dysfunction, protein nitration, and nuclear DNA fragmentation were not investigated. Thus, the aim of the current study was to explore the effects of RSV treatment on these important mechanisms in APAP hepatotoxicity.

2. MATERIALS AND METHODS

2.1 Animals

Male C57Bl/6 mice (8–12 weeks old) were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in an environmentally controlled room with a 12 h light/dark cycle and free access to food and water. All experiments followed the criteria of the National Research Council for the care and use of laboratory animals and were approved by the Institutional animal Care and Use Committee of the University of Kansas Medical Center.

2.2 In vivo experiments

Mice were fasted overnight, then injected i.p. with 300 mg APAP/kg body weight. APAP (Sigma, St Louis, MO) was dissolved in warm saline. At 1.5 h post-APAP, some animals

were i.p. injected with 50 mg/kg trans-resveratrol (RSV) (Cayman, Ann Arbor, MI) dissolved in 40% dimethyl sulfoxide (DMSO) in saline or with the vehicle alone (1.2 mL/kg). Mice were euthanized at 0, 6, 24 or 48 h post-APAP and blood and livers were harvested. Blood was drawn from the caudal vena cava into a heparinized syringe and centrifuged to obtain plasma. The right and caudate liver lobes were used for isolation of mitochondria, as previously described (Xie et al., 2013). One portion from the left lateral lobe was fixed in 10% phosphate-buffered formalin for histology, and the remaining pieces were flash frozen and stored at -80°C for later analysis.

2.3 In vitro experiments

Primary mouse hepatocytes were isolated as previously described (Bajt et al., 2004). After a short incubation period to allow attachment to the culture dish, the cells were washed and treated with 5 mM APAP dissolved in the culture medium. Some cells were co-treated with either 100 μM RSV or DMSO vehicle and the cells were harvested at 0, 4.5 or 8.5 h post-APAP.

2.4 Biochemical assays

Plasma alanine aminotransferase (ALT) activity was measured using a kit, according to the manufacturer's instruction (Pointe Scientific, MI). Lactate dehydrogenase activity (LDH) was measured as previously described (Xie et al., 2013). The JC-1 assay was performed using a kit, according to the manufacturer's instructions (Cell Technology, Fremont, CA). Assays for hepatic GSH and GSSG levels were performed using a modified Tietze assay, as previously described (Jaeschke and Mitchell, 1990). Real time-PCR for mRNA quantification was performed as previously described (Saito et al., 2010b). APAP-protein adducts were measured in liver tissues and mitochondrial pellets as described (Ni et al., 2012b; McGill et al., 2012b).

2.5 Western Blotting

Western blotting was performed, as previously described (Bajt et al., 2000). The primary antibodies were rabbit anti-JNK and anti-phospho-JNK antibodies (Cell Signaling Technology, Danvers, MA), rabbit anti-Bax polyclonal antibody (Cell Signaling Technology, Danvers, MA), rabbit anti-AIF antibody (Cell Signaling Technology, Danvers, MA), rabbit anti-EndoG (ProSci, Poway, CA) and rabbit anti-nitrotyrosine (Upstate, Lake Placid, NY). A horseradish peroxidase-coupled anti-rabbit IgG (Santa Cruz) was used as secondary antibody.

2.6 Histology and immunohistochemistry

Formalin-fixed liver tissues were embedded in paraffin and 5 μm thick sections were cut. Hematoxylin and eosin (H&E) staining was performed for evaluation of tissue necrosis (Gujral et al., 2002). Nitrotyrosine staining was performed for assessment of nitrotyrosine (NT) protein adducts, as previously described (Knight et al., 2002), using the Dako LSAB peroxidase kit (Dako, Carpinteria, CA) and a rabbit polyclonal anti-nitrotyrosine antibody (Life Technologies, Grand Island, NY). Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay was performed for DNA strand break assessment

with the In Situ Cell Death Detection Kit, AP (Roche Diagnostics, Indianapolis, IN), as described in the manufacturer's instructions.

2.7 Statistics

All data were expressed as mean \pm SE. For normally distributed data, statistical significance was evaluated by one-way analysis of variance (ANOVA), followed by Student Newman–Keuls' test. When the data were not normally distributed, ANOVA was performed on ranks, followed by Dunn's multiple comparisons. $P < 0.05$ was considered significant.

3. RESULTS

3.1 RSV protects against APAP hepatotoxicity

To confirm that RSV protects against APAP hepatotoxicity, mice were treated with 300 mg/kg APAP followed by 50 mg/kg RSV 1.5 h later and plasma and livers were collected at 0, 6, 24, and 48 h post-APAP. Consistent with earlier reports, plasma ALT activities were significantly lower in the RSV-treated animals compared to vehicle-treated animals at all time points (Fig. 1A). Similarly, RSV-treated mice had less evidence of necrosis in H&E-stained histology sections (Fig. 1B).

3.2 RSV does not interfere with protein binding but does reduce protein nitration

To determine whether RSV affects the metabolic activation of APAP, we next measured APAP-protein adducts at 6 h. There was no difference in adduct levels in either total liver homogenates or in mitochondrial fractions (Fig. 2), suggesting that RSV does not inhibit NAPQI formation or protein binding when administered 1.5 h after APAP. Consistent with this, there was also no difference in GSH depletion and recovery between vehicle and RSV-treated mice at 6 and 24 h post-APAP (Fig. 3A). However, there were lower glutathione disulfide (GSSG) levels and a reduction in the GSSG-to-GSH ratio at 24 h (Fig. 3B). Although these data could suggest that RSV has antioxidant effects, it is difficult to determine whether or not the reduced GSSG at this late time point is a cause or a consequence of the protection. To further test this, we performed immunostaining for nitrotyrosine in the liver at 6 h post-APAP (Fig. 3C). Protein tyrosine nitration is thought to result from peroxynitrite (ONOO^-) formed within mitochondria as a result of superoxide (O_2^-) reacting with nitric oxide (NO) during APAP hepatotoxicity. Importantly, livers from RSV-treated animals stained much less strongly for nitrotyrosine (Fig. 3C) and lower nitrotyrosine levels were seen by western blotting (Fig. 3D). These data are consistent with the hypothesis that RSV may serve as a direct antioxidant at early time points. A number of natural products have been shown to induce expression of antioxidant genes (Wu et al., 2014). To determine whether or not RSV also prevents oxidative stress by inducing a stress response, we measured expression of several known Nrf2 targets and other antioxidant genes, namely the catalytic subunit of glutamate cysteine ligase (Gclc), heme oxygenase-1 (Ho-1), catalase, superoxide dismutases (Sod) 1 and 2, and metallothioneins 1 and 2 by RT-PCR. Although most of these genes were induced by APAP, none were further induced by RSV co-treatment (Table 1). Thus, the antioxidant effects of RSV did not involve the selective induction of antioxidant genes.

3.3 RSV does not affect JNK activation or the MPT

Because JNK activation during APAP hepatotoxicity is thought to require oxidative stress, we next measured JNK phosphorylation in these animals. Surprisingly, despite the reduced protein nitration, we did not observe a decrease in either activation or mitochondrial translocation of JNK between the vehicle and RSV-treated mice (Fig. 4). In fact, JNK activation appeared to be higher after RSV compared with vehicle treatment. The latter was not an effect of the RSV vehicle, as DMSO did not affect JNK activation or translocation (Suppl. Fig. 1). It is generally accepted that JNK translocation is a critical step in the mitochondrial dysfunction caused by APAP. To determine whether or not mitochondrial dysfunction was prevented by RSV despite the similar JNK activation, primary mouse hepatocytes were co-treated with 5 mM APAP and either vehicle or 100 μ M RSV, and the mitochondrial membrane potential was assessed using the JC-1 assay. We found that RSV provided complete protection against APAP-induced cell death (Fig. 5A) without preventing the loss of the mitochondrial membrane potential (Fig. 5B). Mitochondrial membrane potential declined as early as 4.5 h post-treatment and remained low to at least 8.5 h. Interestingly, RSV alone actually caused a transient loss of the membrane potential, with recovery by 8.5 post-treatment (Fig. 5B). These data show that, although RSV reduces oxidative stress in this model of APAP toxicity, the extent of the reduction may not be enough to prevent mitochondrial dysfunction.

3.4 RSV prevents release of endonucleases from mitochondria

To determine whether or not RSV affects release of endonucleases from the mitochondria and the resulting nuclear DNA fragmentation, AIF and EndoG release into the cytosol were measured by western blotting and TUNEL staining was performed in tissue sections. Interestingly, although RSV did not prevent mitochondrial JNK translocation *in vivo* or the loss of the mitochondrial membrane potential *in vitro*, it reduced both the release of these endonucleases from mitochondria (Fig. 6A) and nuclear DNA fragmentation (Fig. 6B). Because Bax has been shown to be responsible for release of AIF from mitochondria at early time points in APAP hepatotoxicity (Bajt et al., 2008), we next measured translocation of Bax into mitochondria. Surprisingly, there was no difference in mitochondrial Bax translocation between vehicle and RSV-treated animals (Fig. 6A). These data suggest that RSV protects against APAP hepatotoxicity, in part, by inhibiting Bax-independent release of AIF and EndoG from mitochondria and preventing nuclear DNA fragmentation. Interestingly, we found that treatment with DMSO vehicle alone caused a minor increase in endonuclease release as well (Suppl. Fig. 1). Thus, the fact that RSV completely prevented AIF release and reduced EndoG release after treatment with APAP and the RSV vehicle further supports the idea that RSV is an effective inhibitor of endonuclease release.

4. DISCUSSION

The objective of the current investigation was to evaluate the mechanisms of protection of RSV against APAP hepatotoxicity. Previous work has shown that RSV can prevent LPO and inflammation during APAP hepatotoxicity. However, those earlier studies failed to test the effects of RSV on critical upstream events in the basic pathophysiology of APAP-induced liver injury. In contrast, our data suggest that the delayed administration of RSV did

not affect protein adduct formation but appeared to protect by scavenging peroxynitrite and by preventing nuclear DNA fragmentation through inhibiting release of the endonucleases AIF and EndoG from mitochondria.

4.1 Resveratrol as direct scavenger of peroxynitrite

Several groups have provided evidence for peroxynitrite formation during APAP-induced liver injury (Hinson et al, 1998; Knight et al., 2001) and follow-up studies have demonstrated that peroxynitrite is indeed a critical cytotoxic mediator in the pathophysiology (Knight et al., 2002). The oxidant stress is mainly located in mitochondria (Jaeschke, 1990), presumably derived from the impaired respiratory chain (Meyers et al., 1988), and consequently peroxynitrite formation occurs also in mitochondria (Cover et al., 2005). The mitochondrial oxidant stress and peroxynitrite are responsible for the opening of the mitochondrial membrane permeability transition pore (MPT) and necrotic cell death (Kon et al., 2004, 2010). Our data indicated the protective effect of RSV correlated with extensive reduction of nitrotyrosine adducts in the liver at 6 h. Interestingly, there was no significant effect on GSSG formation, which indicated that RSV appeared to mainly scavenge peroxynitrite rather than prevent the oxidant stress during these early time points. In support of this conclusion, RSV did not affect protein adduct formation including adducts in mitochondria and did not affect JNK activation and translocation to mitochondria, which are related to the mitochondrial oxidant stress (Hanawa et al., 2008; Saito et al., 2010a). On the other hand, several studies have shown that RSV can act as a direct scavenger of peroxynitrite. Holthoff et al. (2010) found that RSV prevents serum albumin nitration by 5-amino-3-(4-morpholinyl)-1,2,3-oxadiazolium chloride (SIN-1), which is a donor of both NO and O_2^- and therefore serves as a source of $ONOO^-$, with an EC_{50} of $\sim 23 \mu M$. Importantly, they also observed that RSV could prevent nitrotyrosine formation in cultured cells at concentrations similar to the one used in our study (Holthoff et al., 2010). These data were confirmed by another group (Yang et al., 2012). Although it has been reported that RSV can induce expression of endogenous antioxidant genes (Surh et al., 2008), we were unable to reproduce this in our model. Our findings are more consistent with a recent report showing that RSV failed to induce expression of Nrf2-dependent antioxidant response genes in a high-throughput screening study (Wu et al., 2014). Thus, our data support the hypothesis that RSV protected *in vivo* mainly by directly scavenging peroxynitrite.

4.2 Mitochondrial dysfunction and DNA fragmentation

Due to a lack of caspase activation, caspase-activated DNase cannot be involved in nuclear DNA damage during APAP hepatotoxicity (Jaeschke et al., 2006). Instead it was shown that DNA fragmentation is linked to mitochondrial dysfunction (Cover et al., 2005). More specifically, AIF and EndoG are released from the intermembrane space of mitochondria into the cytosol and translocate to the nucleus to induce DNA damage (Bajt et al., 2006) and generate DNA fragments of various sizes (Cover et al., 2005; Jahr et al., 2001). Importantly, preventing DNA fragmentation reduces the injury (Bajt et al., 2008; 2011). Release of both endonucleases appears to require two steps: the formation of a pore in the outer mitochondrial membrane, and proteolytic cleavage of the inner-membrane-anchored N-terminus of AIF (Arnoult et al., 2003; Otera et al., 2005). The issue of what protease is responsible for the latter is controversial. Conflicting reports suggest that the enzyme is a

inflammatory response with cytokine formation and neutrophil recruitment (Cover et al., 2006; Williams et al., 2010a, 2010b), the pathophysiological relevance of inflammation is controversial (Jaeschke et al., 2012). Sterile inflammation is critically dependent on the release of damage associated molecular patterns (DAMPs) from necrotic cells. Thus, a reduction in cell necrosis will attenuate DAMP release and consequently reduce pro-inflammatory cytokine formation and neutrophil infiltration (Xie et al., 2013). One of the previous studies showed RSV partially protected against APAP toxicity with reduced neutrophil accumulation at 4 h (Sener et al., 2009). However, the 4 h time point is very early during the injury phase and DAMP release making it likely that the reduced injury was the cause of the lower neutrophil infiltration. The second study also showed only a correlation between less liver injury in the RSV-treated group and reduced hepatic TNF- α mRNA expression (Masubuchi et al., 2009). Thus, none of these studies actually demonstrated a direct anti-inflammatory effect of RSV. Together, the preponderance of experimental evidence argues against inflammation being a major contributor to APAP-induced liver injury in experimental animals (Jaeschke et al., 2012) and in humans (Antoniades et al., 2012; Williams et al., 2014).

4.5 Summary and conclusions

In summary, our data suggest that therapeutic administration of RSV protects against APAP-induced liver injury by scavenging peroxynitrite and by preventing AIF and EndoG release from mitochondria and the subsequent nuclear DNA fragmentation. These findings are in contrast to previously reported mechanisms of RSV-mediated protection and illustrate the importance of considering all steps in the basic pathophysiology of APAP toxicity. Importantly, our data strongly emphasize that RSV may be a valid treatment option for APAP overdose.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AIF	apoptosis-inducing factor
ALT	alanine aminotransferase
APAP	acetaminophen
DAMP	damage associated molecular pattern
DMSO	dimethyl sulfoxide

EndoG	endonuclease G
GSH	glutathione
GSSG	glutathione disulfide
<i>gclc</i>	Glutamate-cysteine ligase, catalytic subunit
H&E	hematoxylin& ampeosin
Ho-1	heme oxygenase 1
JNK	c-Jun N-terminal kinase
LDH	lactate dehydrogenase
LPO	lipid peroxidation
MPT	mitochondrial membrane permeability transition
Mt	metallothionein
NAPQI	<i>N</i> -acetyl- <i>p</i> -benzoquinone imine
RSV	resveratrol
SOD	superoxide dismutase
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay

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HIGHLIGHTS

- Resveratrol post-treatment protects against acetaminophen hepatotoxicity
- Resveratrol post-treatment does not affect acetaminophen-protein binding or JNK activation
- Resveratrol reduces oxidative stress and nitrotyrosine staining during acetaminophen toxicity
- Resveratrol reduces release of endonucleases from damaged mitochondria during acetaminophen toxicity

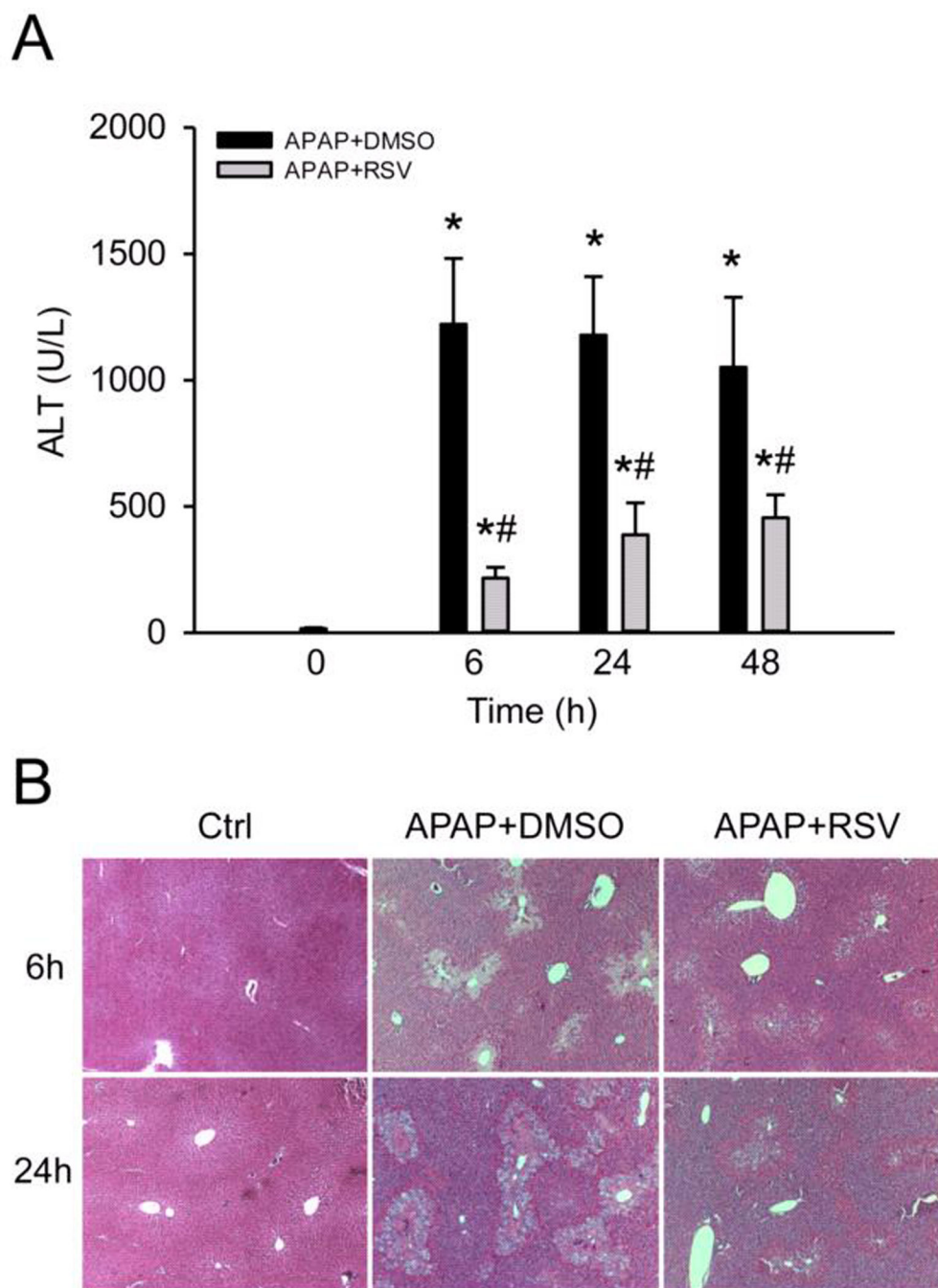


Figure 1. Resveratrol protects against acetaminophen (APAP) hepatotoxicity

Mice were treated with 300 mg/kg APAP followed by dimethyl sulfoxide (DMSO) vehicle or 50 mg/kg resveratrol (RSV) 1.5h later. Plasma and liver tissue were harvested at the indicated time points post-APAP. (A) Plasma alanine aminotransferase (ALT) activity. (B) H&E stained liver sections. Bars represent means \pm SEM for $n = 3-6$ mice. * $P < 0.05$ vs. 0h. # $P < 0.05$ vs. APAP + DMSO treatment.

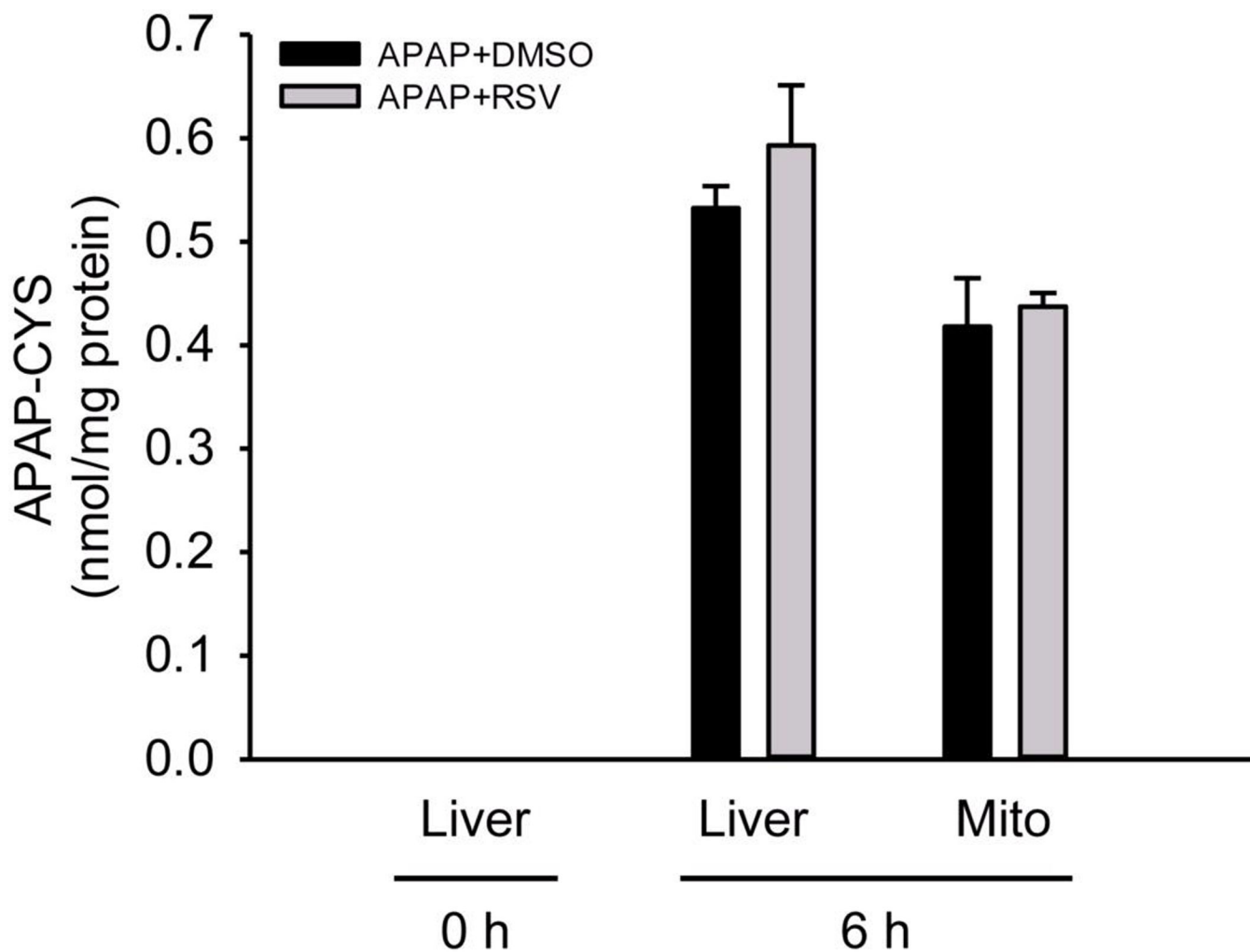


Figure 2. Resveratrol post-treatment does not affect acetaminophen (APAP)-protein binding
Mice were treated with 300 mg/kg APAP followed by dimethyl sulfoxide (DMSO) vehicle or 50 mg/kg resveratrol (RSV) 1.5h later. Liver tissue was harvested at 6h post-APAP. APAP-protein adducts were measured in both whole liver homogenates and isolated mitochondrial fractions. Bar graphs show mean \pm SEM for n = 3–6 mice.

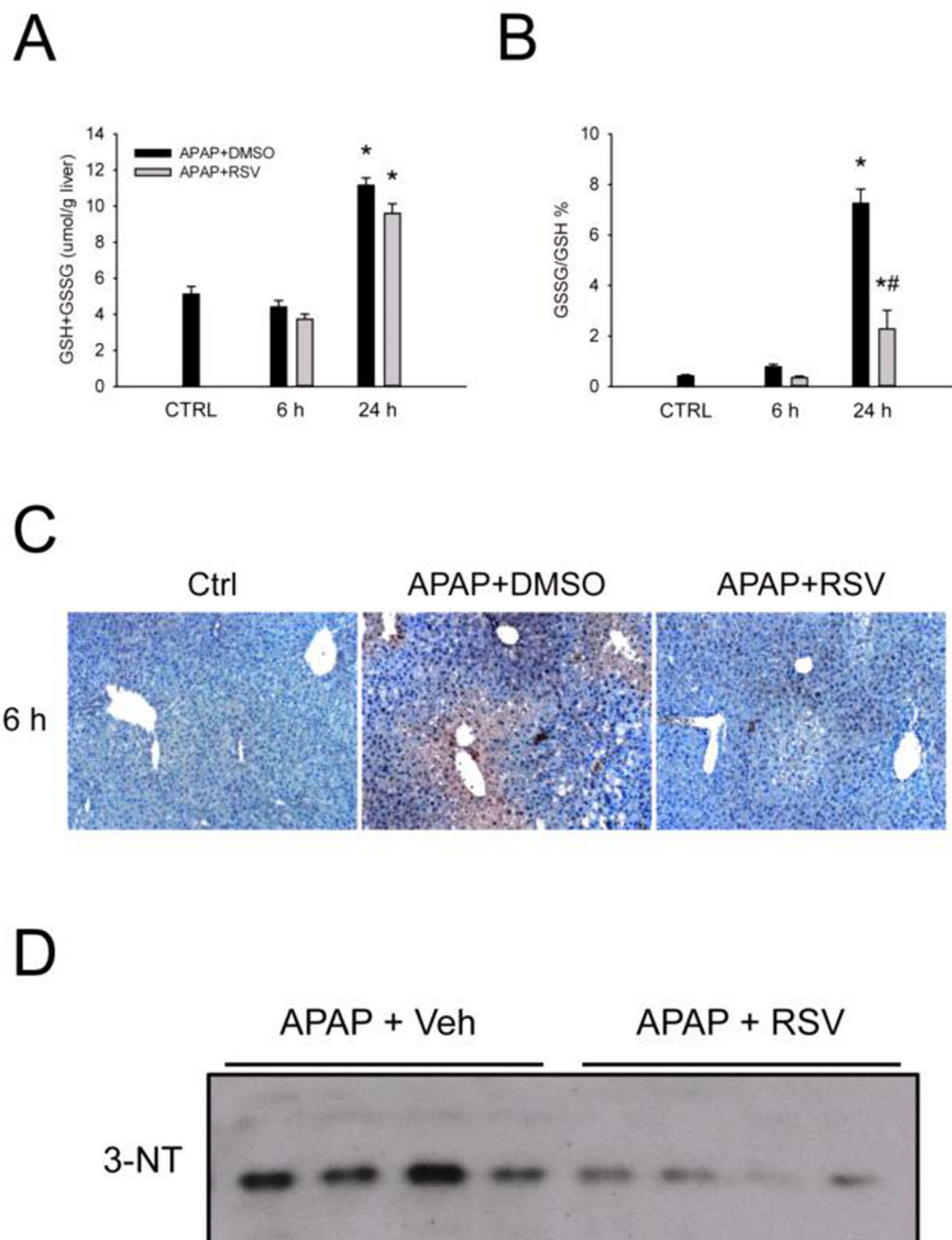


Figure 3. Resveratrol post-treatment does not affect glutathione depletion but does reduce oxidative stress and nitrotyrosine levels in the liver

Mice were treated with 300 mg/kg acetaminophen (APAP) followed by dimethyl sulfoxide (DMSO) vehicle or 50 mg/kg resveratrol (RSV) 1.5h later. Liver tissue was harvested at the indicated time points post-APAP. (A) Total hepatic glutathione (GSH) levels. (B) The percentage of total GSH in the oxidized form (GSSG). (C) Nitrotyrosine staining of liver tissue sections. (D) Western blot of whole liver homogenate using an anti-nitrotyrosine antibody. Bar graphs show mean \pm SEM for $n = 3-6$ mice. * $P < 0.05$ vs. CTRL. # $P < 0.05$ vs. APAP + DMSO treatment.

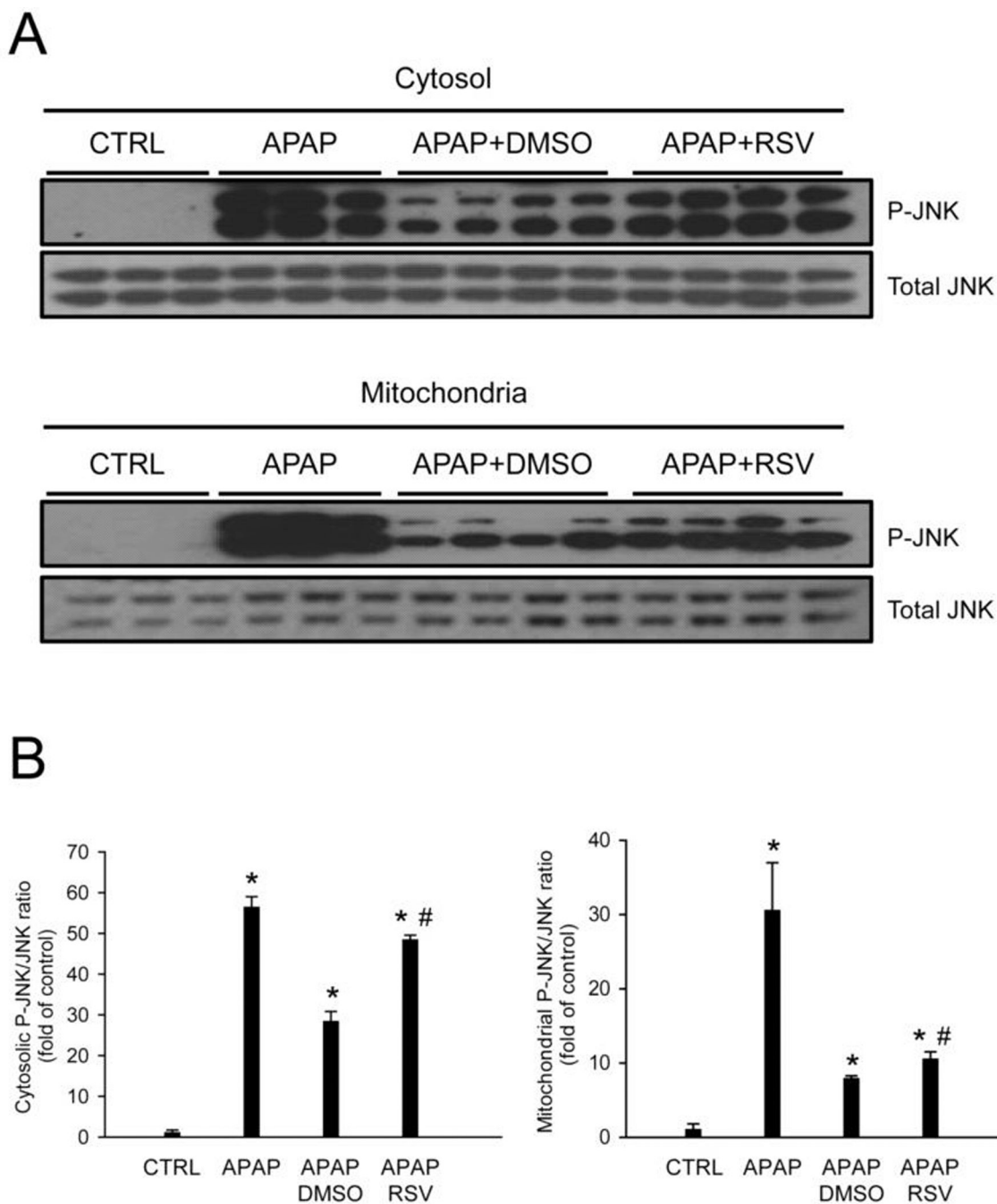


Figure 4. Resveratrol does not prevent c-Jun N-terminal kinase (JNK) activation or mitochondrial translocation

Mice were treated with 300 mg/kg acetaminophen (APAP) followed by dimethyl sulfoxide (DMSO) vehicle or 50 mg/kg resveratrol (RSV) 1.5h later. (A) Liver tissue was harvested at 6 h post-APAP and mitochondrial and cytosol fractions were isolated and probed for the total and phosphorylated forms of the c-Jun N-terminal kinases 1/2. (B) Densitometry. Bar graphs show mean \pm SEM for n = 3–4 mice. *P < 0.05 vs. CTRL. #P < 0.05 vs. APAP + DMSO treatment.

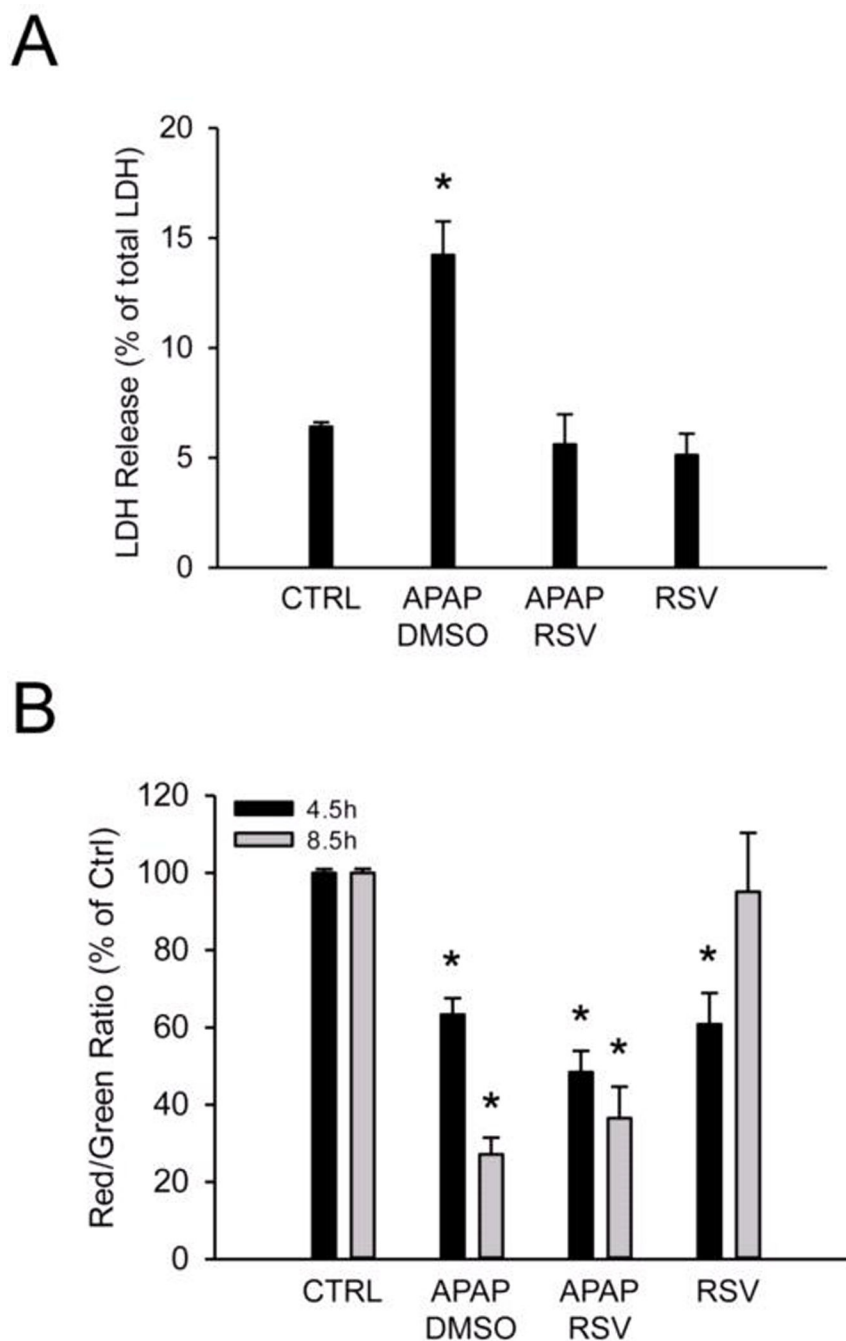
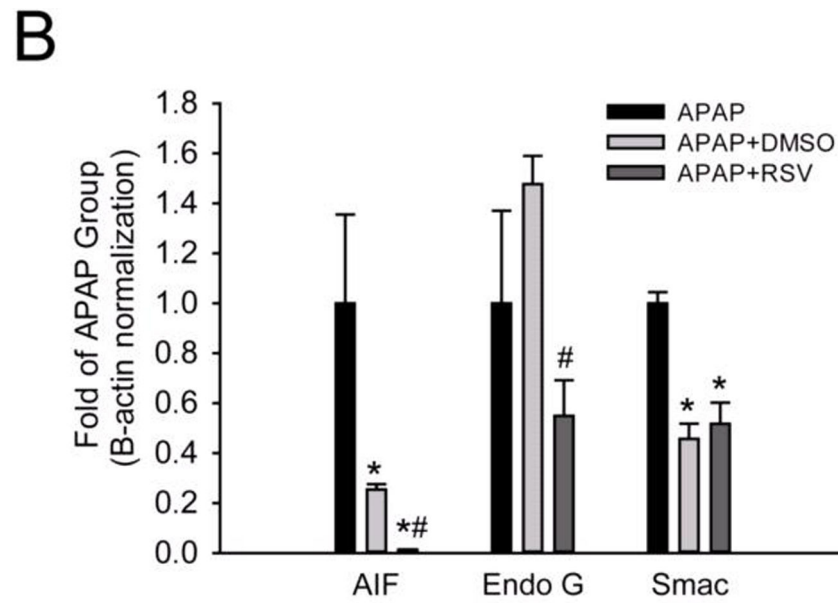
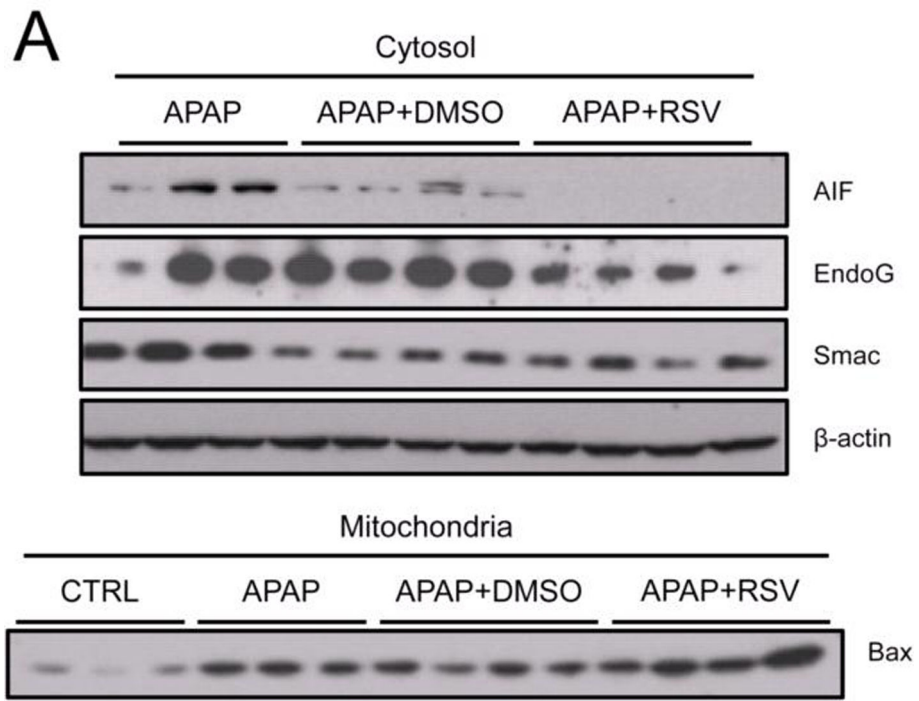


Figure 5. Resveratrol protects against acetaminophen (APAP) toxicity in vitro without preventing loss of mitochondrial membrane potential

Primary mouse hepatocytes were treated with 5 mM APAP and co-treated with either dimethyl sulfoxide (DMSO) vehicle or 100 μ M resveratrol (RSV). (A) Lactate dehydrogenase (LDH) release into the cell culture medium. (B) Mitochondrial membrane potential, based on JC-1 red/green fluorescence ratio. Bar graphs show mean \pm SEM for n = 3 hepatocyte preparations. *P < 0.05 vs. CTRL.



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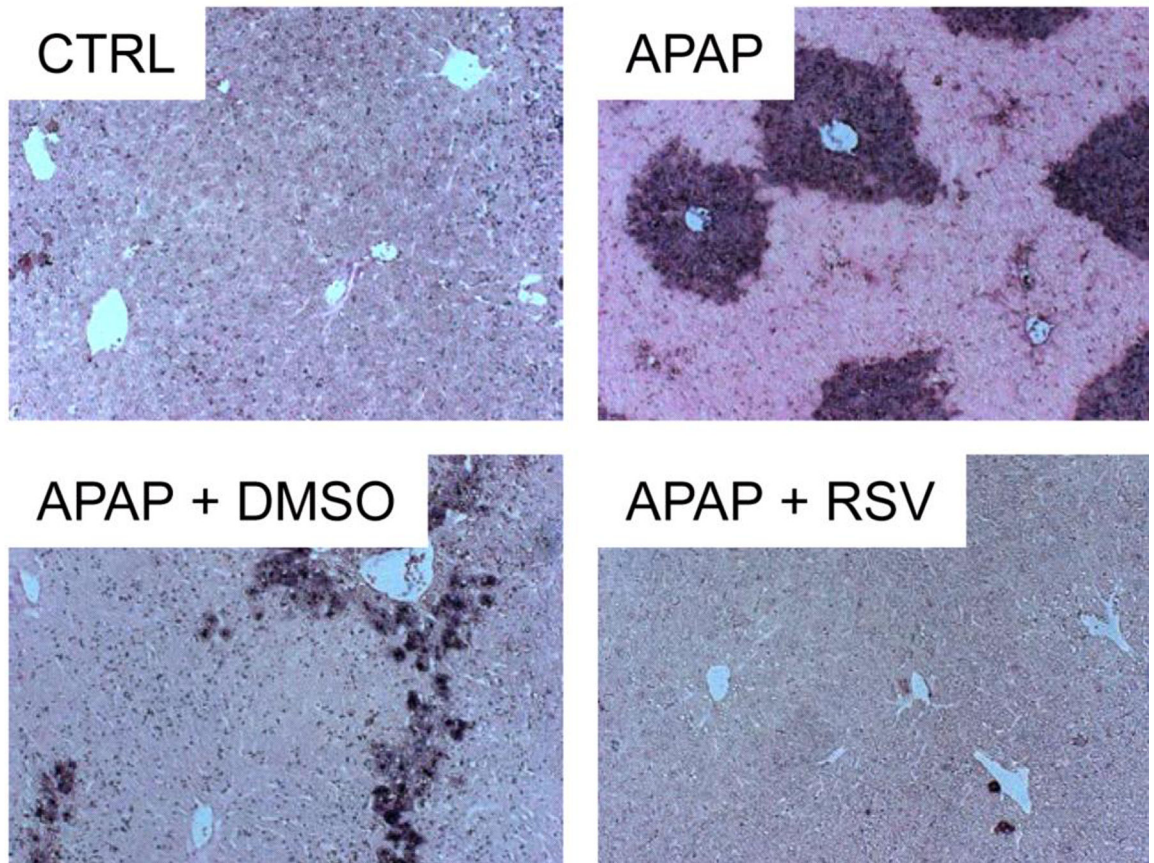


Figure 6. Resveratrol prevents release of endonucleases factor from mitochondria and nuclear DNA fragmentation during acetaminophen (APAP) hepatotoxicity

Mice were treated with 300 mg/kg APAP followed by dimethyl sulfoxide (DMSO) vehicle or 50 mg/kg resveratrol (RSV) 1.5h later. Liver tissue was harvested at 6 h post-APAP and mitochondrial and cytosol fractions were isolated. (A) Western blots for apoptosis-induced factor (AIF), endonuclease G (EndoG), and Bax in subcellular fractions. (B) Densitometry. Bar graphs show mean \pm SEM for $n = 3-4$ mice. * $P < 0.05$ vs. CTRL. # $P < 0.05$ vs. APAP + DMSO treatment. (C) Nuclear DNA fragmentation in liver tissue sections as visualized by the TUNEL assay.

Table 1

Induction of antioxidant genes

Gene	CTRL	APAP	APAP + Veh.	APAP + RSV
Gclc	1.0±0.16	2.0±0.36*	4.1±0.40*	2.7±0.13*
Catalase	1.0±0.14	0.63±0.03	0.58±0.05	0.56±0.03*
Ho-1	1.0±0.16	63.6±4.8*	45.2±3.6*	40.7±3.1*
Sod1	1.0±0.14	1.0±0.11	1.1±0.10	1.3±0.10
Sod2	1.0±0.060	0.72±0.09*	0.73±0.05*	0.75±0.04*
Mt-1	1.0±0.50	14.3±1.4*	15.1±0.71*	19.0±2.5*
Mt-2	1.0±0.69	20.1±3.2*	15.1±0.42*	18.1±0.54*

Data are expressed as mean ± SE for n = 3–6.

* p < 0.05 vs. CTRL.