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# Selenoprotein MsrB1 deficiency exacerbates acetaminophen-induced hepatotoxicity *via* increased oxidative damage

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## Abstract

Acetaminophen (APAP) overdose induces acute liver damage and failure *via* reactive oxygen species production and glutathione (GSH) depletion. Methionine sulfoxide reductase B1 (MsrB1) is an antioxidant selenoenzyme that specifically catalyzes the reduction of methionine *R*-sulfoxide residues. In this study, we used *MsrB1* gene-knockout mice and primary hepatocytes to investigate the effect of MsrB1 on APAP-induced hepatotoxicity. Analyses of histological alterations and serum indicators of liver damage showed that $MsrB1^{-/-}$ mice were more susceptible to APAP-induced acute liver injury than wild-type ($MsrB1^{+/+}$) mice. Consistent with the *in vivo* results, primary $MsrB1^{-/-}$ hepatocytes displayed higher susceptibility to APAP-induced cytotoxicity than $MsrB1^{+/+}$ cells. MsrB1 deficiency increased hepatic oxidative stress after APAP challenge such as hydrogen peroxide production, lipid peroxidation, and protein oxidation levels. Additionally, basal and APAP-induced ratios of reduced-to-oxidized GSH (GSH/GSSG) were significantly lower in $MsrB1^{-/-}$ than in $MsrB1^{+/+}$ livers. Nrf2 nuclear accumulation and heme oxygenase-1 expression levels after APAP challenge were lower in $MsrB1^{-/-}$ than in $MsrB1^{+/+}$ livers, suggesting that MsrB1 deficiency attenuates the APAP-induced activation of Nrf2. Collectively, the results of this study suggest that selenoprotein MsrB1 plays a protective role against APAP-induced hepatotoxicity *via* its antioxidative function.

### Keywords

Methionine sulfoxide; Selenoenzyme MsrB1; Acetaminophen; Hepatic damage; Oxidative stress

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**Conflict of interest**

The authors have declared that no conflicts of interest exist.

## Introduction

Oxidation of methionine occurs mostly in non-enzymatic reactions mediated by reactive oxygen species (ROS), whereas its reduction requires an enzymatic reaction led by methionine sulfoxide reductase (Msr) [1,2]. The cyclic oxidation and reduction of methionine is a defense mechanism that protects cells from oxidative stress [3]. Therefore, Msr is considered as an important antioxidant enzyme, capable of eliminating cellular ROS [4,5]. There are two Msr families, displaying different substrate stereospecificities. The conjunct action of these two types of Msr allows for the complete reduction of any mixture of methionine-(*R,S*)-sulfoxide residues in proteins [6,7]. MsrA is specific to the *S*-form of methionine sulfoxide, whereas MsrB only processes the *R*-form.

Acetaminophen (*N*-acetyl-*p*-aminophenol, APAP) is used worldwide as an analgesic and antipyretic drug. However, APAP overdose can cause severe acute liver damage, including total failure and death [8], and is the most common cause of drug-induced liver injury in many countries, including the United States and the United Kingdom [9]. The reactive metabolite *N*-acetyl-*p*-benzoquinone imine, which is generated through the biotransformation of APAP in the liver, depletes glutathione (GSH), covalently binds to thiol groups of proteins, and induces ROS production, thereby, ultimately, generating hepatotoxicity [10]. An effective therapy for APAP overdose patients is treatment with GSH (or *N*-acetylcysteine) to enhance hepatic GSH levels and thereby, reduce ROS levels [10,11].

Mammalian cells possess three MsrB proteins (MsrB1–MsrB3), located within different cellular compartments [12]. Among these three MsrB enzymes, MsrB1 is a cytosolic and nuclear selenoprotein that contains selenocysteine within its active site [12,13,14]. MsrB1 is highly expressed in liver, representing the main hepatic MsrB enzyme [15,16]. MsrB1 knockout mice exhibit increased protein oxidation, lipid peroxidation, and oxidized GSH levels in liver and kidney [16], suggesting that MsrB1 plays an antioxidative role within these detoxifying organs. Additionally, MsrB1 knockdown in mammalian cells increases ROS production and oxidative stress-induced cell death [17], whereas its overexpression enhances cell survival under oxidative stress conditions [18].

We have previously shown that MsrA knockout mice are more susceptible to APAPinduced acute liver damage than wild-type mice, suggesting that MsrA plays a protective role against APAP-induced hepatotoxicity [19]. Since MsrB is the counterpart of MsrA, it would be of interest to establish whether MsrB produces a similar effect against APAP-induced hepatic damage. In the present study, we used MsrB1-deficient mice and primary hepatocytes to investigate the role of MsrB1, the major hepatic MsrB enzyme, in APAP-induced hepatotoxicity.

## Material and Methods

### Animal preparation

The generation of MsrB1 knockout (*MsrB1*$^{-/-}$) mice is described elsewhere [16]. The breeding lines for *MsrB1*$^{-/-}$ mice were obtained by backcrossing into the C57BL/6N genetic

background for >8 generations, as previously described [20]. *MsrB1*$^{-/-}$ and wild-type *MsrB1*$^{+/+}$ mice at 8–10 weeks of age were used for all the experiments, which were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of Yeungnam University.

### APAP challenge in mice

Age-matched male *MsrB1*$^{-/-}$ and *MsrB1*$^{+/+}$ mice (~10 weeks old) were used to carry out the APAP challenge. After overnight starvation, mice were intraperitoneally administered APAP (300 mg/kg body weight; Sigma–Aldrich) dissolved in warm saline solution or sterile saline control (10 μL/g body weight). Food was immediately returned to the mice following administration. Mice were sacrificed 6 h after APAP treatment to obtain liver tissues and blood from the heart.

### Histology

A slice of liver was fixed in a freshly prepared 4 % paraformaldehyde solution, embedded in paraffin, and then cut into 5 μm thick sections. After deparaffinization and dehydration, liver sections were stained with hematoxylin and eosin (H&E) for microscopic examinations.

### Isolation of primary hepatocytes

Primary hepatocytes were isolated from the livers of male *MsrB1*$^{+/+}$ and *MsrB1*$^{-/-}$ mice (8–10 weeks old) using a two-step collagenase perfusion method, as previously described [21,22]. Isolated cells were seeded onto culture plates in DMEM supplemented with 10 % fetal bovine serum, 100 U penicillin/streptomycin, and 3.2 mg/L insulin. Cells were allowed to attach for 5 h at 37 °C in a 5% $CO_2$ incubator, after which the medium was changed to remove unattached and dead cells.

### Cell viability assay

Primary hepatocytes were seeded into 24-well plates at a density of $5\times10^4$ cells/well, and subsequently treated with or without APAP for 24 h. Cell viability was evaluated using an established colorimetric 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT)-based assay.

### Measurements of ALT, AST, and LDH levels

Alanine transaminase (ALT), aspartate transaminase (AST), and lactate dehydrogenase (LDH) levels were measured in serum or culture medium using assay kits (IVD Lab), according to the manufacturer’s instructions, using a Shimadzu UV visible spectrophotometer.

### Western blot analysis

Antibodies against MsrB1, Nrf2, heme oxygenase-1 (HO-1), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as previously described [23,24]. Antibodies against histone 1 and HSP90 were purchased from Santa Cruz Biotechnology and Cell Signaling Technology, respectively. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) using a NuPAGE 4–12% Bis-Tris gel

(Invitrogen) and transferred onto a polyvinylidene difluoride membrane. Membranes were subsequently probed with primary antibodies, followed by the addition of horseradish peroxidase-conjugated secondary antibodies. GAPDH expression was used as the protein loading control. Quantitative analysis of blot signals was performed using the LAS-4000 imaging system (GE Healthcare Life Sciences) and ImageJ software (open source).

### Measurements of hydrogen peroxide, 4-hydroxynonenal, and protein-carbonyl levels

$H_2O_2$ levels in liver samples were determined using the ferric-sensitive dye, xylenol orange (Sigma–Aldrich), as previously described [25]. 4-Hydroxynonenal (HNE) levels were determined by measuring HNE-protein adducts using anti-HNE antibodies, as previously described [19]. Protein-carbonyl levels were measured using an OxyBlot protein oxidation detection kit (Millipore), according to the manufacturer's recommendations. The blot signals were quantitatively analyzed using the ImageJ software.

### Measurement of GSH contents

Free and total GSH contents were measured using a GSH fluorescent detection kit (Arbor Assays), according to the manufacturer's instructions. Briefly, liver homogenates were prepared in 5 % sulfosalicylic acid to remove proteins. The oxidized GSH (GSSG) contents were calculated by subtracting the measured free GSH from the measured total GSH. The GSH contents were expressed as μmol/g of total protein.

### Nuclear protein fractionation

Nuclear and cytoplasmic proteins from livers were fractionated using an NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Scientific), according to the manufacturer's instructions. Histone 1 and HSP90 were used as nuclear and cytoplasmic marker proteins, respectively.

### Reverse transcription-PCR (RT-PCR)

Total RNA was extracted from livers with the TRI-Solution (Bioscience), and then reverse-transcribed to cDNA using a Reverse Transcription Master Premix kit (ELPIS Biotech). The primers used for RT-PCR were: 5′-GCCAGGTGTCTACGTGTGTG-3′ and 5′-TTCTTTGCCTTTAGGGACGA-3′ for MsrB1; 5′-CTATTCAGCAGAGCGGTTCC-3′ and 5′-ATTTTCGTTGGGACGAACTG-3′ for thioredoxin reductase 1; 5′-GGAGCCAAAAGGGTCATCAT-3′ and 5′-GTGATGGCATGGACTGTGGT-3′ for GAPDH. GAPDH mRNA expression was used as the internal control.

### Statistical analysis

Statistical analysis was conducted with the Prism 5 software (GraphPad) using two-way ANOVA followed by Bonferroni post-tests. In the case of the data with added samples, $p$ values were adjusted using the alpha spending function. Differences were considered significant at a $p$ value $< 0.05$.

## Results

### MsrB1 knockout mice exhibit higher susceptibility to APAP-induced liver injury

*MsrB1*$^{-/-}$ and *MsrB1*$^{+/+}$ mice were challenged with APAP (300 mg/kg body weight) for 6 h, after which liver injury was analyzed, based on levels of liver damage indicators and histological alterations. The serum ALT and AST levels were significantly higher in APAP-treated *MsrB1*$^{-/-}$ than in APAP-treated *MsrB1*$^{+/+}$ mice (Fig. 1A and B). Additionally, *MsrB1*$^{-/-}$ mice challenged with APAP displayed significantly higher serum LDH levels than did *MsrB1*$^{+/+}$ mice challenged with APAP (Fig. 1C). Notably, there were statistically significant interactions between drug and genotype factors from all the serum data. The histological analysis also evidenced an increased susceptibility to APAP-induced liver damage in *MsrB1*$^{-/-}$ mice. While livers from control *MsrB1*$^{-/-}$ mice were histologically normal, similar to those obtained from control *MsrB1*$^{+/+}$ mice, APAP challenge induced stronger liver damage in *MsrB1*$^{-/-}$ than in *MsrB1*$^{+/+}$ mice (Fig. 1D). Indeed, the central lobule area of the *MsrB1*$^{-/-}$ liver displayed more necrotic lesions. Taken together, these results suggest that MsrB1 plays a protective role against APAP-induced liver injury.

### MsrB1-deficient hepatocytes are more susceptible to APAP-induced toxicity

We further investigated the protective role of MsrB1 against APAP-induced hepatotoxicity using primary hepatocytes isolated from livers of *MsrB1*$^{+/+}$ and *MsrB1*$^{-/-}$ mice. Primary *MsrB1*$^{+/+}$ and *MsrB1*$^{-/-}$ hepatocytes were treated for 24 h with various concentrations of APAP (0, 1, 5, and 10 mM). While APAP treatment reduced viability in a dose-dependent manner for both types of cells, MsrB1 deficiency significantly increased APAP-induced cell mortality at 5 and 10 mM concentrations (Supplementary Fig. S1). We chose the 10 mM concentration of APAP to analyze the levels of ALT and AST in the culture media after 24 h. The increased APAP-induced cell mortality was evident in *MsrB1*$^{-/-}$ cells (Fig. 2A). The basal activities of ALT and AST were similar in both media (Fig. 2B and C). The levels of ALT and AST increased in response to APAP in both *MsrB1*$^{-/-}$ and *MsrB1*$^{+/+}$ hepatocytes; however, the culture medium from APAP-treated *MsrB1*$^{-/-}$ cells displayed significantly higher levels of both these hepatocellular damage indicators (Fig. 2B and C). Taken together, these results demonstrate a higher susceptibility to APAP-induced cytotoxicity in MsrB1-deficient hepatocytes, which is consistent with the *in vivo* results.

### MsrB1 depletion increases hepatic oxidative stress

We examined the oxidative stress status of livers upon APAP challenge by measuring the hydrogen peroxide, HNE, and protein-carbonyl levels (Fig. 3A–C). Basal $H_2O_2$ levels were similar in *MsrB1*$^{-/-}$ and *MsrB1*$^{+/+}$ livers. The APAP challenge significantly increased the $H_2O_2$ levels in *MsrB1*$^{-/-}$ livers, while a marginal increase in $H_2O_2$ level was observed in *MsrB1*$^{+/+}$ livers (Fig. 3A). Basal HNE levels were similar in *MsrB1*$^{-/-}$ and *MsrB1*$^{+/+}$ livers. Although the HNE levels significantly increased in both livers after APAP challenge, this increase was significantly higher in *MsrB1*$^{-/-}$ mice (Fig. 3B), suggesting that MsrB1 plays an antioxidative function against lipid peroxidation. Basal protein-carbonyl levels were similar in *MsrB1*$^{-/-}$ and *MsrB1*$^{+/+}$ livers. Although APAP treatment slightly increased the protein-carbonyl levels in both livers, significantly higher levels were observed in *MsrB1*$^{-/-}$

livers (Fig. 3C). Taken together, these results suggest that MsrB1 deficiency increases hepatic oxidative stress after APAP exposure.

### MsrB1 deficiency decreases the ratio of free to oxidized GSH

Since GSH depletion is a well-known mechanism in APAP-induced hepatotoxicity [11], we measured the total hepatic GSH contents in *MsrB1*$^{+/+}$ and *MsrB1*$^{-/-}$ mice (Fig. 3D). Basal GSH levels were similar among both livers. Although APAP challenge significantly depleted GSH in both livers, the reduction in GSH levels was not significantly different in *MsrB1*$^{-/-}$ livers. We also measured free (reduced) GSH contents and the ratios of free to oxidized GSH (GSH/GSSG). Basal free GSH levels was not significantly different between *MsrB1*$^{-/-}$ and *MsrB1*$^{+/+}$ livers (Fig. 3E). The free GSH levels significantly decreased in both livers after APAP challenge; however, a significantly higher decrease was observed in the *MsrB1*$^{-/-}$ livers (Fig. 3E). The basal ratio of GSH/GSSG was significantly lower (2-fold) in *MsrB1*$^{-/-}$ than in *MsrB1*$^{+/+}$ livers (Fig. 3F). APAP exposure significantly decreased the GSH/GSSG ratios in both livers; however, this decrease was significantly larger in *MsrB1*$^{-/-}$ livers (Fig. 3F). These results suggest that MsrB1 deficiency induces a decrease in GSH/GSSG ratios after APAP exposure, as well as at a basal state, through increasing oxidative stress.

### MsrB1 deficiency attenuates APAP-induced Nrf2 activation

Since APAP challenge activates hepatic Nrf2 [26,27], which is a key regulator of the response to oxidative stress, we investigated whether MsrB1 affects the activation of the Nrf2 pathway. The basal Nrf2 protein levels were lower in *MsrB1*$^{-/-}$ than in *MsrB1*$^{+/+}$ livers (Fig. 4A and B). Nrf2 levels significantly increased in response to APAP in both livers. However, the APAP-induced Nrf2 expression levels were not significantly different between *MsrB1*$^{+/+}$ and *MsrB1*$^{-/-}$ livers. We also analyzed the expression of an Nrf2 target gene, *HO-1*. The basal levels of HO-1 were similar in *MsrB1*$^{-/-}$ and *MsrB1*$^{+/+}$ livers (Fig. 4A and C). Although the APAP challenge induced the expression of HO-1 in both livers, the expression levels were significantly lower in MsrB1-deficient livers.

Nrf2 is translocated from cytoplasm to nucleus when activated. We thus analyzed the nuclear accumulation of Nrf2 in response to APAP through the fractionation of cytoplasmic and nuclear proteins from liver tissues. Cytoplasmic Nrf2 proteins were hardly detectable in all samples obtained from control and APAP-treated *MsrB1*$^{+/+}$ or *MsrB1*$^{-/-}$ mice, while nuclear Nrf2 proteins were readily detectable (Supplementary Fig. S2). The basal nuclear Nrf2 protein levels were similar in both *MsrB1*$^{+/+}$ and *MsrB1*$^{-/-}$ mice (Fig. 5A and B). APAP challenge significantly increased the nuclear accumulation of Nrf2 in both *MsrB1*$^{+/+}$ and *MsrB1*$^{-/-}$ livers. However, *MsrB1*$^{-/-}$ mice showed significantly lower nuclear Nrf2 levels than *MsrB1*$^{+/+}$ mice after APAP challenge (Fig. 5A and B). Taken together, these results suggest that MsrB1 deficiency attenuates APAP-induced Nrf2 activation.

### MsrB1 deficiency does not affect APAP-induced expression of thioredoxin reductase 1

Thioredoxin reductase 1 (TXNRD1), a key redox regulator included in the cytosolic thioredoxin system, is involved in the mechanism modulating the susceptibility to APAP-induced hepatotoxicity. Depletion of TXNRD1 induces resistance to APAP-induced toxicity in livers and hepatocytes [22,28,29]. We recently reported that MsrA can modulate hepatic

TXNRD1 expression upon APAP challenge [22]. MsrA deficiency increased APAP-induced TXNRD1 expression levels in hepatocytes, whereas overexpression of MsrA reduced the elevated TXNRD1 levels in APAP-treated MsrA-deficient cells [22]. Therefore, we tested whether MsrB1 deficiency would affect the expression of hepatic TXNRD1 under APAP conditions. RT-PCR analysis showed that APAP challenge increased the TXNRD1 expression levels in both *MsrB1*$^{+/+}$ and *MsrB1*$^{-/-}$ livers, and that no significant difference could be observed between the two liver samples upon APAP challenge (Fig. 6). These results suggest that MsrB1 deficiency does not affect the expression of hepatic TXNRD1 upon APAP challenge.

## Discussion

In this study, we demonstrated the protective role of MsrB1, which is the major hepatic MsrB enzyme, against APAP-induced hepatotoxicity, using MsrB1 knockout mice and primary hepatocytes. MsrB1 deficiency promoted APAP-induced liver injury and cytotoxicity in mice and hepatocytes, respectively. Increased oxidative stress with GSH depletion is thought to be a major mechanism through which APAP induces hepatotoxicity [11]. MsrB1 deficiency led to an increase in APAP-induced oxidative stress processes, such as lipid peroxidation and protein oxidation. In addition, basal GSH/GSSG levels in liver were significantly lower in MsrB1-deficient mice than in wild-type mice, which was also the case for APAP-induced GSH/GSSG levels. Therefore, the results obtained from this study suggest that the antioxidative function of MsrB1 plays a key role in protecting hepatic cells from APAP-induced toxicity. A decrease in basal GSH/GSSG ratios in livers harvested from MsrB1-deficient mice has also been reported in a previous study [16].

We previously demonstrated that a deficiency in MsrA, which is specific to the methionine *S*-sulfoxide reduction process, aggravates APAP-induced acute liver injury in mice [19]. MsrA deficiency significantly accelerates APAP-induced hepatic oxidative stress processes, such as GSH depletion and lipid peroxidation [19]. The present study demonstrates that a deficiency in MsrB1, responsible for methionine *R*-sulfoxide reduction, induces a higher susceptibility to APAP-induced hepatic damage. Therefore, this result suggests that both methionine *S*- and *R*-sulfoxide reduction systems, harboring antioxidative activities, are key toward conferring hepatic protection against APAP-induced toxicity.

Nrf2 is a key transcription factor that modulates the responses to oxidative and electrophilic challenges. Generally, the activation of Nrf2 has a beneficial effect on hepatic protection processes against oxidative challenge. For example, Nrf2-deficient mice exhibit an enhanced susceptibility to liver damage induced by ischemia/reperfusion challenge [30]. Liver-specific TXNRD1-deficient mice show enhanced hepatic Nrf2 activity, providing them with resistance to APAP-induced liver injury [28]. In the present study, APAP-induced nuclear accumulation levels of Nrf2 were shown to be attenuated in *MsrB1*$^{-/-}$ livers. This impact on Nrf2 activation might be responsible for the increased APAP-induced hepatotoxicity in MsrB1-deficient mice.

There have been several reports describing the effects of antioxidant enzymes, including selenoproteins, on APAP-induced hepatotoxicity *in vivo*. For example, mice overexpressing

selenoprotein GSH peroxidase 1 show increased sensitivity to APAP toxicity [31], whereas there is little impact of knockout of this gene [32]. Knockout of cytosolic superoxide dismutase 1 (SOD1) provides mice with resistance to APAP toxicity [32], as does selenoprotein TXNRD1 knockout [28,29]. In contrast, a deficiency in mitochondrial SOD2 aggravates APAP toxicity [33], as does MsrB1 deficiency. Therefore, it is appropriate to conclude that the antioxidant enzymes have different effects on APAP toxicity, which can be protective or deleterious, or neither.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

| | |
|---|---|
| **ALT** | alanine transaminase |
| **APAP** | acetaminophen |
| **AST** | aspartate transaminase |
| **GAPDH** | glyceraldehyde 3-phosphate dehydrogenase |
| **GSH** | glutathione |
| **H&E** | hematoxylin and eosin |
| **HO** | heme oxygenase |
| **HNE** | 4-hydroxynonenal |
| **LDH** | lactate dehydrogenase |
| **Msr** | methionine sulfoxide reductase |
| **ROS** | reactive oxygen species |
| **RT-PCR** | reverse transcription-PCR |
| **TXNRD1** | thioredoxin reductase 1 |

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## Highlights

- MsrB1 deficiency increases APAP-induced hepatotoxicity.
- MsrB1 depletion enhances hepatic oxidative stress including lipid peroxidation levels.
- MsrB1 deficiency accelerates glutathione oxidation in response to APAP.
- MsrB1 deficiency attenuates APAP-induced Nrf2 activation.

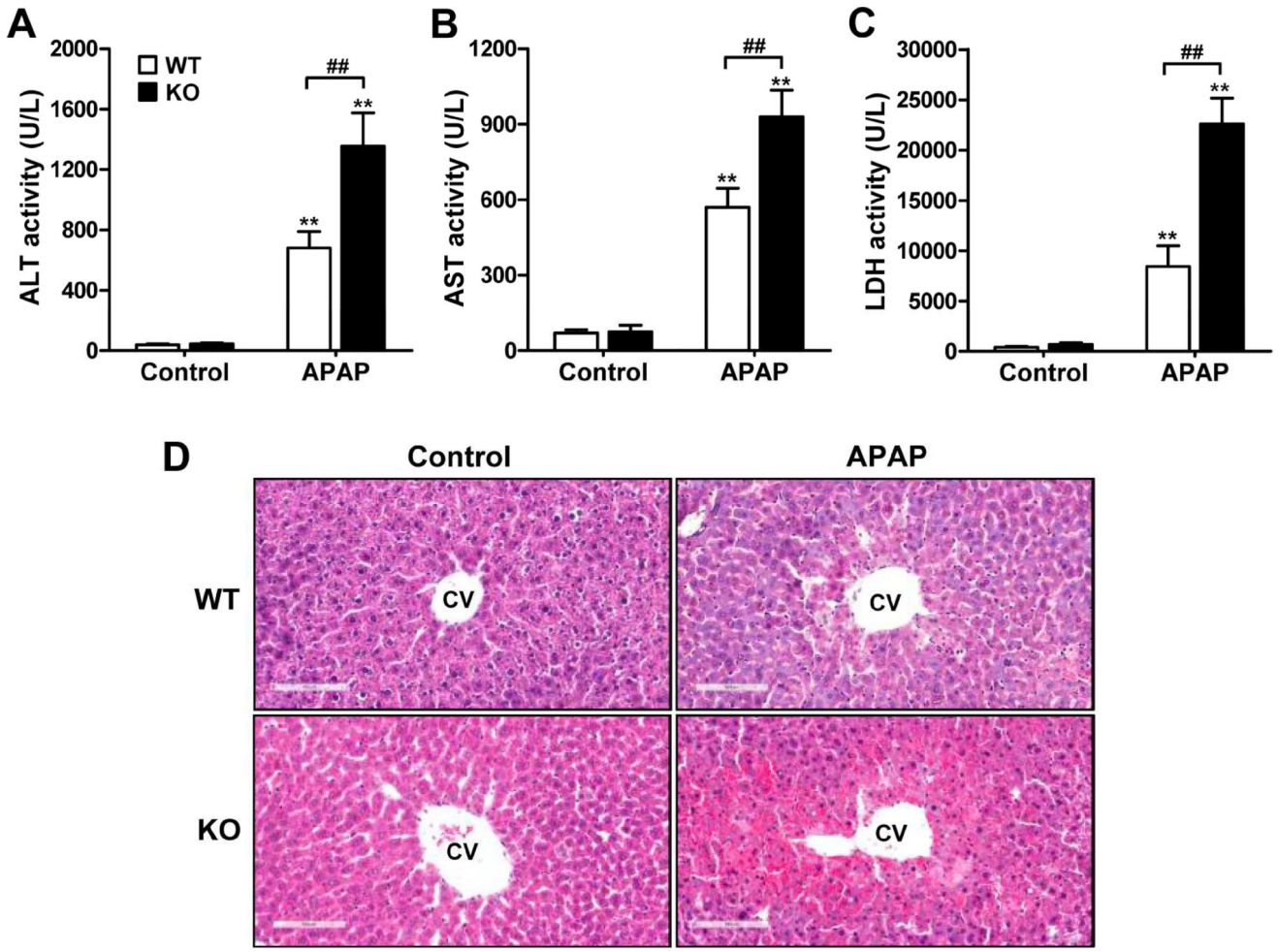


**Fig. 1. Increased susceptibility to APAP-induced acute liver injury in MsrB1-deficient mice**

Male *MsrB1*$^{+/+}$ (WT) and *MsrB1*$^{-/-}$ (KO) mice were intraperitoneally challenged with APAP (300 mg/kg) or saline control for 6 h. (A–C) Serum ALT (A), AST (B), and LDH (C) levels. Representative data from two independent experiments are shown as mean ± SE (n = 6 for each group). $^{**}p < 0.01$ *vs.* WT or KO controls; $^{\#\#}p < 0.01$, based on comparisons with WT mice. (D) Histological analysis. Liver sections were stained with H&E, and representative pictures are shown (magnitude 200×). Livers from control *MsrB1*$^{-/-}$ mice were histologically normal. APAP challenge caused centrilobular hepatotoxicity and central lobular regions contained more necrotic lesions in *MsrB1*$^{-/-}$ mice. CV, central vein.

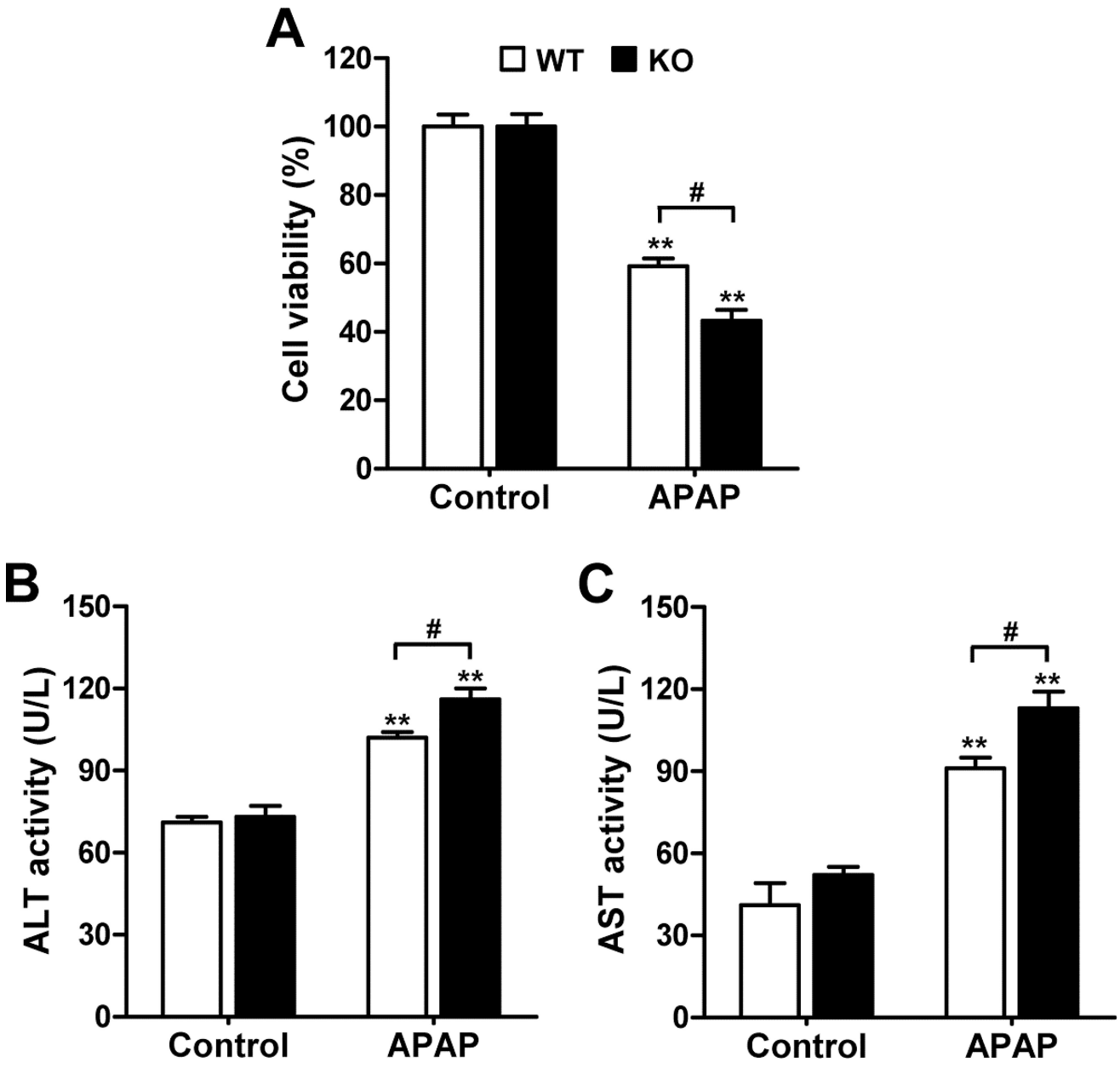


**Fig. 2. Increased susceptibility of MsrB1-deficient hepatocytes to APAP-induced toxicity**

Primary hepatocytes isolated from livers of male *MsrB1*$^{+/+}$ (WT) and *MsrB1*$^{-/-}$ (KO) mice were treated with 10 mM APAP for 24 h. (A) Cell viability. (B, C) Activities of ALT (B) and AST (C) detected in the culture media. Data are representative of at least two independent experiments and expressed as mean ± SE (n = 3 for each group). $^{**}p < 0.01$ *vs.* WT or KO controls; $^{\#}p < 0.05$, based on comparisons with WT cells.

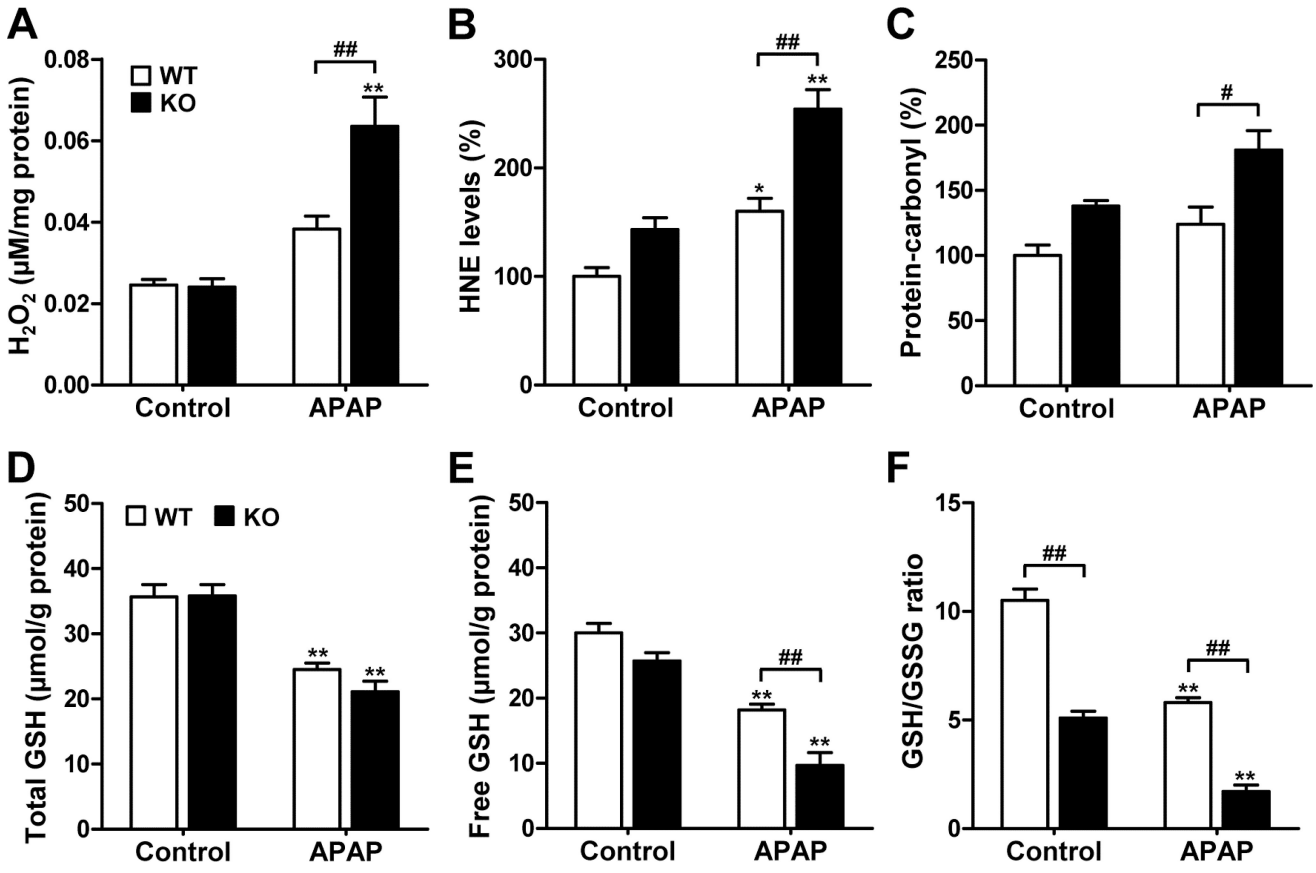


**Fig. 3. Increased oxidative stress and free GSH depletion in MsrB1-deficient liver upon APAP challenge**

Male *MsrB1*$^{+/+}$ (WT) and *MsrB1*$^{-/-}$ (KO) mice were intraperitoneally challenged with APAP (300 mg/kg) or saline control for 6 h. (A) $H_2O_2$ contents. (B) 4-Hydroxynonenal (HNE) levels. (C) Protein-carbonyl levels. HNE and protein-carbonyl were used as lipid peroxidation and protein oxidation markers, respectively. Data are representative of two independent experiments. (D) Total GSH contents. (E) Free GSH (reduced form) contents. (F) GSH (reduced) to GSSG (oxidized) ratios. All data are expressed as mean ± SE (n = 6 for each group in $H_2O_2$ data; in other data, n = 3 for control groups and n = 4 for APAP-treated groups). *$p < 0.05$ and **$p < 0.01$ *vs.* WT or KO controls; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$, based on comparisons with WT mice.

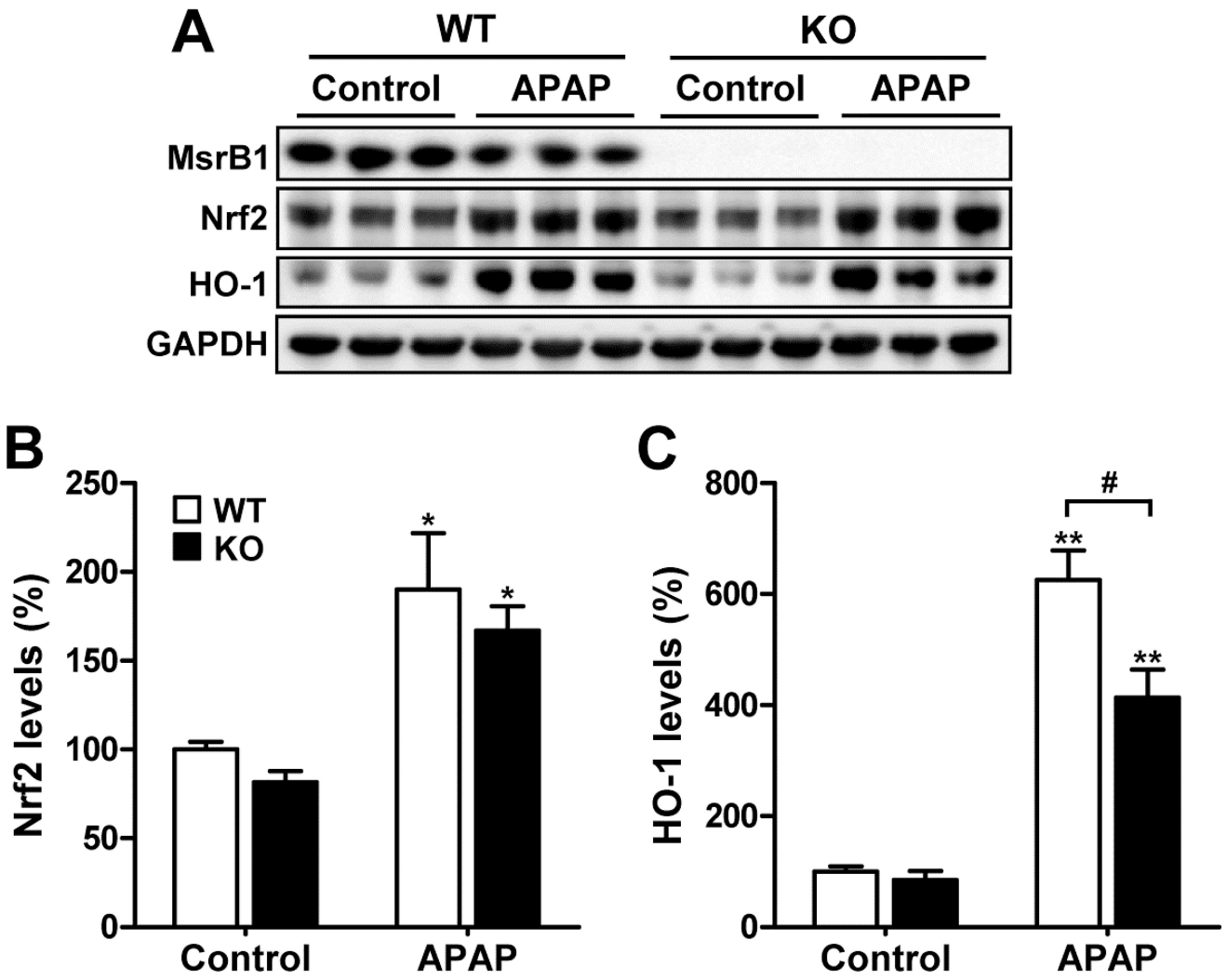


**Fig. 4. Expression levels of Nrf2 and its target protein HO-1**

Male *MsrB1*$^{+/+}$ (WT) and *MsrB1*$^{-/-}$ (KO) mice were intraperitoneally challenged with APAP (300 mg/kg) or saline control for 6 h. (A) Total lysates from livers were subjected to Western blot analysis of Nrf2 and HO-1. GAPDH was used as the loading control. (B, C) Quantitative analysis of Nrf2 (B) and HO-1 (C) levels normalized to GAPDH. Data are expressed as mean ± SE (n = 6 for each group). *$p < 0.05$ and **$p < 0.01$ *vs.* WT or KO controls; $^{\#}p < 0.05$, based on comparisons with WT mice.

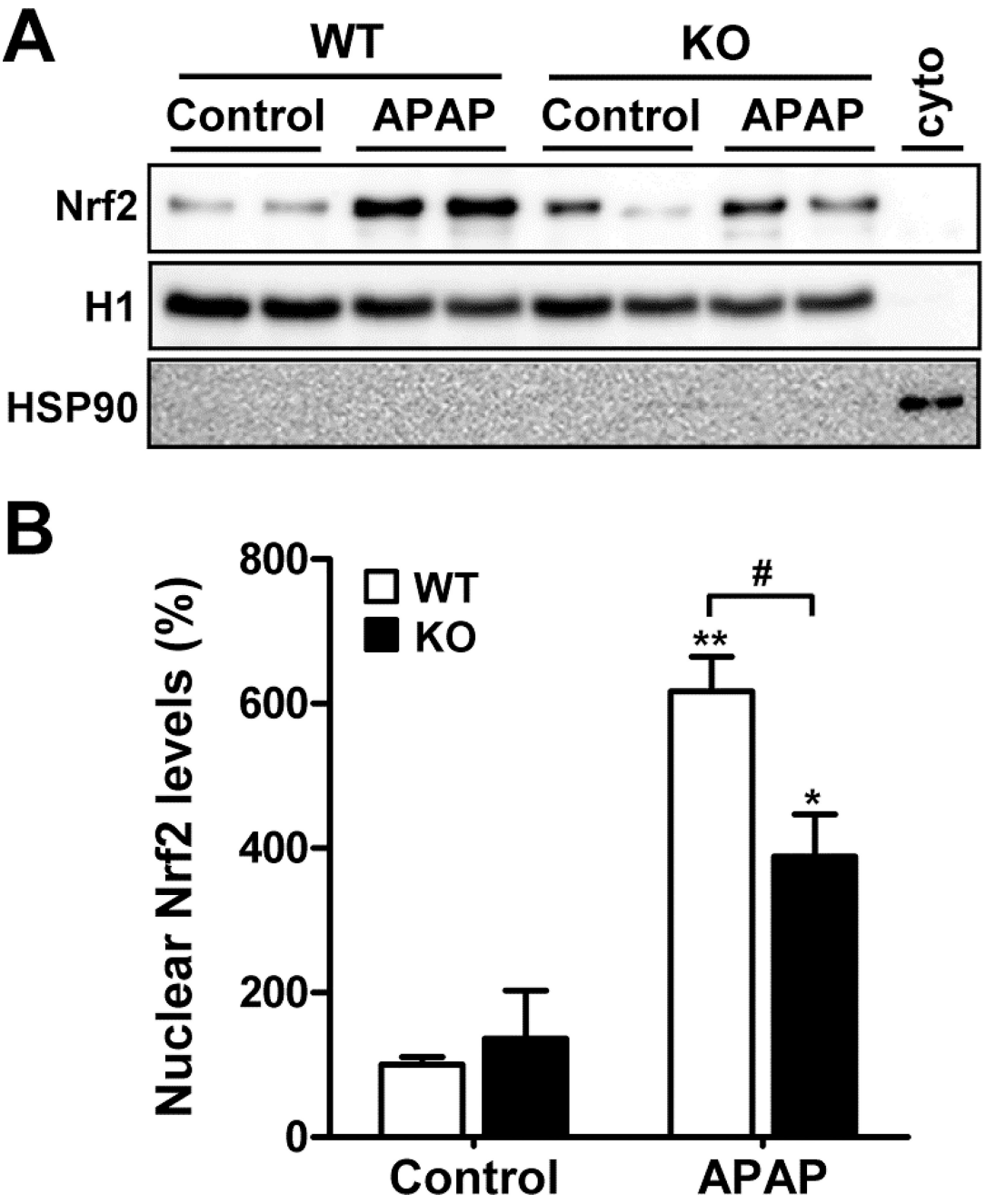


**Fig. 5. Analysis of nuclear accumulation of hepatic Nrf2 upon APAP challenge**

Male *MsrB1*$^{+/+}$ (WT) and *MsrB1*$^{-/-}$ (KO) mice were intraperitoneally challenged with APAP (300 mg/kg) or saline control for 6 h. (A) Cytoplasmic and nuclear proteins were fractionated from livers and the nuclear Nrf2 protein levels were analyzed through Western blot analysis. Ten micrograms of proteins was loaded onto SDS–PAGE. Histone 1 (H1) and HSP90 were used as nuclear and cytoplasmic marker proteins, respectively. Cyto, a cytoplasmic sample of APAP-treated WT liver. (B) Quantitative analysis of nuclear Nrf2 normalized to GAPDH. All data are representative of two independent experiments and

shown as mean ± SE (n = 3 for each group). *$p < 0.05$ and **$p < 0.01$ *vs.* WT or KO controls; $^{\#}p < 0.05$, based on comparisons with WT mice.

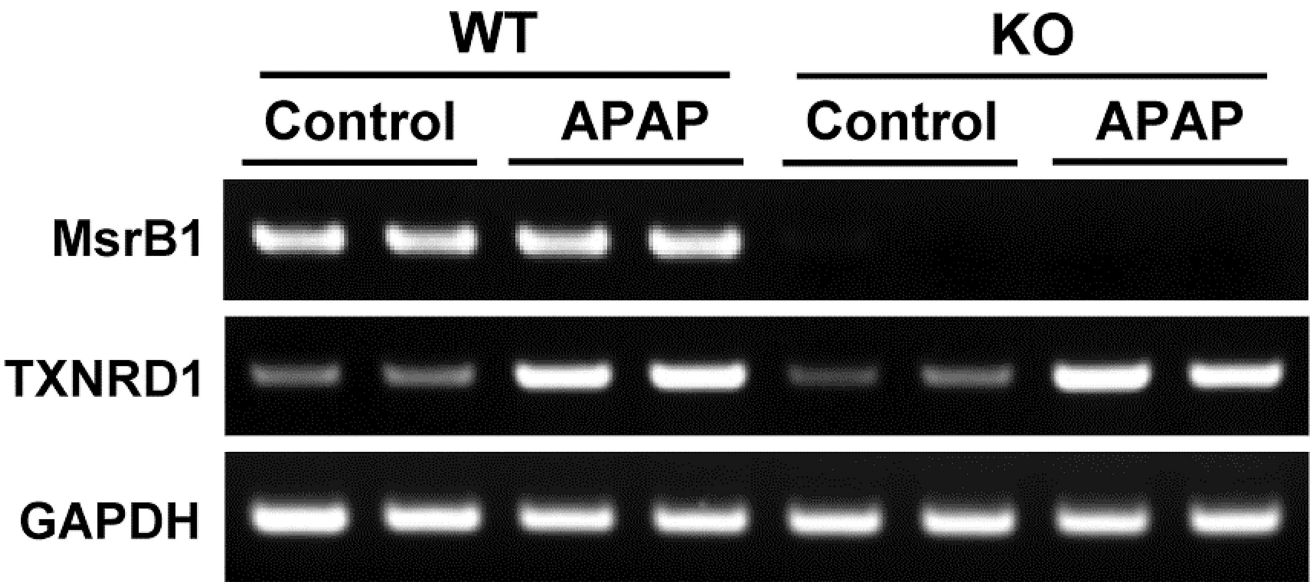


**Fig. 6. Effect of MsrB1 deficiency on APAP-induced thioredoxin reductase 1 (TXNRD1) expression levels**

Liver samples from male *MsrB1*$^{+/+}$ (WT) and *MsrB1*$^{-/-}$ (KO) mice intraperitoneally challenged with APAP (300 mg/kg for 6 h) or saline control were subjected to RT-PCR to assess TXNRD1 expression. GAPDH mRNA expression was used as the internal control.