Hepatoprotective Effect of Neoagarooligosaccharide via Activation of Nrf2 and Enhanced Antioxidant Efficacy

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ABSTRACT

Neoagarooligosaccharides (NAOS) are generated by β-agarases, which cleave the β-1,4 linkage in agarose. Previously, we reported that NAOS inhibited fat accumulation in the liver and decreased serum cholesterol levels. However, the hepatoprotective effect of NAOS on acute liver injury has not yet been investigated. Thus, we examined whether NAOS could activate NF-E2-related factor 2 (Nrf2)-antioxidant response element (ARE) and upregulates its target gene, and has hepatoprotective effect in vivo. In hepatocytes, phosphorylation and subsequent nuclear translocation of Nrf2 are increased by treatment with NAOS, in a manner dependent on p38 and JNK. Consistently, NAOS augmented ARE reporter gene activity and the antioxidant protein levels, resulting in increased intracellular glutathione levels. NAOS antagonized tert-butylhydroperoxide-induced reactive oxygen species (ROS) generation. Moreover, NAOS inhibited acetaminophen (APAP)-induced serum ALT and AST and significantly decreased hepatocyte degeneration and inflammatory cell infiltration. Moreover, ROS production and glutathione depletion by APAP were reversed by NAOS. APAP-mediated apoptotic signaling pathways were also inhibited in NAOS-treated mice. Upregulated hepatic expression of genes related to inflammation by APAP were consistently diminished by NAOS. Collectively, our results demonstrate that NAOS exhibited a hepatoprotective effect against APAP-mediated acute liver damage through its antioxidant capacity.

Keywords: Neoagarooligosaccharide; Acetaminophen; Oxidative stress; Nrf2; Liver
INTRODUCTION

Idiosyncratic drug-induced liver injury (DILI) is recognized as a cause of morbidity and mortality worldwide although it is rare\(^1\). The increased incidence of DILI over the past decade is the major common reason of drug withdrawals from markets. Among drugs that induce DILI, overdose of acetaminophen (APAP), a \(p\)-aminophenol derivative with analgesic and antipyretic activities, prevalently induces acute liver failure and DILI\(^2, 3\). APAP undergoes metabolism such as glucuronidation and sulfation in the liver, catalyzed by UDP-glucuronosyltransferase (UGT) and sulfotransferase (SULT), respectively. When the UGT and SULT pathways are saturated, cytochrome P450s (CYP450s) metabolized APAP to form an immensely electrophilic reactive metabolite \(N\)-acetyl-\(p\)-benzoquinone-imine (NAPQI). NAPQI is an efficiently detoxified by conjugation with glutathione, and this reaction is mediated by glutathione S-transferase (GST). However, excessive NAPQI covalently binds onto cellular macromolecules leading to oxidative stress and cytotoxicity in the liver\(^4\). NF-E2-related factor 2 (Nrf2) regulates the gene transcription associated with APAP detoxification, including UGT\(^5\), SULT\(^6\), and GST\(^7\).

Nrf2 is a crucial regulator of oxidative stress and inflammation\(^8\). Under basal states, Nrf2 is constitutively degraded through the ubiquitin proteasome pathway by binding to the inhibitory Nrf2, Keap1, which is a redox-regulated protein for the Cullin3-dependent E3 ubiquitin ligase complex. Under oxidative stress, Nrf2 is released from Keap1, which in turn leads to its translocation to the nucleus, where it stimulates the expression of a distinct set of target genes by binding to the antioxidant response element(s) (AREs) in target gene promoters. Nrf2 knockout mice was susceptible to APAP administration than wild-type littermates owing to deficient restoration in response to glutathione depletion\(^9\). Consistently, hepatocyte-specific Keap1-deleted mice, which exhibit Nrf2 accumulation, are more resistant to APAP-induced acute liver injury\(^10\). Thus, Nrf2 may serve a promising target molecule for...
the amelioration of APAP-induced acute liver damage 11).

Neoagarooligosaccharides (NAOS) are produced by β-agarase, which specifically cleaves the β-1,4 glycosidic bond of agarose 12). In contrast, α-agarase cleaves the α-1,3 linkage in agarose to produce agarooligosaccharides (AOS). Previously, we prepared enzymatically hydrolyzed NAOS from agarose and showed that it inhibited hepatic steatosis and hypercholesterolemia induced by high cholesterol diet 13). Consistent with our findings, another group confirmed the effects of NAOS on obesity and diabetes 14). Moreover, NAOS exhibits no toxicity up to 5,000 mg/kg body weight/day in acute, 14-, and 91-repeat oral toxicity tests 15). These strongly support the possible use of NAOS in dietary supplements and medications. Although NAOS has been reported to have antioxidant activities in in vitro systems15), the mechanisms underlying the antioxidant efficacy of NAOS remain to be elucidated. Nrf2-ARE signaling pathway is one of the most important defensive signaling pathways in APAP-induced acute liver injury.

In this study, we thus explored whether NAOS protects against APAP-induced acute liver damage through activation of the Nrf2-ARE signaling pathway. Our results demonstrate that NAOS mitigated APAP-induced acute liver injury. Furthermore, NAOS activated Nrf2-ARE through p38 MAPK and JNK. Collectively, our results suggest that NAOS inhibits acute liver injury in vivo and in vitro, suggesting that NAOS could be a novel compound that treats acute liver injury.
Materials and methods

Materials

MTT, acetaminophen, DCFH-DA, compound C (CompC, AMPK inhibitor), lipopolysaccharide (LPS) and dimethylsulfoxide were acquired from Sigma Chemicals (St. Louis, MO). PD98059 (ERK inhibitor), SB 203580 (P38 inhibitor), and SP600125 (JNK inhibitor) were obtained from Millipore (Bedford, MA). Rottlerin (rott, PKCδ inhibitor) was purchased from Calbiochem (San Diego, CA, USA).

Preparation of NAOS

NAOS was prepared as described previously \(^ {13} \). Briefly, \textit{S. lividans} TK24 was grown in RSM3 agar plate at 28°C for 4 days and then inoculated in RSM3 medium at 180 rpm for 60 h. After centrifugation, the supernatant was collected by filtration and DNS assay was used to measure enzyme activity. Ammonium sulfate was added to filtered supernatant while stirring. Once ammonium sulfate was fully dissolved, the supernatant was stirred, subject to centrifugation, and the resulting protein pellet was resuspended in distilled water and again subject to centrifugation. Then, the supernatant was collected by centrifugation and filtered. Agarase activity was measured under standard conditions, as previously described \(^ {13} \). Agar was washed twice with tap water and once with distilled water. Agar (7.5 g) was washed in distilled water, autoclaved, and 1.5% agar solution was cooled to 40°C and then reacted at 100 rpm overnight at 40°C with DagA. Reacted 1.5% agar solution was autoclaved and filtered. Partial purification of 1.5% agar solution was freeze-dried at -50°C and 5 mTorr. Lyophilized product was confirmed by thin-layer chromatography method.

Cell culture

HepG2 cells were provided by American Type Culture Collection (ATCC, Manassas, VA).
Cells were then cultured in DMEM medium supplemented with 10% FBS and 50 units/mL penicillin/streptomycin at 37°C in a humidified 5% CO2 atmosphere.

**Primary hepatocyte isolation**

Primary hepatocytes were isolated by the two-step hepatic portal vein perfusion method described previously 16). Briefly, ICR mice were anesthetized and the portal vein was cannulated under aseptic conditions with a 24 gauge catheter and inferior venae cava cut. The liver was perfused with Ca2+-free Hank’s balanced saline solution (HBSS) at 37°C for 5 min and an additional perfusion medium with HBSS containing 0.05% collagenase for 20 min and Ca2+ at a perfusion flow rate of 10 mL/min. After perfusion, the undigested tissue were minced gently and suspended in PBS. The cell suspension was then passed through a cell strainer and centrifuged at 50×g for 5 min at 4°C. Isolated hepatocytes were plated onto collagen-coated culture flasks and cultured in DMEM containing 50 units/mL penicillin/streptomycin with 10% FBS.

**Animals**

All experimental protocols for the animal studies were approved by the Animal Care and Use Committee of Chosun University. Male ICR mice (6-weeks-old) were purchased from Oriental Bio (Sungnam, Korea) and were given standard food and water at libitum. Mice received NAOS for 5 days by oral administration (po). Before being sacrificed, mice received an intraperitoneal injection (ip) of 300 mg/kg of APAP for 24 h (Fig. 4A). Liver tissue and serum were collected for later biochemical and molecular biological analysis.

**MTT assay**

Cells were seeded into 48-well plates and treated chemicals for 24 h, and cells were stained
with MTT (0.2 mg/ml, 4 h) as previously reported \(^1\). The media were then discarded, and formazan crystals produced were dissolved with the addition of 200 μL of dimethyl sulfoxide. Finally, absorbance was measured at 540 nm with a microplate reader (Spectra Max, Molecular Device, Sunnyvale, CA).

**Plasmid construction and luciferase assay.**

To measure antioxidant response element (ARE)-luciferase activity in a rapid and reproducible manner, we generated a HepG2 cells stably transfected with an NQO1-ARE luciferase gene construct, which contains tandem repeats of three copies of the ARE in the 5′-upstream region of NQO1. Firefly luciferase activity was determined by the addition of Luciferase Assay Reagent II (Promega, Madison, WI) according to the manufacturer's guidance and instructions.

**Blood biochemistry**

Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured with commercial assay kits (Asan Pharmaceutical, Seoul, Korea).

**Histopathology**

Liver tissues dissected were fixed in 10% neutral buffered formalin and dehydrated tissue was then embedded in paraffin, sectioned (3–4 μm). The liver slides were stained with hematoxylin and eosin (H&E). Subsequently, the histopathological changes of each sample were assessed using a light microscope (Zeiss, Germany).
Measurement of ROS production

Cells grown on 12-well plates were loaded with 10 μM DCFH-DA and maintained at 37°C for 30 min, after which they were harvested. ROS levels were determined using a fluorescence-microplate reader (Jemini, Molecular Device, Sunnyvale, CA) with an excitation set at 485 nm and emission at 530 nm. ROS generation was normalized to the protein concentration of each sample and defined relative to vehicle-treated control. For the mice, 10 mg of liver tissue was homogenized with 1 mL of PBS and then DCFH levels were measured as previously reported. In brief, 50 μL of freshly prepared liver homogenate was mixed with 4.85 mL 0.1 M potassium phosphate buffer (pH 7.4) and DCFH-DA was added with at a final concentration of 5 μM for 15 min at 37°C. After centrifuging at 10,000 g for 10 min at 4°C, the pellet was suspended in 5 mL potassium phosphate buffer (pH 7.4) on ice and incubated for 60 min at 37°C. ROS production was estimated as described above.

Immunoblot analysis

Sample preparation, SDS-polyacrylamide gel electrophoresis, and immunoblot analysis were performed as previously described. Briefly, proteins were separated by 7.5% gel electrophoresis and then transferred to nitrocellulose membranes. The membranes were incubated in the presence of relevant primary antibody at 4°C overnight and then incubated with secondary antibody conjugated to horseradish peroxidase. Antibodies against Nrf2, Bax, Bcl-xL, and Poly (ADP-ribose) polymerase (PARP) were provided by Santa Cruz Biotechnology (Santa Cruz, CA). p-Nrf2 (Ser40) antibody was obtained from Novus (EP1809Y). NAD(P)H quinone oxidoreductase (NQO)1, caspase-3, lamin A/C and antibodies were obtained from Cell Signaling (Danvers, MA). Glutamate cysteine ligase (GCL) antibody was from Abcam (Cambridge, MA) and heme oxygenase (HO)-1 antibody was purchased from Enzo Life Sciences (Plymouth Meeting, PA). Bad antibody was obtained from BD
Bioscience (Erembodegem, Belgium). β-actin antibody was acquired from Sigma Chemicals (St. Louis, MO). Immunoreactive protein was visualized by ECL chemiluminescence (GE Healthcare UK Limited). Equal protein loading was verified using β-actin.

**RNA isolation and real-time RT-PCR analysis**

Total RNA was isolated using TRIzol solution (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocols. Total RNA (2 μg) was reverse-transcribed into cDNA using an oligo(dT)$_{18}$ primer using a high-capacity cDNA synthesis kit (Bioneer, Daejon, Korea) with a thermal cycler (Bio-Rad, Hercules, CA). Real-time PCR was carried out with StepOne system (Applied Biosystems, Foster City, CA) using SYBR green premix (Applied Biosystems). The primer sequences used for the PCR are as follows: mouse TNF-α sense 5′-AAGCCTGTAGCCCACGTCGTA-3′ and antisense 5′-AGGTACAACCCATCGGCTGG-3′, mouse IL-1β sense 5′-TGGACGGACCCAAAGATG-3′ and antisense 5′-AGAAGGTGCTCATGTCCTCA-3′, mouse IL-6 sense 5′-GTTCCTCTGGGAAATCGTGGA-3′ and antisense 5′-TGTACTCCAGGTAGCTATGG-3′, mouse GAPDH sense 5′-TGCCCCCATCCTTGTGATG-3′ and antisense 5′-TGTGGTCATGATCCCTTCC-3′. The PCR conditions were as follows: 95 °C for 15 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. The relative levels of the PCR products were determined based on the Ct value by using the 2$^{-ΔΔCt}$ method.

**Enzyme-linked immunosorbent assay (ELISA)**

For the quantitative determination of pro-inflammatory cytokines, including TNF-α, IL-6, and 1β ELISA kits were purchased from BD Bioscience (Becton, Dickinson and Company). Cytokines levels in mice serum were quantified by ELISA using anti-mouse TNF-α, IL-6, or
IL-1β antibodies, respectively, and a Biotin-labeled secondary antibody according to the manufacturer’s instructions.

**Determination of GSH content in cells**

Cellular GSH contents were measured using a GSH determination kit (Cayman Chemical, Ann Arbor, MI) as previously described 20). In brief, HepG2 cells were seeded onto 6-well flasks and treated with 0.3–1.0 mg/mL NAOS for 12 h. The cells were lysed in 5% metaphosphoric acid to precipitate proteins. The cell lysates were centrifuged at 10,000×g for 10 min, and the supernatants were used to determine GSH levels in accordance with the manufacturer's instructions. Absorbance at 410 nm was measured using a microplate reader. GSH content in liver tissue of mice was measured using GSH determination kit (BIOXYTECH GSH-400; Oxis International, Portland, Oregon) as previously reported 18).

**Statistical analysis**

To evaluate the statistical significance of differences among the treatment groups One-way analysis of variance (ANOVA) was adopted. The Newman-Keul test was used to assess the significance of differences between the means of multiple groups. Results are expressed as means ± standard error (S.E.).
Results

*Nrf2 activation by NAOS*

To examine the cytotoxic effects of NAOS, we first treated HepG2 cells with various concentrations of NAOS for 24 h and then checked on the cell viability by MTT assay. Since NAOS had no cytotoxicity of HepG2 cells at concentrations of up to 1 mg/mL in MTT assays (Fig. 1A), and we proceeded our further experiments within the limit of 1 mg/mL of NAOS.

Activated Nrf2 binds the ARE in promoters of its target gene, which in turn, results in the upregulation of antioxidant genes. To examine transactivation by NAOS, we measured NQO1-ARE luciferase activity using NQO1-ARE luciferase constructs stably transfected into HepG2 cells. As shown in Fig. 1B, NQO1-ARE luciferase activity significantly increased following treatment with NAOS. To investigate the effect of NAOS on Nrf2 activity, we assessed the response to NAOS in a time-dependent manner and determined Nrf2 nuclear translocation in response to 1 mg/mL NAOS (Fig. 1C). Nuclear Nrf2 levels increased up 0.5–6 h after NAOS treatment. Next, we treated HepG2 cells with various concentrations of NAOS for 1 h and then examined the nuclear accumulation of Nrf2. Nuclear Nrf2 was increased by NAOS in a dose-dependent manner (Fig. 1D).

*Nrf2 target gene induction by NAOS*

Next, we examined the protein levels of GCL, HO-1, and NQO1, which are target genes of Nrf2, to investigate whether increased NAOS-induced Nrf2 accumulation in the nucleus leads to the expression of its target gene. As expected, NAOS increased GCL, HO-1, and NQO1 expression in a time- and dose-dependent manner in HepG2 cells (Figs. 2A and B). Additionally, we further confirmed the effect of NAOS on GCL and HO-1 expression in primary murine hepatocytes (Fig. 2C). We showed that NAOS significantly increased intracellular GSH levels (Fig. 2D). Since Nrf2 activation regulated biosynthesis of GSH, we
next determined whether NAOS helps to maintain redox homeostasis. Cellular damage and mitochondrial dysfunction caused by ROS lead to oxidative stress \(^{21}\). \textit{tert}-butyl hydroperoxide (\textit{t}-BHP) induces a critical uplift in ROS formation, which was almost completely prevented by NAOS pretreatment (Fig. 2E). Suppression of ROS production by NAOS suggests that NAOS has antioxidant properties against \textit{t}-BHP-induced ROS production.

\textit{Nrf2 target gene induction via MAPK activation by NAOS}

Nrf2 phosphorylation activated separation of Nrf2 from Keap1, and phosphorylated Nrf2 afterwards translocates to the nucleus where it exerts its transcriptional function \(^{22}\). Treatment with NAOS markedly increased Nrf2 phosphorylation, from 15 to 30 min, however expression of total Nrf2 was not affected (Fig. 3A). To determine the mechanism underlying the induction of Nrf2 by NAOS, we examined whether upstream kinases associated with Nrf2 phosphorylation contribute to NAOS-induced Nrf2 target gene expression (GCL and NQO1). We first treated cells with chemical inhibitor of AMPK (compound C) and PKC\(\delta\) (rottlerin) and examined GCL and NQO-1 induction in response to NAOS (Fig. 3B). However, we found that there were no differences in the protein levels of GCL and NQO-1. Conversely, NAOS-induced increases in GCL and NQO-1 were inhibited following treatment with P38 and JNK inhibitor, but not with ERK inhibitor (Fig. 3C and Supplementary Fig. 2). Inhibitory efficiency of each MAPK inhibitors used was checked in the LPS-treated cells (Supplementary Fig. 1). Enhanced Nrf2 phosphorylation by NAOS was blocked by P38 or JNK inhibitor (Fig. 3D), respectively. Moreover, p38 or JNK phosphorylation was increased by NAOS treatment, which was reversed by each inhibitor (Fig 3E). These data indicate that NAOS upregulates Nrf2 target gene expression via MAPK, including P38 and JNK, in hepatocytes.
Protection of NAOS on APAP-induced Acute Hepatitis

Next, we have extended our current in vitro observations concerning the cytoprotective effect of NAOS. Thus, we investigated whether NAOS was effective in antagonism to APAP-induced acute liver injury in mice (Fig. 4A). NAOS-administration significantly suppressed the elevation serum levels of ALT and AST caused by the injection of APAP (Figs. 4B and C). Similar to the result of serum biochemical parameter, H&E staining of liver tissue revealed a significant betterment regarding the development of degenerative regions, inflammatory cell infiltration and abnormal hepatocytes by NAOS (Fig. 4D and Table 1). These observations indicated that NAOS protected against APAP-induced acute hepatitis.

Suppression of APAP-induced oxidative stress and cell death in the liver by NAOS

APAP-induced hepatotoxicity results from oxidative stress induced by ROS production and depleted glutathione (GSH) by CYP450s. To examine the inhibition of APAP-inducible ROS production by NAOS, we measured ROS levels in the liver tissue from mice using DCFH-DA fluorescence dye. As shown in Fig. 5A, treatment with NAOS significantly decreased the APAP-induced increase in ROS levels. Furthermore, we measured the hepatic GSH content and found that NAOS treatment inhibited the GSH depletion caused by single APAP-treatment (Fig. 5B). Next, we examined the expression of proteins associated with cell death and apoptosis to determine the effect of NAOS on APAP-induced cell death in the mice liver. As shown in Fig. 5C, APAP treatment mounted up Bad and Bax expression and declined Bel-xL, precursor-PARP1/2, and pro-caspase3 protein expression. However, administration of NAOS markedly reversed these effects. These data indicate that NAOS has a protective effect in opposition APAP-inducible oxidative stress and was able to improve APAP-mediated cell death in the liver.
**Inhibition of APAP-inducible Hepatic Inflammation by NAOS**

The relation of the inflammatory response in APAP toxicity has been established \(^{23}\). Next, we examined the expression of pro-inflammatory cytokines, including TNF-\(\alpha\), IL-6, and IL-1\(\beta\), to demonstrate the effects of NAOS against the hepatic inflammation induced by APAP. Pro-inflammatory cytokine production was analyzed by ELISA in serum of mice; NAOS treatment was found to markedly decrease the elevation in cytokine release by APAP (Fig. 6A). Finally, we determined the levels of hepatic mRNA of inflammatory genes by real-time RT-PCR analysis. APAP significantly mounted up TNF-\(\alpha\), IL-6, and IL-1\(\beta\) expression in vehicle-treated mice, whereas treatment with NAOS diminished APAP-induced inflammatory gene expression (Fig. 6B). These observations indicated that NAOS inhibited the inflammatory response induced by APAP (Fig. 7).
Discussion

In this study, we examined the effects of NAOS on oxidative stress-mediated hepatic injury in \textit{in vitro} and \textit{in vivo} models. We have shown that treatment of hepatocytes with NAOS increased phosphorylation of p38 and JNK subsequently leading to the activation of the Nrf2-ARE pathway. Consequently, NAOS upregulated the expression of Nrf2 target genes, which increase intracellular GSH levels and inversely reduce ROS levels. Moreover, we found that NAOS administration significantly mitigated APAP-derived acute liver damage by blocking oxidative stress and inflammatory responses in mice.

Nrf2 is an important regulator of detoxification, antioxidative and anti-inflammatory responses, and other cytoprotective mechanism, and plays as a protector against various pathological insults, including ROS and toxic xenobiotics \cite{24}. Owing to high metabolic activity, the liver is specifically vulnerable to oxidative stress-mediated liver disease, such as DILI, viral hepatitis, and alcoholic/nonalcoholic liver disease, even cancer \cite{11,25}. Thus, pharmacological activation of Nrf2 is considered to be an attractive target molecule for the prevention or treatment of oxidative stress-associated liver diseases \cite{25,26}. However, the role of NAOS in Nrf2 activation in hepatocytes has not been examined. The current study demonstrated for the first time that NAOS could activate Nrf2 and induce the expression of its target genes in hepatocytes. In the resting states, Nrf2 is anchored by Keap1 through association with Keap1, BTB-Kelch protein, which plays a role as a substrate adaptor protein for a Cul3-dependent E3 ubiquitin ligase complex \cite{8}. Upon exposure to ROS, Keap1 undergoes conformational change through oxidation of cysteine residues on Keap1. Then, Nrf2 bypasses Keap1 and translocates to the nucleus to activate the ARE, inducing the expression of genes encoding cytoprotective antioxidant proteins.

Additionally, Nrf2 is activated via posttranslational modifications such as phosphorylation and acetylation. Among them, activation by phosphorylation leads to enhanced stability.
and/or nuclear accumulation of Nrf2. Nrf2 contains several serine, threonine and tyrosine residues, which may provide sites for phosphorylation by different kinase. Nrf2 phosphorylation is mediated by PKCδ 27, AMPK 28, or MAPKs 29 and is thought to enhance the stability and/or nuclear accumulation of Nrf2. Among them, PKCδ has been shown to phosphorylate Nrf2 at Ser40, promoting the translocation of Nrf2 into the nucleus 30. Multiple phosphorylated serine or threonine residues (S215, S408, S558, T559 and S577) by MAPKs were also identified 31. Although, MAPK has been implicated in Nrf2 activation by many previous reports, the regulation of MAPK on Nrf2 activity is not clear yet. Moreover, Sun et al. showed that Nrf2 stability is not affected by Nrf2 phosphorylation and concluded that MAPK regulates Nrf2 through indirect mechanisms 31. It was also reported that ERK may contribute to phosphorylation of Nrf2 at Ser40 32,33. Other reports also showed that Nrf2 phosphorylation of Nrf2 at Ser40 by diverse stimuli was also inhibited by p38 or JNK inhibitors 34, 35. Here, we reveal that NAOS increases the Nrf2 phosphorylation at Ser40 residue. By treating hepatocytes with specific inhibitors, we found that p38 and JNK is responsible for Nrf2 phosphorylation. In contrast, AMPK, PKCδ, ERK is not involved in this regulation. Moreover, treatment with NAOS increased phosphorylation of p38 and JNK, which were reversed by each inhibitor. Therefore, the coordinated regulation by p38 and JNK pathways might result in the final outcome of upregulation of antioxidant enzyme expression in NAOS-treated hepatocytes (Fig. 3). Albeit, study regarding role of MAPK on phosphorylation of Nrf2 at Ser40 need to be investigated.

Because Nrf2 can regulate the transcription of genes involved in the biotransformation and excretion of APAP, Nrf2 activation is regarded as a crucial therapeutic target against APAP-induced liver injury 36. Here, we found that treatment with NAOS inhibited the APAP-induced increase in serum transaminase activities and improved histological indices, such as hepatocyte degeneration and inflammatory cell infiltration. Oxidative stress contributes for
the pathophysiology of APAP-induced hepatotoxicity \(^{37}\). Consequently, the increase in free radical formation, mainly hydrogen peroxide and superoxide anion, exacerbate the peroxidation of membrane phospholipids. Treatment with NAOS significantly decreased the APAP-mediated increase in ROS levels concomitantly with the restoration of GSH depletion. Consequently, NAOS treatment suppressed APAP-induced apoptosis. These results suggest that NAOS has protective effects against APAP-inducible oxidative stress and cell death in the liver.

APAP overdose results in necrotic cell death and subsequent inflammation, such as the release of pro-inflammatory cytokines and activation of immune cells \(^{38}\). Resident macrophages (Kupffer cells) and inflammatory cells (neutrophils, monocytes/macrophages) are recruited into the liver by APAP overdose, although the involvement of immune cells in the pathophysiology of APAP is controversial \(^{37}\). As expected, treatment with APAP significantly induced the increased levels of pro-inflammatory cytokines and pro-inflammatory genes, such as TNF-\(\alpha\), IL-6, and IL-1\(\beta\), whereas NAOS prevented the generation of inflammatory cytokines and inflammatory gene expression induced by APAP (Fig. 6).

To summarize, our study revealed, for the first time, that NAOS administration inhibited APAP-induced liver injury, and attenuated hepatic inflammation and oxidative stress. Regarding the molecular mechanisms underlying the hepatoprotective potential of NAOS, we demonstrated that NAOS exhibits an anti-oxidant effect via activation of the Nrf2/ARE pathway. Collectively, these results enabled us to identify NAOS as a beneficial compound that inhibit APAP-induced hepatotoxicity.
Acknowledgments

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Conflict of interest

The authors declare no conflict of interest.

Supplementary Materials

The online version of this article contains supplementary materials.
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Figure 1. The effect of NAOS on Nrf2 activation.

A, HepG2 cells were incubated for 24 h with NAOS (0.1–1 mg/mL) and then measured the cell viability by MTT assay. B, NQO1-ARE luciferase activity. We checked on NQO1-ARE luciferase activity using the lysates of HepG2 cells stably transfected with the NQO1-ARE luciferase construct following exposure to NAOS (0.1–1 mg/mL) for 12 h. Data represent the mean ± S.E. of 3 ingeminate experiments; the statistical significance of differences between each treatment group and the control (***p<0.01). C, Time-dependent nuclear translocation of Nrf2 in HepG2 cells treated with NAOS (1 mg/mL). Nrf2 protein level in the nuclear fractions of cells incubated with NAOS from 30 min to 6 h was determined by immunoblotting. D, Dose-dependent nuclear translocation of Nrf2 in HepG2 cells treated with NAOS for 1 h. Nuclear Nrf2 protein level was immunoblotted in cells treated with NAOS (0.1–1 mg/mL) as get by scanning densitometry. Data represent the mean ± S.E. of 3 ingeminate experiments; *p < 0.05, **p < 0.01, significant in comparison with the vehicle-treated control.
Figure 2. The effect of NAOS on Nrf2 target gene expression.

A, HepG2 cells were incubated for 1 h to 12 h with 1 mg/mL NAOS, and then the cell lysates were immunoblotted for GCL, HO-1, and NQO1. B, Expression of Nrf2 target genes (GCL, HO-1, and NQO1) in HepG2 cells treated with varying concentrations of NAOS for 6 h. Level of GCL, HO-1, and NQO1 proteins in cells treated with NAOS (0.1–1 mg/mL) was
measured using western blot. **C**, The effect of NAOS on Nrf2 target genes expression in mouse primary hepatocytes. Primary hepatocytes were incubated with various concentrations of NAOS (0.1–1 mg/mL) for 6 h. The levels of GCL and HO-1 in the cells as get by scanning densitometry. Data represent the mean ± S.E. of 3 ingeminate experiments; *p < 0.05, **p < 0.01, significant in comparison with the vehicle-treated control. **D**, Increases in the basal levels of intracellular GSH in response to NAOS. Level of GSH concentrations was assured in the lysates of cells treated with NAOS (0.3–1 mg/mL) for 12 h. Data represent the mean ± S.E of 3 ingeminate experiments; the statistical significance of differences between each treatment group and the control (**P<0.01). **E**, The effect of NAOS on t-BHP-induced ROS production. HepG2 cells were incubated with 500 μM t-BHP and/or 0.3–1 mg/mL NAOS. And cells were stained with DCFH-DA (10 μM) for 30 min at 37°C. The intensity of intracellular fluorescence by ROS was measured using a fluorescence microplate reader. Data represent the mean ± S.E. of 3 ingeminate experiments; **p<0.01, significant in comparison with the vehicle-treated control; ###p<0.01, significant versus t-BHP alone.
Figure 3. The effect of NAOS on Nrf2 phosphorylation and target gene expression.

A, Phosphorylation of Nrf2 in HepG2 cells treated with NAOS was checked by immunoblot. Levels of Nrf2 phosphorylation were measured in cell lysates following incubation with 1 mg/mL of NAOS from 15 min to 6 h. Results were confirmed in ingeminate experiments. B, HepG2 cells were treated with NAOS and/or AMPK and PKCδ inhibitor, and then immunoblotted for GCL and NQO1 proteins to determine the effects of NAOS. Cells were treated with 1 mg/mL of NAOS in combination with 5 μM compound C (CompC, AMPK inhibitor) or 2.5 μM rottlerin (rott, PKCδ inhibitor) for 6 h. C, The effects of NAOS on Nrf2 target gene expression via the activation of MAPK. HepG2 cells were treated with 1 mg/mL of NAOS and/or MAPK inhibitors (ERK, P38, and JNK inhibitor; each of 10 μM) for 6 h. D, HepG2 cells were treated with NAOS (1 mg/mL for 30 min) and/or p38 and JNK inhibitor, and then immunoblotted for p-Nrf2. E, The effects of NAOS on p38 and JNK phosphorylation. HepG2 cells were treated with 1 mg/mL of NAOS and/or MAPK inhibitors (P38 or JNK inhibitor) for 30 min. Data represent the mean ± S.E. of 3 ingeminate experiments; *p < 0.05, **p < 0.01, significant in comparison with the vehicle-treated control; #p < 0.05, ##p < 0.01, significant in comparison with NAOS-treated.
Figure 4. Inhibition of APAP-induced acute liver injury by NAOS.

A. Study design. ICR mice were treated with NAOS (250–500 mg/kg; po) for a period 5 days. In the past the mice were sacrificed, liver injury was induced by a single intraperitoneal injection (i.p.) of acetaminophen (APAP) (300 mg/kg) for 24 h. B-C, ALT and AST activities were measured by routine clinical assays using commercial kits. All values are expressed as means ± S.E. of 10 mouse serum samples (significant in comparison with the vehicle-treated
control, **p<0.01; significant in comparison with APAP, ##p<0.01). **D**, Representative histological sections of the mice liver. Mice liver samples were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned, and then stained with H&E for prevalent histological observations (Scale bar = 120 μm). CV = central vein; PT = portal triad. (Color figure can be accessed in the online version.)
Figure 5. Inhibition of APAP-induced oxidative stress by NAOS.

A, Measurement of ROS generation *in vivo*. Frozen mice livers (n=5) were homogenized and stained with 5 μM DCFH-DA, and level of ROS was checked using a fluorescence microplate reader. B, GSH measurements (n=5). GSH levels were measured in NAOS-administered mice following APAP treatment. GSH concentrations were measured in mouse liver homogenates. C, The level of proteins related in death of cell in the presence and
absence of NAOS and/or APAP was measured by western blots analyzing. Bcl-xL, precursor-PARP, pro-Caspase3, Bad, and Bax protein levels in the livers of mice were determined by immunoblotting. Data represent the mean ± S.E.; **p < 0.01, significant in comparison with vehicle-treated control; ##p < 0.01, significant in comparison with APAP.
Figure 6. Suppression of APAP-induced inflammatory responses by NAOS.

A, ELISA. Levels of TNF-α, IL-6, and IL-1β of mice serum (n=5) were measured by ELISA.

B, RT-PCR analysis. mRNA levels of TNF-α, IL-6, and IL-1β were analyzed by real-time RT-PCR in frozen mice livers tissue (n=5). All values are expressed as mean ± S.E. (significant in comparison with the vehicle-treated control, *p<0.05, **p<0.01; significant in comparison with APAP, #p<0.05, ##p<0.01).
Figure 7. A schematic diagram of the molecular mechanisms underlying NAOS-induced activation of antioxidant genes via Nrf2 activation and NAOS-induced protection cope with APAP-derived oxidative stress and acute liver damage.
Table 1. General histomorphometrical analysis of hepatic tissues, taken from vehicle or APAP-treated mice

| Groups      | Index                  | Degenerative regions (%/mm²) | Infiltrated inflammatory cells (%/mm²) | Abnormal hepatocytes (cells/1000 hepatocytes) |
|-------------|------------------------|------------------------------|---------------------------------------|---------------------------------------------|
| Controls    |                        |                              |                                       |                                             |
| vehicle     | 1.83±0.88              | 36.60±16.77                  | 18.00±10.70                           |                                             |
| APAP        | 77.94±13.18**          | 467.20±114.88**              | 770.00±146.26**                       |                                             |
| NAOS        |                        |                              |                                       |                                             |
| 250 mg/kg   | 32.56±11.40##          | 201.60±52.93##               | 304.40±119.48##                       |                                             |
| 500 mg/kg   | 14.01±3.93##           | 88.20±27.26##                | 132.20±40.71##                       |                                             |

Values are expressed as mean ± SD of six hepatic histological fields (** p<0.01 as compared with vehicle-treated control mice or ## p<0.01 as compared with APAP-treated mice)