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Oleanolic acid activates Nrf2 and protects from acetaminophen hepatotoxicity via Nrf2-dependent and Nrf2-independent processes

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ABSTRACT

Oleanolic acid is a plant-derived triterpenoid, which protects against various hepatotox-icants in rodents. In order to determine whether oleanolic acid activates nuclear factor erythroid 2-related factor 2 (Nrf2), a transcription factor known to induce various antiox- idant and cytoprotective genes, wild-type and Nrf2-null mice were treated with oleanolic acid (90 mg/kg, i.p.) once daily for 3 days. Oleanolic acid increased nuclear accumulation of Nrf2 in wild-type but not Nrf2-null mice, as determined by Western blot and immuno- fluorescence. Oleanolic acid-treated wild-type mice had increased hepatic mRNA expres- sion of the Nrf2 target genes NAD(P)H:quinone oxidoreductase 1 (Nqo1); glutamate–cysteine ligase, catalytic subunit (Gclc); heme oxygenase-1 (Ho-1); as well as Nrf2 itself. In addition, oleanolic acid increased protein expression and enzyme activity of the prototypical Nrf2 target gene, Nqo1, in wild-type, but not in Nrf2-null mice. Oleanolic acid protected against acetaminophen hepatotoxicity in wild-type mice but to a lesser extent in Nrf2-null mice. Oleanolic acid-mediated Nrf2-independent protection from acetaminophen is, in part, due to induction of Nrf2-independent cytoprotective genes, such as metallothionein. Collec- tively, the present study demonstrates that oleanolic acid facilitates Nrf2 nuclear accumu- lation, causing induction of Nrf2-dependent genes, which contributes to protection from acetaminophen hepatotoxicity.

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1. Introduction

Oleanolic acid is a natural triterpenoid that is a constituent of the leaves of *Olea europaea*, *Viscum album* L., and other plants, and is an aglycone of many saponins. Oleanolic acid is used in Chinese medicine for the treatment of liver disorders, such as viral hepatitis, and has been shown to

protect mice from various hepatotox-icants that cause oxidative and electrophilic stress, including carbon tetra- chloride, acetaminophen, bromobenzene, and thioaceta- mide. In mice pretreated with oleanolic acid, and then exposed to one of the above chemical hepatotox-icants, serum alanine transaminase concentrations and centrilob- ular necrosis were diminished [1–4].

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Abbreviations: ALT, alanine transaminase; ARE, antioxidant response element; ATF-4, activating transcription factor-4; Cul3, Cullin3; Gclc, glutamate cysteine ligase catalytic subunit; Ho-1, heme oxygenase-1; MT, metallothionein; Nrf2, nuclear factor-erythroid 2-related factor 2; Keap1, kelch-like ECH-associated protein 1; Maf, musculo-aponeurotic fibrosarcoma; Nqo1, NAD(P)H:quinone oxidoreductase 1; NAPQI, N-acetyl-p-benzoquinoneimine; OA, oleanolic acid.

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Acetaminophen is a widely used antipyretic drug that causes hepatotoxicity at high doses. At therapeutic doses, most acetaminophen is conjugated with either glucuronic acid in a reaction with UDP-glucuronosyltransferases or sulfate in a reaction with sulfotransferases. However, at higher doses, UDP-glucuronosyltransferases and sulfotransferases become saturated and more of the toxic metabolite, *N*-acetyl-*p*-benzoquinoneimine (NAPQI), is formed via a reaction with cytochrome p450s. If NAPQI is not detoxified via conjugation with glutathione or if glutathione becomes depleted, NAPQI can bind to cellular proteins and form adducts, which leads to hepatotoxicity [5].

Nuclear factor erythroid 2-related factor 2 (Nrf2), and its repressor kelch-like ECH-associated protein 1 (Keap1), have been characterized over the past decade as an important endogenous cellular mechanism for coping with oxidative stress [6]. Nrf2 is a transcription factor that binds to antioxidant response elements (AREs) in upstream promoter regions of various cytoprotective enzymes, the induction of which helps restore the intracellular balance between oxidants and antioxidants. Under conditions when oxidative/electrophilic stress is low, Keap1 sequesters Nrf2 in the cytosol, by acting as an adaptor for Cullin (Cul) 3-based E3 ligase, to target Nrf2 for proteasomal degradation [7]. Cul3 and other Cullin-based E3 ligases regulate the turnover of important transcription factors by acting as scaffold proteins to help facilitate ubiquitination, marking proteins for proteasomal degradation [8]. When oxidative/electrophilic stress is increased in the cell, Nrf2 is able to avoid Keap1-mediated proteasomal degradation by Cul3, and translocate into the nucleus [9]. Once in the nucleus, Nrf2 heterodimerizes with a small musculo-aponeurotic fibrosarcoma (Maf) protein, c-jun, or activating transcription factor-4 (ATF-4) and binds to AREs, promoting transcription of various cytoprotective genes [10–12].

Nrf2 target genes include, but are not limited to, glutathione-S-transferases, NAD(P)H:quinone oxidoreductase 1 (Nqo1), glutamate–cysteine ligase catalytic subunit (Gclc), and heme oxygenase-1 (Ho-1). Nqo1 catalyzes the reduction of quinones, protecting cells against redox cycling and oxidative stress [13]. In addition, Nqo1 can reduce the toxic metabolite of acetaminophen back to the parent compound *in vitro* [14]. Gclc is the rate-limiting enzyme in the synthesis of glutathione, a major antioxidant molecule in cells, which is critical for the maintenance of cellular redox homeostasis and detoxification of electrophiles, including NAPQI [15]. Ho-1 catalyzes the breakdown of heme into bilirubin, carbon monoxide, and iron. Bilirubin is an antioxidant, and its generation following Ho-1 induction reduces cytotoxicity caused by oxidative stress [16]. In addition, small quantities of carbon monoxide are cytoprotective, anti-apoptotic, vasorelaxant, and anti-inflammatory [17]. Also, heme-mediated induction of Ho-1 or pretreatment with biliverdin (a bilirubin precursor) protects rats against acetaminophen-induced hepatotoxicity [18].

The group of enzymes under the transcription control of Nrf2 are referred to as the “Nrf2 regulon” and provide antioxidative protection [19]. Nrf2-null mice have compromised antioxidant protection and are highly susceptible to target-organ injury from acetaminophen [20], benzo[*a*]pyrene [21], diesel exhaust [22], hyperoxia [23], hydrogen peroxide [24], and many other oxidative-type pathologies. In contrast,

mice with a hepatocyte-specific deletion of Keap1 have marked increases in Nrf2 target genes and are protected from acetaminophen hepatotoxicity [25].

Previous studies have suggested that oleanolic acid is unable to activate the Nrf2–Keap1 pathway because oleanolic acid is a natural compound and lacks necessary functional groups (Michael acceptors) possessed by its synthetic derivatives, including CDDO-Im [26,27]. Nqo1 is a prototypical target gene of Nrf2 activation; therefore, Nqo1 mRNA expression and enzyme activity are used as biomarkers of Nrf2 activation [28]. In previous studies, low doses of oleanolic acid were unable to induce Nqo1 activity *in vitro* in Hepa1c1c7 murine hepatoma cells [26]. In addition, a low, single, oral dose of oleanolic acid (9 mg/kg) failed to induce hepatic Nqo1 mRNA expression in mice [27].

However, in a recent study, dosing oleanolic acid (~25 mg/kg, s.c.) once daily for 4 days, induced many cytoprotective genes, such as the Nrf2-dependent genes Nqo1, Gclc, and Ho-1 (three to fivefold over vehicle) [29]. However, in this study by Liu et al. nuclear translocation of Nrf2 protein was implied but not actually shown. Furthermore, Nrf2-null mice were not used by Liu et al. [29] to demonstrate definitively whether induction of Nqo1, Gclc, and Ho-1 was caused by Nrf2 activation, as other transcription factors and mechanisms are capable of inducing these genes [30–33]. Therefore, the purpose of the present study was to investigate whether oleanolic acid (90 mg/kg, i.p.), administered once daily for 3 days, as was used previously in hepatoprotection studies [34], activates the Nrf2–Keap1 pathway using wild-type and Nrf2-null mice, and whether activation of Nrf2 is responsible for oleanolic acid-mediated protection against acetaminophen hepatotoxicity.

2. Methods

2.1. Reagents

Oleanolic acid was a gift from Dr. Jie Liu (NIEHS, Research Triangle Park, NC). Nqo1 and β -actin antibodies were purchased from Abcam (Ab2346, Ab8227, respectively, Cambridge, MA). Nrf2 antibody was purchased from Santa Cruz Biotechnology (sc-30915, Santa Cruz, CA). All other chemicals, unless otherwise specified, were purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Animals and husbandry

Eight-week-old male C57BL/6 mice were purchased from Charles River Laboratories, Inc. (Wilmington, MA). Nrf2-null mice on a mixed C57BL/6 and AKR background were obtained from Dr. Jefferson Chan (University of California, Irvine, Irvine, CA) and backcrossed seven generations into C57BL/6 mice to >99% congenicity, as was determined by Jackson Laboratories (Bar Harbor, ME). Animals were housed in a temperature-, light-, and humidity-controlled environment and fed Teklad Rodent Diet #8604 (Harlan Laboratories, Madison, WI) *ad libitum*. The housing facility is an American Animal Association Laboratory Animal Care-accredited facility at the University of Kansas Medical Center, and all procedures were preapproved in accordance with the Institutional Animal Care and Use Committee guidelines.

2.3. Animal treatment

Male wild-type and Nrf2-null mice were dosed with oleanolic acid (90 mg/kg, i.p.) or vehicle (2% Tween 80 in sterile saline, 10 mL/kg, i.p.) in the morning, once daily for 3 days. After cervical dislocation on the morning of the fourth day, livers were removed, cut into small pieces, frozen in liquid nitrogen, and stored at -80°C for later use for quantification and analysis of mRNA, protein, and enzyme activity. For immunofluorescent staining of Nrf2, a section of liver was frozen in isopentane and stored at -80°C .

For the hepatoprotection study, wild-type and Nrf2-null mice were dosed with oleanolic acid (90 mg/kg, i.p.) or vehicle (2% Tween 80, 10 mL/kg, i.p.) in the morning, once daily for 3 days. On the fourth day, mice were administered acetaminophen (500 mg/kg, i.p.) in saline (pH 8) in a volume of 20 mL/kg. Blood and livers were collected 8 h later. Portions of the liver were fixed in 10% zinc formalin for 48 h.

2.4. Total RNA isolation

Total liver RNA was isolated using RNA-Bee reagent (Tel-Test, Inc., Friendswood, TX) according to the manufacturer's protocol. Total RNA concentrations were determined spectrophotometrically at 260 nm. The RNA samples were diluted to

500 ng/ μL with diethyl pyrocarbonate-treated deionized water.

2.5. Branched DNA signal amplification (bDNA) analysis

The mRNA expression of Nqo1, Gclc, and Ho-1 in mouse livers was quantified using the bDNA assay (Quantigene 1.0 bDNA signal amplification kit; Panomics, Inc., Fremont, CA) with modifications [35]. Gene sequences of interest were accessed from GenBank. Target sequences were analyzed using Probe-Designer software v1.0 (Chiron Diagnostics Corp., Walpole, MA) to design oligonucleotide probe sets (capture, label, and blocker probes). All probes were designed with a melting temperature of 63°C , enabling hybridization conditions to be held constant (i.e., 53°C) during each hybridization step. Probe sets for Ho-1 and Nqo1 were previously described [36]. Probe sets for Gclc and metallothionein (MT) are listed in Table 1. Total RNA was added to each well of a 96-well plate containing 50 μL of each diluted probe set. RNA was allowed to hybridize with the probe sets overnight at 53°C . Subsequent hybridization steps were carried out according to the manufacturer's protocol, and luminescence was quantified with a Synergy 2 Multi-Detection Microplate Reader interfaced with Gen5 Reader Control and Data Analysis Software (Biotek, Winoosky, VT). Data are presented as relative light units (RLU) normalized to control.

Table 1 – Oligonucleotide probes used for analysis of mouse gene expression by bDNA signal amplification assay.

Gene	Accession #	Target ^a	Function ^b	Probe sequence
Gclc	NM_012815	1548–1566	CE	atggctcggagctggtctgTTTTctcttggaaagaaagt
		1996–1724	CE	cttaattagcttcaggtatgtcagaataTTTTctcttggaaagaaagt
		1725–1749	CE	tcattagtctccagatgctctctTTTTctcttggaaagaaagt
		1848–1870	CE	tcattagtctccagatgctctctTTTTctcttggaaagaaagt
		1896–1917	CE	acttcgcttttctaaagcctgaTTTTctcttggaaagaaagt
		1528–1547	LE	ggccttgctacacccatccaTTTTtaggcataggaccgtgtct
		1567–1588	LE	atgagcgtgtactcctctgcagTTTTtaggcataggaccgtgtct
		1589–1612	LE	ccattgatgatggtgtctatgtctTTTTtaggcataggaccgtgtct
		1656–1678	LE	tcgaactccatgttttcaaggtaTTTTtaggcataggaccgtgtct
		1679–1696	LE	ctgcatcgggtgtccacgTTTTtaggcataggaccgtgtct
		1750–1770	LE	ctctcatccacctggcaacagTTTTtaggcataggaccgtgtct
		1771–1794	LE	agtcaggatggttgcataaactTTTTtaggcataggaccgtgtct
		1822–1847	LE	tttcaaatgaggctatgtgtatctTTTTtaggcataggaccgtgtct
		1918–1939	LE	gggtcgttttaccctcactgtTTTTtaggcataggaccgtgtct
		1613–1632	BL	caggaaacacgcctctcttc
		1633–1655	BL	ggagttcagaatgggatgagtc
		1795–1821	BL	catcagttattacactgtctgtgt
		1871–1895	BL	tccaagtaactctggacattcacac
MT	NM_013602	72–92	CE	ggtccattccgagatctggtgTTTTctcttggaaagaaagt
		170–190	CE	ggagcagcagctctcttgcaTTTTctcttggaaagaaagt
		130–147	LE	caggcgcaggagctggtgTTTTtaggcataggaccgtgtct
		191–207	LE	cagcccacgggacagcaTTTTtaggcataggaccgtgtct
		227–244	LE	ggcgcttgcagacacaTTTTtaggcataggaccgtgtct
		245–262	LE	gcacgtgcactgtccgcTTTTtaggcataggaccgtgtct
		263–283	LE	ctgttcgtcacatcaggcacaTTTTtaggcataggaccgtgtct
		284–302	LE	tttacagtggtggcagcgTTTTtaggcataggaccgtgtct
		303–323	LE	cgtcgggttggtccgatactTTTTtaggcataggaccgtgtct
		93–112	BL	ggtggagcaggagcagttgg
		113–129	BL	caagtcaggagccgcc
		148–169	BL	ggaggtgcacttcagttcttg
		208–226	BL	gccctgggcacattggag

^a Target refers to the sequence of the mRNA transcript as enumerated in the GenBank file.

^b Function refers to the use of the oligonucleotide probe in the assay (CE, capture extender; LE, label extender; BL, blocker probe).

2.6. Nrf2 and Nqo1 protein expression

Livers were homogenized in sucrose–Tris buffer (0.25 mol/L sucrose, 10 mmol/L Tris–HCl, pH 7.4) and centrifuged at $100,000 \times g$ for 60 min at 4 °C. The resulting supernatant, containing the cytosolic fraction, was used to assay for Nqo1 protein. Nuclear extracts were prepared with the NE-PER nuclear extraction kit according to the manufacturer's directions (Pierce Biotechnology, Rockford, IL). Nuclear extracts were used for Nrf2 immunoblotting. Protein concentrations were determined with BCA Assay Kit from Pierce Biotechnology (Rockford, IL).

Cytosolic and nuclear proteins (40 µg protein/lane) were electrophoretically resolved using polyacrylamide gels (4% stacking and 12% resolving for Nqo1; 4% stacking and 10% resolving for Nrf2). Gels were transblotted overnight at 4 °C onto a nitrocellulose membrane for Nqo1, and a polyvinylidene fluoride membrane for Nrf2. Membranes were then washed with PBS-buffered saline containing 0.05% Tween-20 (PBS–T). Membranes were blocked for 1 h at room temperature with 5% non-fat milk in PBS–T. Blots were then incubated with primary antibody (1:1000 dilution for Nqo1 and Nrf2, in 2% non-fat milk in PBS–T) for 3 h at room temperature. Blots were then washed in PBS–T and incubated with secondary antibody conjugated with horseradish peroxidase (1:2000 dilution for Nqo1 and Nrf2) in 2% non-fat milk in PBS–T buffer for 1 h at room temperature. Blots were then washed with PBS–T. Protein–antibody complexes were detected using an enhanced chemiluminescent kit (Pierce Biotechnology, Rockford, IL) and exposed to X-ray film (Denville Scientific, Metuchen, NJ). Intensity of protein bands was quantified using the Discovery Series Quantity One 1D Analysis software (Bio-Rad Laboratories, Hercules, CA). Intensity values were normalized to β -actin and expressed as relative protein expression.

2.7. Immunofluorescence

Nrf2 was detected as previously described [37]. Briefly, isopentane frozen liver was embedded in optimal cutting temperature (OCT) compound prior to cryosectioning. Cryosections were air-dried at room temperature for 5 min and fixed with 4% paraformaldehyde. The sections were blocked at room temperature for 30 min with 5% donkey serum/phosphate-buffered saline with 0.2% Triton X-100 (PBS–Tx), and then incubated overnight with Nrf2 antibody diluted 1:50 in 5% donkey serum/PBS–Tx. A fluorescein isothiocyanate-labeled secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:200 was used, along with rhodamine-labeled phalloidin (Invitrogen, Carlsbad, CA) diluted 1:200 in 5% donkey serum/PBS–Tx. 4,6-Diamidino-2-phenylindole (Invitrogen, Carlsbad, CA) staining was performed to define nuclear regions. Antibody solutions were filtered through 0.22 µm membrane syringe-driven filter units (Osmonics Inc., Minnetonka, MN) prior to use. Frozen liver sections were stained and imaged under uniform conditions for each antibody. Negative controls without primary antibody were also included in the analysis (data not shown). Images were captured on an Olympus BX41 fluorescent microscope with a DP70 camera and DP Controller software (Olympus, Melville, NY).

2.8. Nqo1 activity assay

Cytosolic Nqo1 enzyme activity was determined by quantifying the reduction of 2,6-dichlorophenol–indophenol (DCPIP) as described previously [38,39].

2.9. Serum alanine transaminase (ALT) concentrations

Serum ALT concentrations were determined as a biochemical indicator of hepatocellular injury using Pointe Scientific Liquid ALT Reagent Set (Canton, MI) according to the manufacturer's protocol.

2.10. Histopathology

Liver samples were fixed in 10% neutral-buffered zinc formalin prior to routine processing and paraffin embedding. Liver sections (5 µm) were stained with hematoxylin and eosin. Liver sections were analyzed and assigned one of six scores of severity of necrosis: 0, none; 1, minimal (>2 foci of single cell necrosis per section); 2, mild (at least 5 areas of focal necrosis per section); 3, moderate (at least five foci of zonal necrosis per section); 4, severe (lobular damage, with many viable lobules per section); and 5, global (severe lobular damage, few areas of viability per section). A section was defined as a single viewpoint at low power (100 \times) magnification.

2.11. Statistical analysis

An $n = 5$ for all groups was used. Data were analyzed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test ($p \leq 0.05$). Histopathological data were rank ordered prior to ANOVA analysis, which was followed by Newman–Keuls multiple range test ($p \leq 0.05$).

3. Results

3.1. Nrf2 nuclear translocation in oleanolic acid-treated wild-type and Nrf2-null mice

Oleanolic acid (90 mg/kg, i.p.), given once daily for 3 days, increased Nrf2 protein 432% in nuclear extracts from livers of wild-type mice (Fig. 1, upper). Nrf2-null mice treated with vehicle or oleanolic acid did not have detectable levels of Nrf2 protein in the nuclear fractions. Indirect immunofluorescent analysis revealed minimal Nrf2 staining (green) in frozen sections from wild-type mice (Fig. 1, lower). The actin cytoskeleton was stained in red. Nrf2 staining of liver sections from oleanolic acid-treated wild-type mice was strong and localized to the nucleus (blue). No Nrf2 staining was observed in vehicle- or oleanolic acid-treated Nrf2-null mice.

3.2. Nrf2, Nqo1, Gclc, and Ho-1 mRNA expression in oleanolic acid-treated wild-type and Nrf2-null mice

Oleanolic acid increased mRNA expression of Nrf2 (347%), Nqo1 (1108%), Gclc (521%), and Ho-1 (955%) in livers of wild-type mice (Fig. 2). Nrf2-null mice treated with oleanolic acid showed no increase in Nrf2, Nqo1, Gclc, or Ho-1 mRNA expression.

3.3. Nqo1 protein expression and enzyme activity in oleanolic acid-treated wild-type and Nrf2-null mice

Because Nqo1 is considered the prototypical Nrf2-target gene [40–43], Nqo1 protein and enzyme activity were evaluated.

Wild-type mice treated with oleanolic acid had increased Nqo1 protein expression (79%) as determined by Western blot (Fig. 3, upper). Vehicle treated Nrf2-null mice had much lower Nqo1 protein expression (–73%) than wild-type mice. Furthermore, oleanolic acid did not change Nqo1 protein expression in Nrf2-

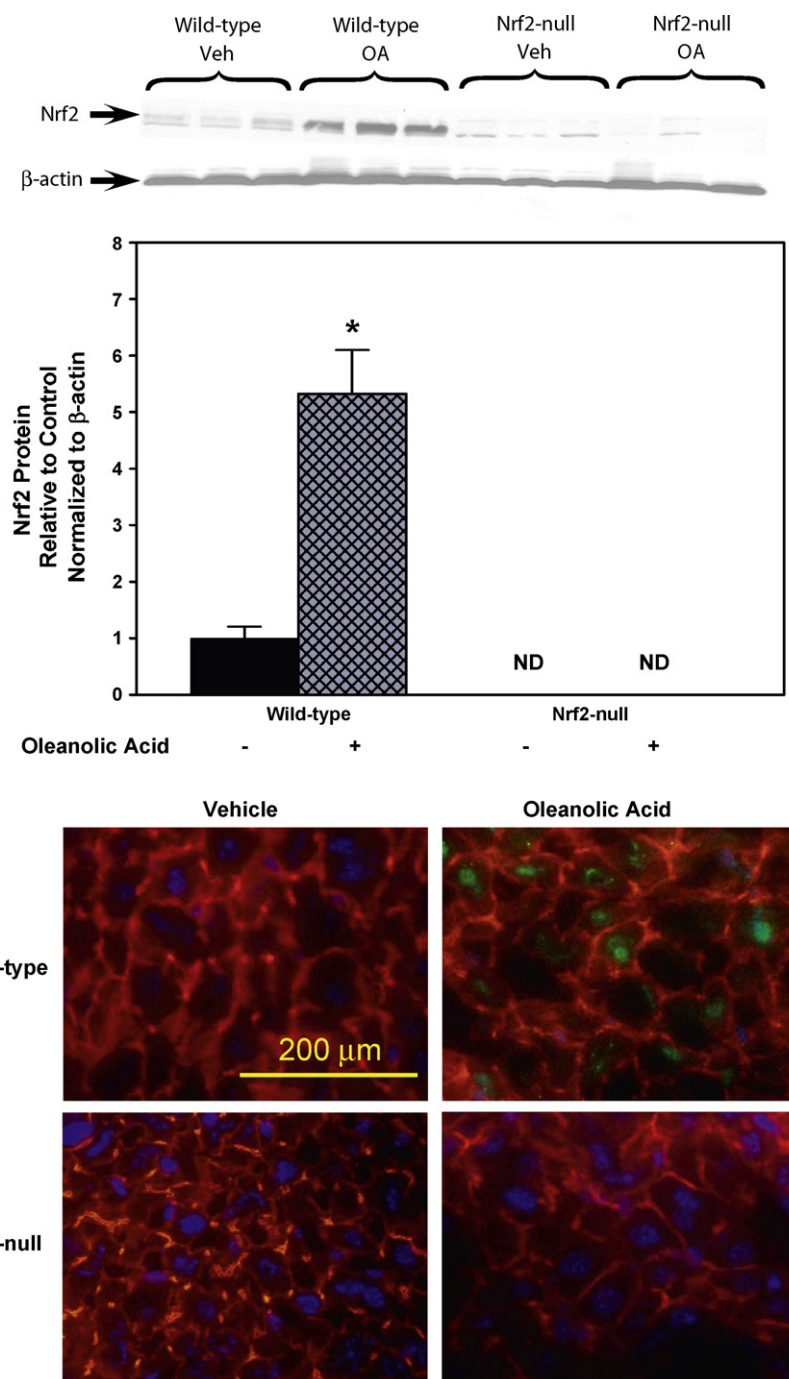


Fig. 1 – Upper: Western blot of liver nuclear fractions for Nrf2 after treatment with oleanolic acid (90 mg/kg, i.p.) once daily for 3 days. Also shown is the quantification of specific band intensity, normalized to β -actin, and expressed relative to control as mean \pm S.E.M. **Abbreviations:** ND, not detected. Asterisk (*) indicates a statistically significant difference from wild-type mice treated with vehicle ($p \leq 0.05$). **Lower:** Immunofluorescent localization of Nrf2 in livers from wild-type mice after vehicle or oleanolic acid. Indirect immunofluorescence to detect Nrf2 (green) and actin (red) was performed on liver cryosections (5 μ m) from wild-type mice after oleanolic acid (90 mg/kg, i.p.) treatment once daily for 3 days. Sections were mounted in Prolong Gold containing DAPI for nuclear staining (blue). Representative images are shown at high-power magnification (400 \times). Bar represents 200 μ m.

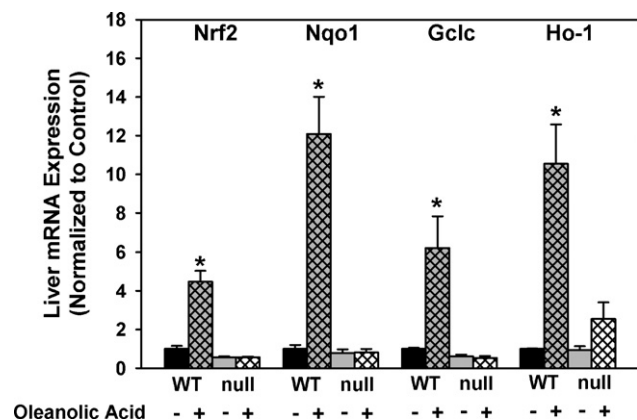


Fig. 2 – Messenger RNA expression of Nqo1, Gclc, and Ho-1 in livers of wild-type and Nrf2-null mice after treatment with oleanolic acid (90 mg/kg, i.p.) once daily for 3 days. Messenger RNA was quantified by the bDNA assay. Data is expressed relative to wild-type controls as mean \pm S.E.M. Abbreviations: WT, wild-type; null, Nrf2-null. Asterisk (*) indicates a statistically significant difference from wild-type mice receiving vehicle ($p \leq 0.05$).

null mice. Oleanolic acid induced Nqo1 enzyme activity 154% in wild-type mice (Fig. 3, lower). Nqo1 activity is less in Nrf2-null mice (–47%) than in wild-type mice and is not induced by oleanolic acid. Of note, in wild-type mice treated with oleanolic acid, even though Nqo1 mRNA expression was induced over 1100%, Nqo1 protein expression and enzyme activity did not increase more than 150%.

3.4. Oleanolic acid-mediated protection against acetaminophen in wild-type and Nrf2-null mice

In this study, serum ALT concentrations corresponded with histopathological necrosis grading. Oleanolic acid did not alter serum ALTs (baseline of 20–30 IU/L) or liver histology in wild-type or Nrf2-null mice (data not shown). Oleanolic acid-pretreatment decreased acetaminophen-induced hepatotoxicity in wild-type mice, as indicated by an 82% reduction in serum ALTs (Fig. 4) and reduction in necrosis grade (Table 2). Acetaminophen produced more severe hepatotoxicity in Nrf2-null mice (increases in serum ALTs [250%] and necrosis grade) than wild-type mice, similar to a previous report of increased sensitivity of Nrf2-null mice [20]. Oleanolic acid-pretreatment of Nrf2-null mice protected against acetaminophen hepatotoxicity (reduction in serum ALTs [65%] and necrosis grade), but less so than in wild-type mice. Of note, one Nrf2-null mouse pretreated with vehicle and challenged with acetaminophen died before completion of the study (8 h).

3.5. MT mRNA expression in oleanolic acid-treated wild-type and Nrf2-null mice

Because oleanolic acid produced some protection from acetaminophen hepatotoxicity in Nrf2-null mice, mRNA expression of MT, a Nrf2-independent gene [44] that has been shown to protect against acetaminophen hepatotoxicity [45], was quantified in oleanolic acid-treated wild-type and Nrf2-

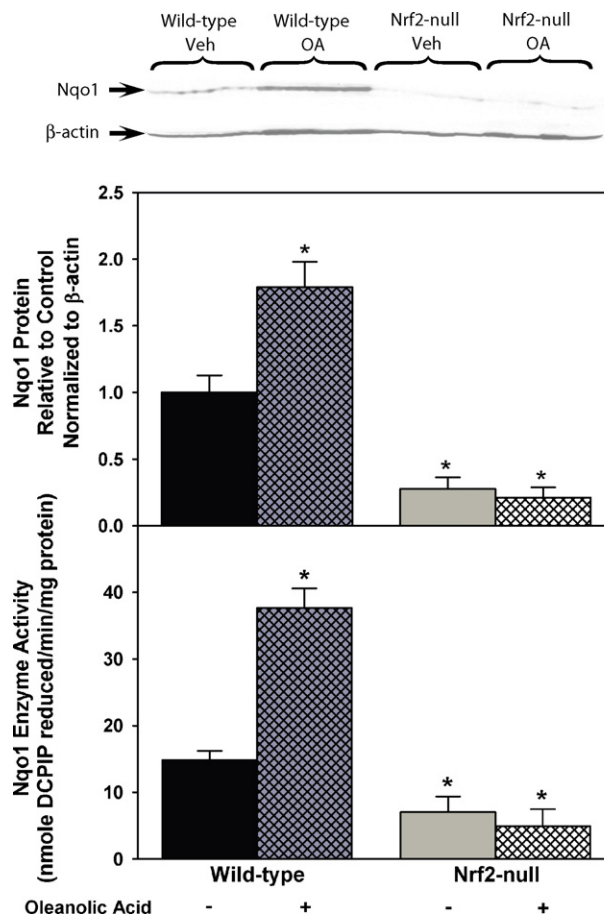


Fig. 3 – Upper: Protein expression of Nqo1 determined by Western blot in livers of wild-type and Nrf2-null mice after treatment with oleanolic acid (90 mg/kg, i.p.) once daily for 3 days. Also shown is the quantification of specific band intensity, normalized to β -actin, and expressed relative to control as mean \pm S.E.M. Lower: Nqo1 enzyme activity in livers of wild-type and Nrf2-null mice after treatment with oleanolic acid (90 mg/kg, i.p.) once daily for 3 days. Units are in nmole DCPIP reduced per min per mg of protein expressed as mean \pm S.E.M. Abbreviations: Veh, vehicle; OA, oleanolic acid. Asterisk (*) indicates a statistically significant difference from wild-type mice receiving vehicle ($p \leq 0.05$).

null mice (Fig. 5). It has been hypothesized that the high sulfhydryl content of MT can scavenge the electrophiles and free radicals formed during the pathogenesis of acetaminophen hepatotoxicity [45]. Indeed, oleanolic acid induced hepatic MT mRNA expression in both wild-type (31-fold) and Nrf2-null mice (22-fold).

4. Discussion

Because it has previously been shown that oleanolic acid (90 mg/kg, i.p.) given once daily for 3 days is protective against various hepatotoxins, which cause toxicity, in part, through oxidative or electrophilic stress mechanisms [34],

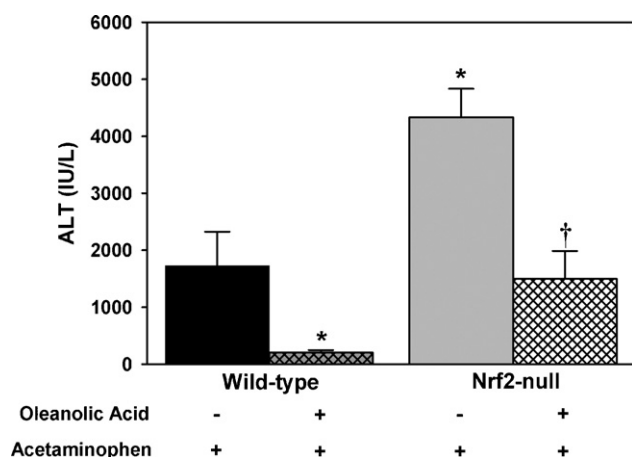


Fig. 4 – Serum alanine transaminase (ALT) levels in wild-type and Nrf2-null mice pretreated with oleanolic acid (90 mg/kg, i.p.) once daily for 3 days ($n = 5$ for all groups except Nrf2-null mice receiving vehicle, $n = 4$), administered acetaminophen (500 mg/kg, i.p.) on the fourth day, and sacrificed 8-h later. Units are in International Units/Liter (IU/L) expressed as mean \pm S.E.M. A minus sign (–) represents that the animal was administered the appropriate vehicle for the compound specified. A plus sign (+) represents that the animal was administered the compound specified. Asterisk (*) indicates a statistically significant difference from wild-type mice receiving vehicle ($p \leq 0.05$). Dagger (†) indicates a statistically significant difference from Nrf2-null mice receiving vehicle ($p \leq 0.05$).

the present studies were undertaken to establish whether oleanolic acid is an activator of the Nrf2–Keap1 pathway. Oleanolic acid (90 mg/kg, i.p.), given once daily for 3 days, increases nuclear accumulation of Nrf2 in wild-type, but not in Nrf2-null mice, as determined by both Western blot of nuclear fractions and immunofluorescence. Also, the oleanolic acid-dosing regimen used in the current studies, increases mRNA of Nrf2, as well as three characteristic Nrf2-target genes (Nqo1, Gclc, and Ho-1) in wild-type, but not in Nrf2-null mice.

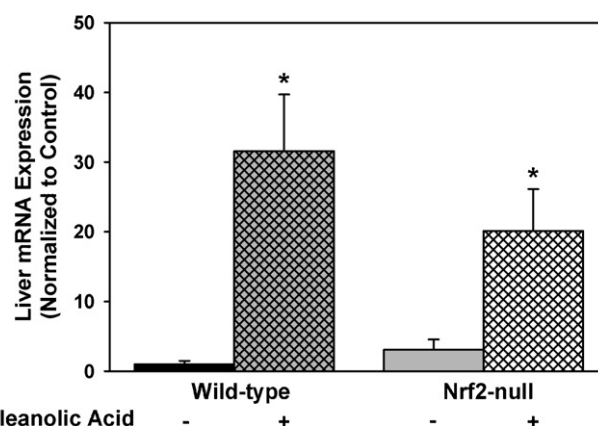


Fig. 5 – Messenger RNA expression of MT from livers of wild-type and Nrf2-null mice after treatment with oleanolic acid (90 mg/kg, i.p.) once daily for 3 days. Messenger RNA was quantified by the bDNA assay. Data is expressed relative to wild-type controls as mean \pm S.E.M. Asterisk (*) indicates a statistically significant difference from wild-type mice receiving vehicle ($p \leq 0.05$).

In addition to mRNA expression, Nqo1 protein expression and activity were quantified because Nqo1 is a prototypical Nrf2-target gene [40–43]. Oleanolic acid increased protein expression of Nqo1 in wild-type mice (Fig. 4). As expected, Nrf2-null mice have lower Nqo1 protein expression than their wild-type counterparts, as reported previously [46]. Oleanolic acid was not able to increase Nqo1 protein expression in Nrf2-null mice, which correlates with the mRNA expression data. In addition, oleanolic acid increased Nqo1 enzyme activity in wild-type, but not Nrf2-null mice.

The functional involvement of Nrf2 in oleanolic acid-mediated hepatoprotection was investigated using acetaminophen as a model hepatotoxicant. Acetaminophen produced liver injury in both genotypes, but the injury was more severe in Nrf2-null mice. Enhanced susceptibility of Nrf2-null mice to acetaminophen is consistent with previous reports [20]. Oleanolic acid-pretreatment of wild-type mice

Table 2 – Histological analysis of livers from vehicle- and OA-pretreated wild-type and Nrf2-null mice after AA challenge.

Treatment group	Histological grade						$p \leq 0.05$
	0	1	2	3	4	5	
Wild-type VC-AA	0	0	0	2	3	0	
Wild-type OA-AA	0	3	2	0	0	0	*
Nrf2-null VC-AA	0	0	0	0	0	5	*
Nrf2-null OA-AA	0	0	0	1	4	0	†

Grade of liver injury in wild-type and Nrf2-null mice pretreated with OA (90 mg/kg, i.p.) once daily for 3 days ($n = 5$ for all groups except Nrf2-null mice receiving vehicle, $n = 4$), administered acetaminophen (500 mg/kg, i.p.) on the fourth day, and sacrificed 8-h later. Wild-type and Nrf2-null mice administered OA or vehicle only did not have any histological abnormalities. Liver sections were analyzed at low power (100 \times) with six scores of severity: 0, none; 1, minimal (>2 foci of single cell necrosis per section); 2, mild (at least five areas of focal necrosis per section); 3, moderate (at least five foci of zonal necrosis per section); 4, severe (lobular damage, with many viable lobules per section); and 5, global (severe lobular damage, few areas of viability per section). Values are expressed as mean \pm S.E.M. Abbreviations: WT, wild-type; null, Nrf2-null; VC, vehicle control; OA, oleanolic acid; AA, acetaminophen.

* Statistically significant difference from wild-type mice receiving vehicle (VC-AA) ($p \leq 0.05$).

† Statistically significant difference from Nrf2-null mice receiving vehicle (VC-AA) ($p \leq 0.05$).

decreased acetaminophen hepatotoxicity. Oleanolic acid-pretreatment also decreased acetaminophen hepatotoxicity in Nrf2-null mice, although the reduction was less than in wild-type mice.

Because oleanolic acid also had some hepatoprotective effects against acetaminophen toxicity in Nrf2-null mice, the possibility that oleanolic acid might induce MT in Nrf2-null mice and produce protection by this mechanism was evaluated. MT is a low-molecular-weight, cysteine-rich, metal-binding protein that is suggested to be capable of trapping reactive oxygen and nitrogen species, as well as electrophiles [47], which all contribute to the pathogenesis of acetaminophen hepatotoxicity [48]. MT has at least one ARE in its promoter region and is induced by Nrf1 (a transcription factor in the same family as Nrf2), but not Nrf2 [44]. Oleanolic acid induces MT in both mice and rats [29], and oleanolic acid-mediated MT induction protects against cadmium toxicity [49]. In addition, MT-null mice are more susceptible than wild-type mice to acetaminophen-mediated hepatotoxicity [49]. Oleanolic acid induced hepatic MT mRNA expression in both wild-type and Nrf2-null mice (Fig. 5), which suggests that that other mechanisms, especially MT induction, in addition to Nrf2 activation might contribute to protection from oleanolic acid-mediated hepatoprotection from acetaminophen.

Other genes that may contribute to Nrf2-independent hepatoprotection by oleanolic acid include a 70 kDa heat shock protein (Hsp70) and the G protein-coupled bile acid receptor 1 (Gpbar1 or Tgr5). Hsp70 mRNA is induced by oleanolic acid [29] and acts to protect cells by reducing the ability of oxidized proteins to aggregate, permitting them time to refold and return to a functional conformation. Furthermore, Hsp70-null mice are more sensitive to acetaminophen hepatotoxicity [50]. Tgr5 mRNA is also induced by oleanolic acid [51] and is a bile acid responsive and cell surface G protein-coupled receptor that when activated suppresses macrophage proinflammatory cytokine production, particularly interleukin-1 α (IL-1 α), IL-1 β , and tumor necrosis factor α (TNF α) [52,53]. Induction of IL-1 and TNF α lead to induction of inducible nitric oxide synthase (iNos) [54,55]. iNos contributes to acetaminophen hepatotoxicity by leading to increased production of nitric oxide and subsequent peroxynitrite formation. Thus, iNos suppression, possibly mediated by oleanolic acid, may contribute to the observed hepatoprotection from acetaminophen in Nrf2-null mice [5,56]. Thus, oleanolic acid has Nrf2-dependent and Nrf2-independent effects, both of which likely contribute to oleanolic acid-mediated hepatoprotection from acetaminophen.

Collectively, increased nuclear accumulation of Nrf2, increased mRNA expression of Nrf2-target genes, and up-regulation of Nqo1 protein and activity provide strong evidence that oleanolic acid activates the Nrf2-Keap1 pathway. In addition oleanolic acid protected from acetaminophen hepatotoxicity in wild-type but less so in Nrf2-null mice, suggesting that oleanolic acid also activates Nrf2-independent cytoprotective mechanisms. Thus, this study establishes oleanolic acid as a Nrf2 activator; however, Nrf2-independent mechanisms that contribute to oleanolic acid-mediated hepatoprotection from acetaminophen require further study.

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