Nrf2 is a transcription factor that regulates important antioxidant and phase II detoxification genes. Arachidonic acid (AA) causes CYP2E1-dependent toxicity in HepG2 cells. The ability of Nrf2 to protect against CYP2E1-dependent AA toxicity and its possible mechanism was evaluated. AA activates Nrf2 in CYP2E1-expressing HepG2 cells (E47 cells), increasing Nrf2 protein and mRNA levels, Nrf2 nuclear translocation, and Nrf2-ARE binding activity. These increases in Nrf2 are associated with elevated expression of Nrf2-regulated antioxidant genes. Overexpression of Nrf2 by transient transfection of plasmid Nrf2 confers resistance of E47 cells against AA toxicity. Blocking Nrf2 with SiRNA-Nrf2 potentiates the CYP2E1-dependent AA toxicity. This enhanced toxicity is accompanied by decreases of cellular GSH levels and increases in production of reactive oxygen species and lipid peroxidation. There is also a potentiation of mitochondrial damage in the presence of Si-RNA-Nrf2. The protective effects of Nrf2 against CYP2E1-dependent toxicity can be blocked by L-buthionine-(S,R)-sulfoximine (BSO), a specific inhibitor of glutamate cysteine ligase (GCL) which is a rate-limiting enzyme in the synthesis of GSH and is regulated by Nrf2. Elevation of GSH by supplementing with GSE can partially reverse the enhanced AA toxicity by SiRNA-Nrf2. Moreover, in contrast to AA, BSO toxicity is not prevented by plasmid Nrf2 probably because protective GSH can not be synthesized. Together, these results suggest that Nrf2, probably through up-regulation of GCL and increase of GSH levels, protects against CYP2E1-dependent AA toxicity.

Polyunsaturated fatty acids (PUFA) such as arachidonic acid (AA) or its metabolites play an important role in a variety of biological processes, such as signal transduction, chemotaxis, and cell proliferation and differentiation (1-3). PUFA also play an important role in alcoholic liver injury (4-6). In the intragastric infusion model of ethanol feeding, liver injury occurs when the rats consume diets containing polyunsaturated fatty acid but not saturated fatty acid (7-8). This model is associated with induction of high levels of CYP2E1 and greatly increased lipid peroxidation, which appear to contribute to the liver injury. AA induced toxicity in HepG2 E47 cells, a cell line that expresses CYP2E1 but not control HepG2 cells, which do not express CYP2E1 (9). AA also induced toxicity in pyrazole-induced rat hepatocytes with high levels of CYP2E1 but not saline control hepatocytes (10). This CYP2E1-dependent AA toxicity was prevented by inhibitors of CYP2E1 and by antioxidants (9-10).

Several genes encoding detoxifying and antioxidative stress enzymes are coordinately induced on exposure to
Nrf2 protection against CYP2E1-dependent AA toxicity

electrophiles and reactive oxygen species (ROS) (11-12). This coordinated response is regulated through a cis-acting element called the antioxidant-responsive element (ARE) within the regulatory region of target genes (13-14). Genes encoding a subset of drug metabolizing enzymes, such as glutathione-S-transferases (GSTs) (13) and NAD(P)H-quinone oxidoreductase 1 (NQO1) (14) have been shown to be under ARE regulation, along with a subset of antioxidant genes, such as heme oxygenase 1 (HO-1) (15) and glutamate cysteine ligase (GCL) (16). The signaling system leading to ARE activation has been partly elucidated, and nuclear factor erythroid 2-related factor 2 (Nrf2) is identified as the key transcriptional factor that transmits the inducer signal to ARE (17). Nrf2 is normally sequestered in the cytoplasm by Kelch-like ECH-associated protein 1 (Keap1) (18). After activation it dissociates from Keap1 and translocates into the nucleus where it complexes with other nuclear factors and binds to ARE, activates transcription of many antioxidant genes and phase II detoxification genes which have ARE elements in their promoter regions (17). Nrf2 is constitutively and ubiquitously expressed in a number of tissues and cell lines and is responsible for the low-level expression of its target genes observed under physiological conditions. However, in cells exposed to oxidative stress, Nrf2 activity is increased, further driving the transcriptional activation of genes whose expression is essential to protect cells against loss of viability (19).

The goal of the current study was to evaluate whether Nrf2 may offer protection against AA plus CYP2E1-dependent toxicity, and if so, to assess possible mechanisms involved in the protection. Nrf2 levels in E47 cells were enhanced by transfection with plasmid expressing Nrf2 or decreased by SiRNA-Nrf2 treatment. The consequences of modulating cellular Nrf2 levels on AA-induced oxidative stress and E47 cell viability were determined.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection Experiments - HepG2 cells which constitutively express CYP2E1 (E47 cells) or control HepG2 cells (C34 cells) which have undetectable P450 activity (20) were cultured in minimal essential medium (MEM) containing 10% fetal bovine serum (FBS) and 0.5 mg/ml of G418 supplemented with 100 units/ml of penicillin and 100 µg/ml of streptomycin and 2 mM L-glutamine in a humidified atmosphere in 5% CO₂ at 37°C. Cells were plated and incubated in MEM overnight, the culture medium was replaced with fresh medium and the different treatments were initiated. SiRNA-Nrf2 and SiRNA-Control (a non-targeting SiRNA) (Santa Cruz Biotech) were transfected using the SiRNA transfection regeant according to the producer’s protocol. Plasmid pEF/Nrf2 and pEF (21) were transfected with Lipofectamine 2000 reagent (Invitrogen).

General Methodology - Cell viability was measured by the MTT assay (20). ROS were determined by flow cytometry with DCF-DA (22). Lipid peroxidation was determined by measurement of the concentration of thiobarbituric acid reactive species (TBARS) in cell lysates (23). GSH was determined as described previously (24). Mitochondrial membrane potential was analyzed by flow cytometry after double staining with 5 µg/ml rhodamine 123 (Rh123) and 5 µg/ml of propidium iodide (PI) (25). C57BL6 Nrf2(-/-) mice were obtained from Dr. Masayuki Yamamoto (University of Tsukuba) and wild type C57BL6 mice were purchased from Charles River Breeding Laboratories (Boston, MA). Activity of serum alanine aminotransferase (ALT) was assayed using diagnostic kits (Thermo Electron, Louisville, CO).

Western Blotting - Nrf2, GCLC, HO-1, and β-actin proteins were detected by Western blotting (26). Sample proteins from whole cell lysates (50 µg), cytoplasm (100 µg) or nuclear extracts (25 µg) were loaded on a 12% denaturing polyacrylamide gel and electroblotted onto 0.2 µm nitrocellulose membranes. Protein concentration was
determined using the Protein DC-20 Assay Kit (Bio-Rad). Protein immunoblot analysis was carried out using the following: anti-human HO-1 (1:5000) monoclonal antibody (StressGen Biotech); anti-human Nrf2 (1:5000) polyclonal antibody (Santa Cruz); anti-human GCLC (1:5000) polyclonal antibody (Lab Vision Corp.); and anti-human β-actin (1:10000) monoclonal antibody (Sigma) as primary antibodies, and horseradish peroxidase-conjugated goat anti-mouse IgG (1:4000) or goat anti-rabbit IgG (1:10000) (Sigma) as secondary antibody. Blots were developed using the enhanced chemiluminescence immunoblot-detecting reagent (Amersham).

**Northern Blotting** - Total RNA was isolated using the TRIzol reagent (Life Technologies). 10 µg of RNA were electrophoresed under denaturing conditions in 0.9% agarose/formaldehyde gels, transferred onto nylon membranes, and hybridized to random-primed 32P-labeled Nrf2, GCLC, HO-1, or GAPDH cDNA probes (22). The mouse Nrf2 cDNA was produced by digesting the plasmid pEF/Nrf2 (21) with Not1 and Bgl II. The human HO-1 cDNA was produced by digesting the plasmid pHHO1 (22) with Xho1 and Xba1. The human GCLC cDNA, and the human GAPDH cDNA were purchased from American Type Culture Collection.

**Electrophoretic Mobility Shift Assay (EMSA)** - Crude nuclear extracts were prepared as described previously (27). The standard binding reaction mixture (12.5 µl) contained 18 mM Hepes (pH 7.9), 80 mM KCl, 2 mM MgCl2, 10 mM DTT, 10% glycerol, 0.2 mg/ml bovine serum albumin, 160 µg/ml poly(dI-dC), 20,000 cpm [γ-32P]ATP-labeled probe, and 2 µg of nuclear extract. Reaction mixtures were incubated at 25 °C for 20 min and analyzed by native 5% polyacrylamide gel electrophoresis and autoradiography as described previously (28). A double-stranded oligonucleotide corresponding to a GCLC ARE (5'-GGCGCGCACCAGCTCCGACTCAGCGCTTTGTGCCG-3', core ARE sequence underlined) was used as probe. In supershift assays, 1 µl of preimmune IgG or anti-Nrf2 antibody was added to the reaction mixture and incubated for 20 min at room temperature prior to electrophoresis.

**Statistics** - Student’s t-test for unpaired data was used to evaluate the differences between the compared groups.

**RESULTS**

**SiRNA-Nrf2 potentiates the toxicity caused by AA in E47 cells** - The ability of AA and 1-buthionine-(S,R)-sulfoximine (BSO) (Sigma) to cause CYP2E1-dependent toxicities has been documented in previous studies (29). To study whether Nrf2 protects HepG2 cells from CYP2E1-dependent toxicity, HepG2 cells which express CYP2E1 (E47 cells) and HepG2 cells which do not express CYP2E1 (C34 cells) were used. AA and BSO cause toxicity in E47 cells but not in C34 cells (29). Blocking Nrf2 by SiRNA-Nrf2 alone did not cause toxicity in C34 cells (Fig. 1A), but did cause a mild toxicity in E47 cells (Fig. 1B) (cell viability 80±7.0%, p<0.05). AA and BSO treatment did not affect cell viability of C34 cells transfected with SiRNA-Control (Fig. 1A), but caused moderate toxicity in E47 cells transfected with SiRNA-Control (Fig. 1B). Blocking Nrf2 by SiRNA-Nrf2 did not significantly affect cell viability of C34 cells treated with AA, but decreased cell viability of C34 cells treated with BSO (Fig. 1A). SiRNA-Nrf2 dramatically enhanced the toxicity of AA and BSO in E47 cells, as cell viability decreased from 62.5±8.5% to 10.7±1.2% and from 63.5±6.8% to 7.9±0.9%, respectively (Fig. 1B). Morphological changes of E47 cells after treatment of AA are shown in Fig. 2. After AA treatment, most of the E47 cells transfected with SiRNA-Nrf2 were swollen or dead and floated to the top (arrow). In comparison only a few E47 cells transfected with SiRNA-Control and treated with AA were swollen (arrow) (Fig. 2).

**SiRNA-Nrf2 enhances the decrease of GSH and increase of ROS and lipid peroxidation of E47 cells treated with AA** - To study whether the enhanced AA toxicity produced by SiRNA-Nrf2 involved...
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oxidative stress, the effects of SiRNA-Nrf2 on the cellular GSH level, ROS production and lipid peroxidation after AA treatment were assayed. E47 cells transfected with SiRNA-Nrf2 or SiRNA-control were incubated with or without 50 µM AA for 24 h. After incubation, cells were collected and GSH, ROS and TBARS were determined. AA treatment decreased the GSH level (Fig. 3A) of E47 cells transfected with SiRNA-Control and increased the levels of ROS (Fig. 3B) and TBARS (Fig. 3C). Blocking Nrf2 with SiRNA-Nrf2 significantly enhanced these changes, as ROS level further increased from 170±18 to 325±29 arbitrary unit/mg protein, TBARS increased from 1.5±0.2 to 3.2±0.4 nmol/mg protein, and GSH levels further declined from 6.5±0.7 to 3.2±0.3 nmols/mg protein (Fig. 3A, 3B, 3C). Even in the absence of AA, the SiRNA-Nrf2 produced a decrease in cellular GSH and modest increases in ROS production and TBARS formation.

**SiRNA-Nrf2 enhances the decline of mitochondrial potential caused by AA in E47 cells** - Damage to the mitochondria is a key target in the AA plus CYP2E1-dependent toxicity (25, 30). Mitochondrial membrane potential was assayed by flow cytometry after double staining with rhodamine 123 (Rh123) and propidium iodide (PI). Rh123 uptake into the mitochondria is proportional to the mitochondrial membrane potential. PI diffuses into the cells and binds to cellular DNA when the integrity of the plasma membrane is lost. As shown in Fig. 4, most of the E47 cells transfected with SiRNA control appear on the low PI and high Rh123 -fluorescence field (lower right quadrant), indicative of intact, viable cells with high mitochondrial membrane potential. AA treatment decreased the mitochondrial membrane potential, the number of cells in the lower left quadrant (M1 zone) increased from 3.82±0.28% to 15.82±2.67%). The largest decline in MMP was found in E47 cells transfected with SiRNA-Nrf2 and treated with AA as the number of cells in the M1 zone increased to 38.82±3.86%, p<0.01 (Fig. 4).

**AA treatment increases Nrf2 nuclear translocation and ARE-binding activity** - To study whether AA treatment can activate Nrf2, we examined the Nrf2 protein nuclear translocation and Nrf2-ARE binding activity in E47 cells. Figure 5A shows that the level of Nrf2 protein in the nuclei of E47 cells was significantly increased while Nrf2 protein in the cytoplasm was significantly decreased after AA treatment, suggesting the translocation of Nrf2 from the cytoplasm into the nucleus. To determine whether Nrf2 binding activity to the ARE was affected by AA, nuclear extracts of E47 cells were prepared and Nrf2 binding activity was determined by EMSA with a double-strand DNA containing the ARE sequence as probe. ARE-binding activity was increased after AA treatment compared with untreated control E47 cells. Nrf2 antibody can supershift this complex (Fig. 5B), indicating that it contains Nrf2. Together, these results suggest that Nrf2 is activated by AA treatment in E47 cells.

**SiRNA-Nrf2 blocks the antioxidant gene (GCLC and HO-1) response caused by AA in E47 cells** - Glutamate cysteine ligase (GCL) and heme oxygenase-1 (HO-1) are important antioxidant genes regulated by Nrf2 (15-16). To determine possible changes of these genes after AA treatment, both mRNA and protein expression of Nrf2, HO-1 and the heavy subunit of GCL (GCLC) were studied. E47 cells were grown overnight, the medium changed, and cells were further incubated with or without 50 µM AA for 24 h. Cells were collected and Nrf2, GCLC, HO-1, and β-actin proteins were determined by Western blot and Nrf2, GCLC, HO-1, and GAPDH mRNA levels were detected by Northern blot. In the untreated E47 cells, SiRNA-Nrf2 decreased Nrf2, GCLC and HO-1 basal mRNA and protein expression levels (Fig. 6A, 6B), validating the effectiveness of the SiRNA to decrease Nrf2 and its antioxidant responsive
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genes. AA treatment increased the Nrf2 mRNA and protein level, as well as GCLC and HO-1 mRNA and protein levels in E47 cells transfected with SiRNA-Control. SiRNA-Nrf2 blocked this response of E47 cells to AA treatment, as Nrf2, GCLC and HO-1 mRNA and protein levels in E47 cells transfected with SiRNA-Nrf2 were all lower than E47 cells transfected with SiRNA-Control in response to AA treatment (Fig. 6A, 6B). We believe that the increase in Nrf2 protein levels reflects an adaption by the E47 cells to the increased oxidative stress produced by AA in the E47 cells. If so, antioxidants should prevent the AA-induced increase in Nrf2 protein levels. Fig.6C shows that treatment with 50 µM α-tocopherol, or 2 mM glutathione ethyl ester, or 5 mM N-acetylcysteine indeed lower the AA-induced levels of Nrf2 protein to the basal levels found in the absence of AA.

Overexpression of Nrf2 confers E47 cell resistance to AA toxicity - To confirm the protective effects of Nrf2 against AA toxicity in E47 cells, E47 cells were transiently transfected with pEF/Nrf2 or an empty plasmid vector pEF as control. Twenty-four hours after transfection, cells were incubated with or without 50 µM AA for 48 h. Cell viability was determined by the MTT assay. E47 cells transfected with pEF/Nrf2 had higher levels of Nrf2 as well as GCLC and HO-1 protein expression compared with E47 cell transfected with the control vector plasmid (Fig. 7A). Cell viability of E47 cells transfected with pEF was decreased about 50% by AA treatment and 75% by treatment with BSO. Overexpressing Nrf2 by pEF/Nrf2 transfection protected E47 cells against AA toxicity, as cell viability increased from 50% to about 80% (Fig. 7B). However, BSO toxicity was not prevented by pEF/Nrf2 (Fig. 7B).

The GCL inhibitor BSO blocks the protective effects of overexpression of Nrf2 on AA toxicity - Many phase II enzymes such as NQO1 and GST, and antioxidant genes like GCL and HO-1 are regulated by Nrf2 (13-16). To evaluate which Nrf2-dependent genes play an important role in the protection against AA toxicity, specific inhibitors of GCL (BSO), HO-1 (chromium mesoporphyrin, CrMP, Porphyrin Products Inc), NQO1 (dicumarol, DI, Sigma), and GST (cibacron blue, CB, Biovision) were used to block the activity of these enzymes. E47 cells (5×10⁴ cells/well) were grown on 24-well plates overnight. After changing the medium, cells were transiently transfected with plasmid pEF or pEF/Nrf2. Twenty-four hours after transfection, cells were further incubated with 50 µM AA for 24 h in the absence or presence of either BSO (100 µM), CrMP (20 µM), DI (50 µM), or CB (5µM) which were added into the culture medium 1 h before addition of AA. Only the GCL inhibitor BSO blocked the protective effects of overexpression of Nrf2 against AA toxicity, suggesting that GCL plays a key role in this protection (Fig. 8). The HO-1 inhibitor CrMP, NQO1 inhibitor DI, and GST inhibitor CB did not prevent the protection by Nrf2 (Fig. 8), suggesting that HO-1, NQO1, and GST may be not important in mediating the Nrf2 protection. In the pEF transfected cells, BSO and CrMP but not DI or CB enhanced the toxicity of AA (Fig. 8).

SiRNA-Nrf2 enhanced AA toxicity can be reversed by supplementation of GSH through GSSE - GCL is the rate-limiting enzyme in the synthesis of GSH. To further prove the protective effects of GCL and GSH, exogenous GSH was supplied by addition of 2 mM GSSE to the culture medium. E47 cells (5×10⁴ cells/well) were grown on 24-well plates overnight. After changing the medium, cells were transiently transfected with SiRNA-control and SiRNA-Nrf2. Thirty hours after transfection, cells were further incubated with or without 50 µM AA for 24 h. GSSE was added 1 h before addition of AA. Cell viability was determined by a MTT assay. The results showed that the toxicity of SiRNA-Nrf2 plus AA was partially reversed by addition of GSSE (Fig. 9). The small toxicity of SiRNA-Nrf2 alone in E47 cells was also abolished by addition of GSSE (Fig. 9).

Induction of CYP2E1 by pyrazole increases serum ALT level in Nrf2 knock-out
mice – To induce liver CYP2E1 levels, Nrf2(-/-) and wild type Nrf2(+/-) mice (18-22g) were injected with pyrazole (i.p., 150 mg/kg) once a day for two days. After treatment blood samples were collected and serum ALT activity was determined. Induction of CYP2E1 by pyrazole in Nrf2(-/-) mice caused a significant increase of serum ALT levels, however, the pyrazole treatment had no effect in wild type Nrf2(+/-) mice (Fig. 10).

DISCUSSION

Nrf2 levels were previously found to be increased in livers from rats chronically fed with ethanol and rats treated with pyrazole, an inducer of CYP2E1 (26). The functional significance of this increase is not clear. CYP2E1-dependent AA toxicity in E47 cells has been characterized in previous studies (29). In this study we found that the transcription factor Nrf2 is important in protecting E47 cells against AA toxicity. Overexpression of Nrf2 confers resistance of E47 cells to AA toxicity. Blocking Nrf2 by SiRNA-Nrf2 significantly increases the AA toxicity to E47 cells. Blocking Nrf2 enhances the decrease of the cellular GSH level and increases the production of ROS and lipid peroxidation in E47 cells after AA treatment. These changes caused by SiRNA-Nrf2 further enhance the mitochondrial damage caused by AA, which leads to the dramatic increase in cell death.

Nrf2 is activated by AA in E47 cells. After AA treatment, total levels of Nrf2 protein and mRNA are increased. The Nrf2 protein in the cytoplasm is translocated into the nucleus and Nrf2-ARE binding activity is significantly increased. More important, Nrf2 regulated genes HO-1 and GCLC are up-regulated by AA. Activation of Nrf2 by AA may be an adaptive response to the increased oxidative stress caused by AA in E47 cells since AA treatment cause significant increases of ROS and lipid peroxidation in these cells. Moreover, treatment with antioxidants lowers these elevated levels of Nrf2.

The protective effects of Nrf2 against CYP2E1-dependent AA toxicity may be mediated by the Nrf2-dependent antioxidant genes. The antioxidant genes GCL and HO-1, and phase II enzymes NQO1 and GST are all regulated by Nrf2 (13-16). GCLC and HO-1 gene expressions are increased by AA treatment in E47 cells. The up-regulation of these antioxidant genes by AA are Nrf2 dependent since overexpression of Nrf2 increases GCLC and HO-1 expression, and blocking Nrf2 with SiRNA-Nrf2 also blocks the increased expression of these genes by AA. E47 cells overexpressing Nrf2 are more resistant to AA toxicity. This protection of Nrf2 is blocked only by BSO, the specific inhibitor of GCL, suggesting that GCL plays an important role in mediating the protection by Nrf2. Although the antioxidant gene HO-1 has been shown to be protective against CYP2E1-dependent toxicity (30), the specific inhibitor of HO-1, CrMP, does not significantly affect the protection by Nrf2, suggesting that HO-1 may be not important in mediating the protection effects of Nrf2 against CYP2E1-dependent AA toxicity. Phase II enzymes NQO1 and GST do not play an important role in mediating the protective action of Nrf2 against AA, since specific inhibitors of these enzymes did not show any effect on the protective action of Nrf2 against AA toxicity.

GSH may play a key role in the protective effects of Nrf2 against AA toxicity in E47 cells. BSO, the specific inhibitor of GCL, reduces the cellular GSH level. Overexpression of Nrf2 protects E47 cells against AA but not BSO toxicity. GSH can not be synthesized in the presence of BSO. Moreover, this protection of Nrf2 against AA toxicity is blocked by BSO, an inhibitor of GCL. GCL is the rate-limiting enzyme in the synthesis of GSH. Increasing GSH by addition of GSSE reversed the toxicity of SiRNA-Nrf2 itself in E47 cells, as well as the enhanced AA toxicity by SiRNA-Nrf2. Together, these results suggest that GSH level may play a key role in the protective effect of Nrf2 against CYP2E1-dependent AA toxicity. By analogy, activation of Nrf2 and increased production...
of GSH may be important in protecting against the toxicity by various chemicals metabolized by CYP2E1 to reactive toxic species e.g. acetaminophen, CCl4, benzene, nitrosamines (31-35) and under various pathophysiological conditions in which CYP2E1 is elevated e.g. alcohol liver disease, non-alcohol fatty liver disease, diabetes (6-7,36-38). Nrf2 null mice are sensitive to the toxicity of elevated CYP2E1 levels by pyrazole treatment, which suggests the importance of Nrf2 protection in vivo against CYP2E1-dependent toxicity.

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FOOTNOTES

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Abbreviations: AA: arachidonic acid; ALT: alanine aminotransferase; ARE: antioxidant-responsive element; BSO: l-buthionine-(S,R)-sulfoximine; CB: cibacron blue; CrMP: chromium mesoporphyrin; CYP2E1: cytochrome P450 2E1; C34 cells: HepG2 cell line established after transfection with pCI-neo; DCF-DA: 2’7’ dichlorofluorescin diacetate; DI: dicumerol; E47 cells: HepG2 cell line established after transfection with pCI-neo-CYP2E1; GCL: glutamate cysteine ligase; GCLC: the catalytic subunit of GCL; GSH: glutathione; GSSE: glutathione ethyl ester; GST: glutathione-S-transferase; HO: heme oxygenase; Keap 1: Kelch-like ECH-associated protein 1; NQO1: NAD(P)H-quinone oxidoreductase 1; Nrf2: nuclear factor erythroid 2-related factor 2; PI: propidium iodide; PNP: p-nitrophenol; PUFA: Polyunsaturated fatty acids; Rh123: rhodamine 123; ROS: reactive oxygen species. TBARS: thiobarbituric acid reactive species.

FIGURE LEGEND

Figure 1. SiRNA-Nrf2 potentiates the toxicity caused by AA and BSO in E47 cells. E47 cells (5×10^4 cells/well) were grown on 24-well plates overnight. After changing the medium, cells were transiently transfected with SiRNA-control and SiRNA-Nrf2. Thirty hours after transfection, cells were further incubated with or without 50 µM AA or 100 µM BSO for 24 h. Cell viability was determined by a MTT assay. Results are expressed as average ± S.E. n=3; *, p<0.05; **, p<0.01 compared with the control C34 or E47 cells transfected with SiRNA-Control.

Figure 2. Morphological changes of E47 cells treated with AA. E47 cells (5×10^4 cells/well) were grown on 24-well plates overnight. After changing the medium, cells were transiently transfected with SiRNA-control and SiRNA-Nrf2. Thirty hours after transfection, cells were further incubated with or without 50 µM AA for 24 h. Cell morphology was visualized under a light microscope (magnification 400×).

Figure 3. SiRNA-Nrf2 enhances the decrease of GSH and increase of ROS and lipid peroxidation of E47 cells treated with AA. E47 cells (5×10^6 cells/well) were grown on 100 mm plates overnight. After changing the medium, cells were further incubated with or without 50 µM
AA for 24 h. After this incubation, cells were collected and GSH, ROS and lipid peroxidation were determined as described in Materials and Methods. Results are expressed as average ± S.E. n=3; **, p<0.01 compared with the control E47 cells transfected with SiRNA-Control; ##, p<0.01, compared with the control E47 cells transfected with SiRNA-Nrf2.

Figure 4. **SiRNA-Nrf2 enhances the decline of mitochondrial membrane potential in E47 cells caused by AA.** E47 cells were transfected with SiRNA-Control or SiRNA-Nrf2 as described in Materials and Methods. Thirty hours after transfection, mitochondrial membrane potential was detected by flow cytometry analysis using Rh123 and PI. Data for cells with low mitochondrial membrane potential are presented in left lower part of the representative image of each group. Results are expressed as average ± S.E. n=3; **, p<0.01, compared with the control E47 cells transfected with SiRNA-Control; ##, p<0.01, compared with the AA treated E47 cells transfected with SiRNA-Control.

Figure 5. **Nuclear Nrf2 levels and Nrf2-ARE binding activity are increased by AA treatment.** (A) Nrf2 is translocated into nucleus after AA treatment. E47 cells (2×10^8 cells in 150mm dishes) were incubated with or without 50 µM AA for 0, 1, and 2 h. Nrf2 protein in the cytoplasm (100µg total protein) and nuclear extracts (15µg total protein) was detected by Western blotting and quantified. Results are expressed as arbitrary densitometry units under the Nrf2 bands and represent average ± S.E. (n=3). **, p<0.01 versus Nrf2 protein in cytoplasm or nucleus at time 0. (B) Nrf2-ARE binding activity is increased by AA treatment. E47 cells (2×10^8 cells in 150 mm dishes) were incubated with or without 50 µM AA for 2 h. Nuclear extracts were prepared and electrophoretic mobility shift assays were carried out in the absence or presence of anti-Nrf2 antibody as described in Materials and Methods.

Figure 6. **SiRNA-Nrf2 blocks the antioxidant gene (GCLC and HO-1) response caused by AA in E47 cells.** E47 cells (1×10^6 cells/well, 6-well plates) were transfected with SiRNA-Control or SiRNA-Nrf2 as described in Materials and Methods. After transfection, cells were further incubated with or without 50 µM AA for 16 h. After incubation cells were collected and Nrf2, GCLC, HO-1, and β-actin proteins were determined by Western blot (A) and Nrf2, GCLC, HO-1, and GAPDH mRNA levels were detected by Northern blot (B). The amount of Nrf2, GCLC or HO-1 protein was normalized to β-actin protein and the amount of Nrf2, GCLC, HO-1 mRNA was normalized to the GAPDH mRNA. Results are expressed as fold induction compared to that of control E47 cells transfected with SiRNA-Control. Data expressed are average ± S.E. (n=3). *, p<0.05; **, p<0.01 versus control E47 cells transfected with SiRNA-Control. ##, p< 0.01 versus AA treated E47 cells transfected with SiRNA-Control. (C) Antioxidants decrease the AA-mediated elevation in Nrf2 protein level. E47 cells were incubated with or without 50 µM α-tocopherol (Toco), or 2 mM glutathione ethyl ester (GSSE), or 5 mM N-acetylcysteine (NAC) in the presence or absence of 50 µM AA for 24 h. After treatment cells were collected and levels of Nrf2, and β-actin proteins in the whole cell lysates were determined by Western blot. Data expressed are average ± S.E. (n=3). **, p<0.01 versus E47 cells in the absence of AA; #, p<0.05, versus E47 cells in the presence of AA.

Figure 7. **Overexpression of Nrf2 confers E47 cells resistance to AA but not to BSO toxicity.** (A) Overexpression of Nrf2 increases GCLC and HO-1 proteins in E47 cells. E47 cells (1×10^6 cells/well, 6-well plates) were transfected with pEF or pEF/Nrf2 as described in Materials and Methods. After transfection, cells were collected and Nrf2, GCLC, HO-1, and β-actin proteins were determined by Western blot. The amount of Nrf2, GCLC or HO-1 protein was normalized to β-actin protein and the results are expressed as fold induction compared to that of E47 cells transfected with pEF. (B) Overexpression of Nrf2 confers E47 cells resistance to AA but not to BSO toxicity. E47 cells (5×10^4 cells/well) were grown on 24-well plates and transfected with 250
ng/well of plasmid pEF/Nrf2 or empty plasmid vector pEF for 24 h. After transfection, cells were further incubated with or without 50 µM AA or 100 µM BSO for 48 h. Cell viability was determined by a MTT assay. Data expressed are average ± S.E. (n=3). **, p<0.01 versus control E47 cells transfected with pEF. ##, p<0.01 versus AA treated E47 cells transfected with pEF.

Figure 8. **GCL inhibitor BSO blocks the protective effects of overexpression of Nrf2 on AA toxicity.** E47 cells (5×10⁴ cells/well) were grown on 24-well plates and transfected with plasmid pEF/Nrf2 or empty plasmid vector pEF for 24 h. After transfection, cells were further incubated with 50 µM AA for 48 h. BSO (100 µM), CrMP (20 µM), DI (50 µM), or CB (5 µM) were added into the culture medium 1 h before addition of AA. Cell viability was determined by a MTT assay. Data expressed are average ± S.E. (n=3). **, p<0.01 versus AA treated E47 cells transfected with pEF. ##, p<0.01 versus AA treated E47 cells transfected with pEF/Nrf2.

Figure 9. **siRNA-Nrf2 enhanced AA toxicity can be reversed by supplement of GSH through GSSE.** E47 cells (5×10⁴ cells/well) were grown on 24-well plates overnight. After changing the medium, cells were transiently transfected with SiRNA-control and SiRNA-Nrf2. Thirty hours after transfection, cells were further incubated with or without 50 µM AA for 24 h. GSSE (2 mM) was added 1 h before addition of AA. Cell viability was determined by a MTT assay. Data expressed are average ± S.E. (n=3). *, p<0.05; **, p<0.01; versus control E47 cells transfected with SiRNA-Control. ##, p<0.01 versus AA treated E47 cells transfected with SiRNA-Nrf2.

Figure 10. **Induction of CYP2E1 by pyrazole increases serum ALT level in Nrf2 knock-out mice.** Nrf2(-/-) and wild type Nrf2(+/+) mice (18-22g) were injected with pyrazole (i.p., 150 mg/kg) once a day for two days. After treatment blood samples were collected and serum ALT activity was determined. Data expressed are average ± S.E. (n=3). **, p<0.01; versus all other groups.
Fig. 1

A

![Graph showing cell viability in C34 Cells with different conditions: SiRNA-Control vs. SiRNA-Nrf2.](image)

B

![Graph showing cell viability in E47 Cells with different conditions: SiRNA-Control vs. SiRNA-Nrf2.](image)
Fig. 2

SiRNA-Control

Control

AA

SiRNA-Nrf2
Fig. 3

A

![Bar graph showing GSH levels](image)

Control AA

|          | SiRNA-Control | SiRNA-Nrf2 |
|----------|---------------|------------|
| Control  | 10            | **         |
| AA       | 5             | **         |

B

![Bar graph showing ROS levels](image)

Control AA

|          | SiRNA-Control | SiRNA-Nrf2 |
|----------|---------------|------------|
| Control  | 100           | **         |
| AA       | 400           | **/##      |

C

![Bar graph showing TBARS levels](image)

Control AA

|          | SiRNA-Control | SiRNA-Nrf2 |
|----------|---------------|------------|
| Control  | 0.5           | **         |
| AA       | 3.0           | **/##      |
Fig. 4

SiRNA-Control

Control

3.82±0.28%

AA

19.52±2.83%

SiRNA-Nrf2

15.82±2.67%

38.82±3.86%
Fig. 5

A

|       | Cytoplasm |       | Nucleus |       |
|-------|-----------|-------|---------|-------|
|       | 0         | 1     | 2       | 0     | 1     | 2       |
| Nrf2  | 1.0±0.1   | 0.3±0.1** | 0.3±0.1** | 1.0±0.1 | 1.6±0.1** | 1.7±0.1** |
| Actin |           |       |         |       |       |         |

B

| Nrf2 antibody | C  | AA |
|---------------|----|----|
| -             |    |    |
| +             |    |    |

[Image shows a western blot with bands labeled Nrf2 and Actin, and asterisks indicating statistical significance.]
Fig. 6

A

|                | SiRNA-Control |                  |                  |
|----------------|---------------|------------------|------------------|
|                | Control       | AA               | AA               |
| Nrf2           | 1.0 ± 0.1     | 1.3 ± 0.1*      | 0.5 ± 0.1**      |
| GCLC           | 1.0 ± 0.1     | 2.0 ± 0.2**     | 0.4 ± 0.1**      |
| HO-1           | 1.0 ± 0.1     | 2.5 ± 0.2**     | 0.7 ± 0.1*       |
| Actin          | 1.0 ± 0.1     | 1.0 ± 0.1       | 0.4 ± 0.1**      |

B

|                | SiRNA-Control |                  |                  |
|----------------|---------------|------------------|------------------|
|                | Control       | AA               | AA               |
| Nrf2           | 1.0 ± 0.1     | 1.5 ± 0.1*      | 0.5 ± 0.1**      |
| GCLC           | 1.0 ± 0.1     | 2.0 ± 0.2**     | 0.4 ± 0.1**      |
| HO-1           | 1.0 ± 0.1     | 2.5 ± 0.2**     | 0.5 ± 0.1**      |
| GAPDH          | 1.0 ± 0.1     | 1.0 ± 0.1       | 0.6 ± 0.1**      |

C

|                | Con | Toco | GSSE | NAC | Con | Toco | GSSE | NAC |
|----------------|-----|------|------|-----|-----|------|------|-----|
| Nrf2           | 1.00| 0.85 | 0.89 | 0.87| 1.44| 1.05 | 1.01 | 1.08|
| Actin          | 1.00| ± 0.10| ± 0.08| ± 0.09| ± 0.10| ± 0.12**| ± 0.10##| ± 0.10#|

by guest on July 10, 2020
Fig. 7

A

| Protein | pEF | pEF/Nrf2 |
|---------|-----|----------|
| Nrf2    | 1.0±0.1 | 1.5±0.1** |
| GCLC    | 1.0±0.1 | 1.5±0.1** |
| HO-1    | 1.0±0.1 | 2.0±0.1** |
| Actin   |        |          |

B

Cell viability (%)

- **pEF**
- **pEF/Nrf2**
Fig. 9

Cell viability (%) by group:

- **SiRNA-Control**
- **SiRNA-Nrf2**

Categories:
- Control
- GSSE
- AA
- GSSE+AA

Significance:
- * p-value
- ** p-value
- *** p-value
Fig. 10

**Nrf2+/+**                       **Nrf2−/−**

ALT (U/L)

Control  Pyrazole  Control  Pyrazole

Nrf2+/+  Nrf2−/−
Transcription factor NRF2 protects HEPG2 cells against CYP2E1 plus arachidonic acid-dependent toxicity
Pengfei Gong and Arthur I. Cederbaum

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