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Oxidative Stress-responsive Transcription Factor NRF2 is Not Indispensable For The Human Hepatic Flavin-Containing Monooxygenase-3 (*FMO3*) Gene Expression in HepG2 Cells

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Abstract

The flavin-containing monooxygenases (FMOs) are important for the oxidation of a variety of endogenous compounds and xenobiotics. The hepatic expression of *FMO3* is highly variable and until recently, it was thought to be uninducible. In this study, human *FMO3* gene regulation by the oxidative stress transcription factor, nuclear factor (erythroid-derived 2)-like 2 (NRF2) was examined. Constitutive *FMO3* gene expression is repressed in HepG2 cells, thus this cell can be a good model for *FMO3* gene regulation studies. Over-expression of NRF2 in HepG2 cells increased NRF2 target gene expression, heme oxygenase-1 (*HMOX1*) and NAD(P)H:quinone oxidoreductase-1 (*NQO1*), but did not alter *FMO3* gene expression. Co-transfection studies with NRF2 or its cytosolic regulatory protein, Kelch-like ECH-associated protein 1 (KEAP1), expression vectors, along with FMO3 promoter luciferase reporter constructs of various lengths (5Kb or 6Kb), did not change FMO3 reporter gene activity significantly. Furthermore, treatment with tert-butyl hydroperoxide (tBHP) and tert-butyl hydroquinone (tBHQ) did not alter FMO3 reporter construct activity. In summary, *in vitro* results suggest that the transcriptional regulation of FMO3 might not involve the NRF2-KEAP1 regulatory pathway.

Keywords

Flavin-containing monoxygenase-3; Oxidative stress; NRF2; KEAP1; tert-butyl hydroperoxide; tert-butyl hydroquinone

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Conflict of interest
The authors declare that there are no conflicts of interest.

Introduction

Flavin-containing monooxygenases (FMOs) are enzymes that catalyze oxidation of a variety of nitrogen-, sulphur- and phosphorous-containing xenobiotics to their respective oxides. In human liver, constitutive *FMO3* expression and developmental expression patterns have been extensively studied (Klick and Hines, 2007, Klick et al., 2008, Shimizu et al., 2008). Briefly, Klick and Hines (2007) demonstrated that binding of transcription factors such as nuclear factor Y (NFY), upstream stimulatory factor 1 (USF1), YYI and an unidentified GC box to the regulatory domains upstream of the transcription start site are important for regulating constitutive *FMO3* transcription (Klick and Hines, 2007). The pre-B-cell leukemia factor 2 (Pbx2) as a heterodimer with an unidentified homeobox (Hox) isoform contributes to *FMO3* developmental- and tissue-specific regulation (Klick and Hines, 2007). Another factor, CCAAT enhancer-binding protein β (C/EBPβ), binding more distal to the transcription start site, is important for the *FMO3* developmental expression pattern (Klick et al., 2008). Finally, Shimuzu et al. (2008) reported that HNF-4 and NF-Y modulates *FMO3* gene expression in the Japanese population. Because xenobiotic-induced *FMO3* gene expression was not identified, transcriptional regulation during toxicant exposure is not well characterized.

Acetaminophen (APAP)-induced hepatotoxicity results in *Fmo3* over-expression in mice (Rudraiah et al., 2014b). Additionally, we also showed that toxic alpha-naphthyl isothiocyanate (ANIT) treatment and bile duct ligation (BDL) induced *Fmo3* gene expression (Rudraiah et al., 2014a). Most hepatotoxicants that induce *Fmo3* also induce oxidative stress. Interestingly, gene expression changes of detoxification enzymes in Nrf2 knockout mice in comparison with wild-type mice by Affymetrix arrays demonstrated a significant decrease in FMO3 expression in knockout mice liver (Li et al., 2004). Furthermore, promoter analysis using MatInspector software (Genomatix, Munich, Germany) revealed two binding sites for Bach-Maf heterodimers at about 5.3 and 6 kb from the transcription start site on the human *FMO3* 6 kb promoter. Bach-Maf heterodimers function as repressors of the gene expression by binding to Maf recognition elements (MAREs) and by recruiting transcriptional co-repressors (Dohi et al., 2008). Under oxidative stress conditions, Bach1 is inhibited, allowing for the activation of hemeoxygenase-1 (Hmox1) by transcriptional activators such as nuclear factor (erythroid-derived 2)-like 2 (NRF2) (Igarashi and Watanabe-Matsui, 2014). It is plausible that NRF2 activates *FMO3* transcription via MAREs under oxidative stress conditions. Thus, the goal of the current study was to investigate whether NRF2 regulates *FMO3* gene expression.

FMO3 constitutive expression is repressed in HepG2 cells making this cell line useful for studying *FMO3* regulation using recombinant *FMO3* promoter-directed reporter constructs. Thus, all *in vitro* studies were performed in HepG2 cells. Reporter constructs containing *FMO3* promoter sequences of various lengths (5Kb or 6Kb) directing luciferase expression were used in the current study. Co-transfection studies with *FMO3* promoter luciferase reporter constructs and NRF2 or its cytosolic retainer Kelch-like ECH-associated protein 1 (KEAP1) did not significantly alter luciferase reporter gene activity. These results suggest that the transcriptional regulation of *FMO3* might not involve NRF2-KEAP1 regulatory pathway.

Materials and Methods

Chemicals

Tert-butyl hydroperoxide and tert-butyl hydroquinone were purchased from Sigma-Aldrich (St Louis, MO). All other reagents were of reagent grade or better and commercially available.

Cell Culture and Transient Transfection Reporter Gene Assay

HepG2 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 mM glucose, 10% fetal bovine serum and 1% antibiotic-antimycotic (100 units/mL penicillin G sodium, 100 μg/mL streptomycin sulfate, and 0.25 μg/mL amphotericin B) in a 5% CO_2 and humidified environment (95% relative humidity) at 37°C. Expression plasmids containing variable lengths of FMO3 promoter sequences (5905bp:pRNH970 or 4908bp:pRNH1013) directing luciferase expression were prepared using a Genome Walker Kit (BD Biosciences) as described previously (Klick and Hines, 2007). Expression plasmid p3XFLAG-myc-CMV-26 (EV26) was obtained from Sigma-Aldrich (St Louis, MO). NRF2 or KEAP1 ORF (Open Biosystems, Waltham, MA) was cloned into EV26. For transient transfection, HepG2 cells were seeded in a 12-well plate at approximately 30% confluence. The following day, transient transfection (NRF2 or KEAP1 expression plasmids) or co-transfections (*FMO3* promoter luciferase reporter constructs and transcription factor expression plasmids) were performed using Lipofectamine (Invitrogen) according to the manufacturer's manual. For tert-butyl hydroperoxide (tBHP) and tert-butyl hydroquinone (tBHQ) treatment, culture media (complete DMEM) was replaced with media containing 100 μM tBHP or 100 μM tBHQ 6 h after transfection. For 5-aza-2'-deoxycytidine (AZA) treatment, HepG2 cells were treated with AZA (Sigma–Aldrich, St. Louis, MO) for 48 h with daily media/drug replacement. Cells were harvested for RNA isolation or to determine luciferase activity using the luciferase assay system (Promega) according to the manufacturer's protocol. If not otherwise indicated, the amount of lipofectamine used was 2.5 μL/well, the amount of transfected plasmids was 0.3 μg/well for transcription factors and 0.5 μg/well for reporter genes.

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

TRIzol reagent (Life Technologies, Carlsbad, CA) was used to extract total RNA from cells transiently transfected with expression plasmids containing NRF2 or KEAP1. cDNA was prepared using the M-MLV RT kit (Invitrogen, Carlsbad, CA). NRF2, KEAP1, HMOX1, NQO1 and FMO3 mRNA expression was quantified by the ΔΔCT method and normalized to the housekeeping gene, β-actin. Primer pairs were synthesized by Integrated DNA Technologies (Coralville, IA) and are presented in Table 1. Amplification was performed using an Applied Biosystems 7500 Fast Real-Time PCR System. Amplification was carried out in a 20 μL reaction volume containing 8 μL diluted cDNA, Fast SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and 1 μM of each primer.

Isolation of Nuclear Fraction from HepG2 Cells

HepG2 cells were plated at 8×10^5 cells per dish in a 60 mm dish overnight. The following day, cells were treated with 100 μM tBHP in serum free DMEM for 2 h. After 2 h, cells were removed from the incubator, scraped and collected in 1 mL cold PBS and transferred into microcentrifuge tubes. The cell suspension was then centrifuged at 12000 g for 1 min. Cell pellets were resuspended in 400 μL cold hypotonic buffer [10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT and 0.2 mM phenylmethylsulfonylfluoride (PMSF)] and incubated for 20 min on ice, vortexed for 10s and then centrifuged at 1000 g for 10 min at 4°C. The resulting pellet was again resuspended in hypertonic buffer (20 mM HEPES, pH 7.9, 1.5 mM MgCl2, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT, 25% glycerol and 0.2 mM PMSF) and incubated on ice for 20 min for high-salt extraction. Samples were vortexed for 10s and centrifuged at 1000 g for 10 min. The supernatant fraction containing nuclear proteins was measured for protein concentration.

Western Blot Analysis

For western blot analysis of NRF2, 40 μg nuclear protein was mixed with Laemmli's buffer, incubated at 90°C for 10 min, and electrophoretically resolved using 10% SDS polyacrylamide gels and transblotted onto PVDF-Plus membrane (Micron Separations, Westboro, MA). A custom rabbit anti-Human NRF2 primary antibody (Cell Signaling Technology, Inc. Danvers, MA) was used to detect NRF2 with TFIIB as a loading control. Blots were then incubated with HRP conjugated secondary antibodies against rabbit IgG (Sigma-Aldrich, St Louis, MO). Protein-antibody complexes were detected using a chemiluminescent kit (Thermo Scientific, IL) with visualization using GeneMate blue autoradiography film (Bioexpress, Kaysville, UT).

LDH Leakage

Percent lactate dehydrogenase (LDH) leakage was determined via the Tox-7 kit (Sigma-Aldrich, St. Louis, MO) as a measure of cytotoxicity following tBHP treatment. The assay was performed according to manufacturer's instructions.

DCFDA - Cellular Reactive Oxygen Species Detection Assay

Cellular ROS levels in HepG2 cells were measured as described previously (Lee et al., 2014). Briefly, HepG2 cells were plated in a black 24-well plate (Wallac Oy, Turku, Finland) at 90% confluence. Cells were incubated with 100 μM tBHP for 2, 24 and 48 h. At appropriate timepoints, cells were further incubated with 5 μM dichlorofluorescein (Sigma, St. Louis, MO) for 30 min and fluorescence was read at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The data are expressed as relative fluorescent units (RFU) per μg of cell protein.

Lipid Hydroperoxide (LPO)

Lipid peroxidation results in the formation of highly reactive and unstable hydroperoxides of both saturated and unsaturated lipids. Hydroperoxides generated were determined using a commercially available Lipid Hydroperoxide Assay Kit (Cayman Chemical, Ann Arbor, MI) according to manufacturers instructions. Briefly, HepG2 cells were plated in a 24-well

plate at 90% confluence. Twenty-four hours after plating, cells were incubated with 100 μM tBHP for 2, 24 and 48 h. Cell were collected in 0.5 ml HPLC-grade water and sonicated for cell lysis. Lipid hydroperoxides were extracted from the lysate using chloroform and the amount of hydroperoxide in the chloroform extract was determined using chromogen at 500 nm. Hydroperoxide concentration in samples is expressed in μM.

Statistical Analysis

Results are expressed as mean ± standard error (SE). Data were analyzed using the student's t-test, one-way ANOVA with Dunnett's post-hoc test or two-way ANOVA followed by the Bonferroni's post-hoc test. All statistical analysis was performed using GraphPad Prism version 4.00 for Macintosh (GraphPad Software, Inc., San Diego, CA). A p value of <0.05 was considered statistically significant.

Results

NRF2 and KEAP1 Over-expression did not Significantly alter FMO3 mRNA Expression in HepG2 Cells

To examine the possible role of the NRF2-KEAP1 regulatory pathway in controlling *FMO3* gene expression, FMO3 mRNA levels were quantified in cells over-expressing NRF2 or its cytosolic regulatory protein, KEAP1. The results in Figure 1 show that transfection with the NRF2 expression vector results in a 2.7±0.2-fold change in NRF2 mRNA levels compared to empty vector-transfected cells. Transfection with KEAP1 expression vector results in a 1.4±0.15-fold change in KEAP1 mRNA levels compared to empty vector controls. Over-expression of NRF2 results in significantly higher expression of the NRF2 target genes, NQO1 and HMOX1, by 3.1±0.7-fold and 3.4±0.4-fold, respectively, compared to the empty vector. No significant change in FMO3 mRNA expression is observed with NRF2 over-expression. AZA, a DNA methyl transferase inhibitor treatment has been shown to induce FMO3 mRNA expression in HepG2 cells (Klick et al., 2008). Treatment of AZA (as a positive control) resulted in a significant induction of FMO3 mRNA levels (2.9±0.7-fold). Furthermore, no significant change in NQO1, HMOX1 and FMO3 mRNA expression is seen with KEAP1 over-expression (Figure 1).

A Subtoxic dose of tBHP results in NRF2 Nuclear Translocation and Expression of NRF2 Target Genes in HepG2 Cells

In the current study, 100 μM tBHP treatment was used to activate endogenous NRF2. To determine whether 100 μM tBHP (at the concentration used) activates NRF2 nuclear translocation and subsequent downstream gene transcription, we measured nuclear NRF2 protein levels as well as HMOX1 and NQO1 mRNA levels in HepG2 cells after tBHP treatment at 2 and 24 h. In response to a 100 μM tBHP treatment for 24 h, the percent LDH leakage from HepG2 cells did not change (Figure 2A). Treatment with this subtoxic dose of tBHP (100 μM) for 2 h results in an increase in NRF2 nuclear localization (Figure 2B). The increased nuclear localization of NRF2 is associated with significant increases in the expression of the NRF2 target genes, HMOX1 and NQO1 by 1.8±0.3- and 1.7±0.06-fold, respectively, compared to vehicle treated controls (Figure 2C and 2D).

Effect of tBHP treatment on Cellular ROS Generation and Lipid Peroxidation

To investigate whether tBHP treatment (ROS inducer) at the concentration (100 μM) used in the current study results in ROS induction, cellular ROS levels were measured using dichlorofluorescin diacetate (DCFDA) and lipid hydroperoxide assays (Figure 3). Treatment with 100 μM tBHP for 2, 24 and 48 h significantly increased cellular ROS levels at 2 and 24 h to 10.5±1.65 and 17±2.2 RFU/mg protein, but the levels returned to control levels by 48 h (Figure 3A). Lipid peroxidation results in the formation of highly reactive and unstable hydroperoxides of both saturated and unsaturated lipids and its quantification is important to assess the role of oxidative injury. tBHP treatment significantly increased hydroperoxide formation to 5±0.75, 3.2±0.34 and 0.68±0.22 μM at 2, 24 and 48 h respectively, compared to control treatment (Figure 3B).

Effect of NRF2 or KEAP1 Over-expression on FMO3-directed Reporter Gene Activity

To determine whether the transcription factor, NRF2, activates *FMO3* gene expression, co-transfection studies with NRF2 or KEAP1 expression vectors and FMO3 promoter luciferase reporter constructs (pRNH1013 and pRNH970) were performed. Co-transfection of reporter genes with NRF2 or KEAP1 did not significantly alter FMO3-directed reporter gene activity (Figure 4A). Furthermore, NRF2 or KEAP1 over-expression studies also were carried out using a subtoxic dose of the pro-oxidant and Nrf2 activator, tBHP. Results are shown in Figure 4B. No significant change in reporter gene activity is seen with KEAP1 co-transfection. However, tBHP treatment combined with NRF2 expression plasmid and *FMO3* promoter luciferase reporter construct co-transfection, significantly decreased luciferase activity.

Effect of Pro-oxidant-induced NRF2-KEAP1 Regulatory Pathway Activation on FMO3-directed Reporter Gene Activity

FMO3 promoter luciferase reporter constructs (pRNH1013 & pRNH970) or empty vector were transiently expressed in HepG2 cells and exposed to tBHP or tBHQ. No significant change in reporter gene activity is observed after treatment (Figure 5).

Discussion

Mammalian FMOs have long been considered non-inducible by xenobiotics (Cashman and Zhang, 2002, Krueger and Williams, 2005). Thus, the transcriptional regulation of FMO involving stress activated transcription factors has not been studied. Recently, Celius et al. (2008, 2010) showed that activation of the Aryl hydrocarbon (Ah) receptor by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induced Fmo3 mRNA expression in mice. The authors also showed that increased Fmo3 mRNA levels in response to 3-methylcholanthrene (3MC) and benzo(a)pyrene (BaP) treatment, but not by TCDD, in Hepa-1 cells was mediated by p53 binding to a response element in the *Fmo3* promoter region (Celius et al., 2010). A gene array analysis performed in our laboratory also identified increased *Fmo3* gene expression after APAP treatment (O'Connor et al., 2014). We have also demonstrated enhanced *Fmo3* gene expression after treatment with other hepatotoxicants such as alpha-naphthyl isothiocyanate and after bile duct ligation (Rudraiah et al., 2014b, Rudraiah et al., 2014a). Given that all three of these latter treatments results in oxidative stress, the goal of the

present study was to determine whether *FMO3* regulation under oxidative stress conditions might involve NRF2-KEAP1 regulatory pathway activation, which is known to mediate many oxidative-stress induced changes in gene expression.

Over-expression of NRF2 induced expression of its known target genes, HMOX1 and NQO1 (Aleksunes et al., 2008, Aleksunes and Manautou, 2007, Aleksunes et al., 2006). With over-expression of KEAP1 we anticipated seeing a significant decrease in HMOX1 and NQO1 expression, but no such changes were observed. HMOX1 has also been shown to be regulated by hypoxia-inducible factor-1α (HIF-1α) and JNK-1 (Yeligar et al., 2010). Similarly, AhR has been shown to regulate NQO1 (Yeligar et al., 2010, Wang et al., 2013). It is possible that during over-expression of KEAP1, transcription factors other than Nrf2 play a role in maintaining expression of HMOX1 and NQO1. Over-expression of NRF2 or KEAP1 did not significantly alter *FMO3* gene expression, suggesting that *FMO3* gene regulation does not involve NRF2-KEAP1. Klick et al. (2008) demonstrated that treatment of HepG2 cells with 5-aza-2'-deoxycytidine (DNA methyl transferase inhibitor) resulted in detectable *FMO3* expression (Klick et al., 2008). Consistent with this, AZA treatment significantly increased *FMO3* expression in our experimental conditions, suggesting that HepG2's are an appropriate model for performing such inducing studies.

To further investigate possible NRF2-mediated *FMO3* gene regulation, co-transfection studies using two FMO3 promoter luciferase reporter constructs (pRNH1013 and pRNH970) were performed. Co-transfection of NRF2 or KEAP1 expression vectors along with the FMO3 reporter constructs did not significantly alter reporter gene activity. However, co-transfection of the NRF2 expression vector and the FMO3 reporter constructs, in addition to activation of NRF2 nuclear translocation by a treatment with a subtoxic dose of tBHP, did reduce reporter gene activity significantly. Although, this is suggestive of a potential NRF2 suppressive effect on *FMO3* gene transcription, it could also be due to a synergistic interaction between the transfection process and the stressor agent. The one other major possibility worth evaluating is the activator protein-1 (AP-1) mediated regulation of *FMO3*. Oxidative stress is known to alter signaling through c-Jun N-terminal Kinase (JNK) and related pathways, thus activating AP-1 (Enomoto et al., 2001, Li and Jaiswal, 1992, Elsby et al., 2003). In addition, promoter analysis discovered four binding sites for AP-1 at about 0.7, 0.9, 4.9 and 5.7 kb from the transcription start site on the human *FMO3* promoter (data not shown).

FMO3 reporter construct over-expression along with NRF2 activation by tBHP or tBHQ treatment also failed to alter reporter gene activity. The tBHP dose used in this study results in oxidative stress, as evidenced by increased NRF2 nuclear translocation and expression of the known NRF2 target genes, HMOX1 and NQO1, without producing noticeable cytotoxicity. Collectively, these data suggest that *FMO3* transcriptional regulation does not involve the NRF2-KEAP1 signaling pathway.

Acknowledgements

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Abbreviations

FMO3	Flavin-containing monoxygenase-3
NRF2	Nuclear factor (erythroid-derived 2)-like 2
KEAP1	Kelch-like ECH-associated protein 1
NQO1	NAD(P)H:quinone oxidoreductase-1
HMOX1	heme oxygenase-1
tBHP	tert-butyl hydroperoxide
tBHQ	tert-butyl hydroquinone

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Highlights

- FMO3 gene expression by transcription factor NRF2 is investigated.
- NRF2 over-expression did not change FMO3 gene expression in HepG2 cells.
- NRF2 over-expression did not alter FMO3-directed reporter gene activity.
- NRF2 over-expression along with activation did not change reporter gene activity.
- FMO3 transcriptional regulation might not involve NRF2-KEAP1 regulatory pathway.

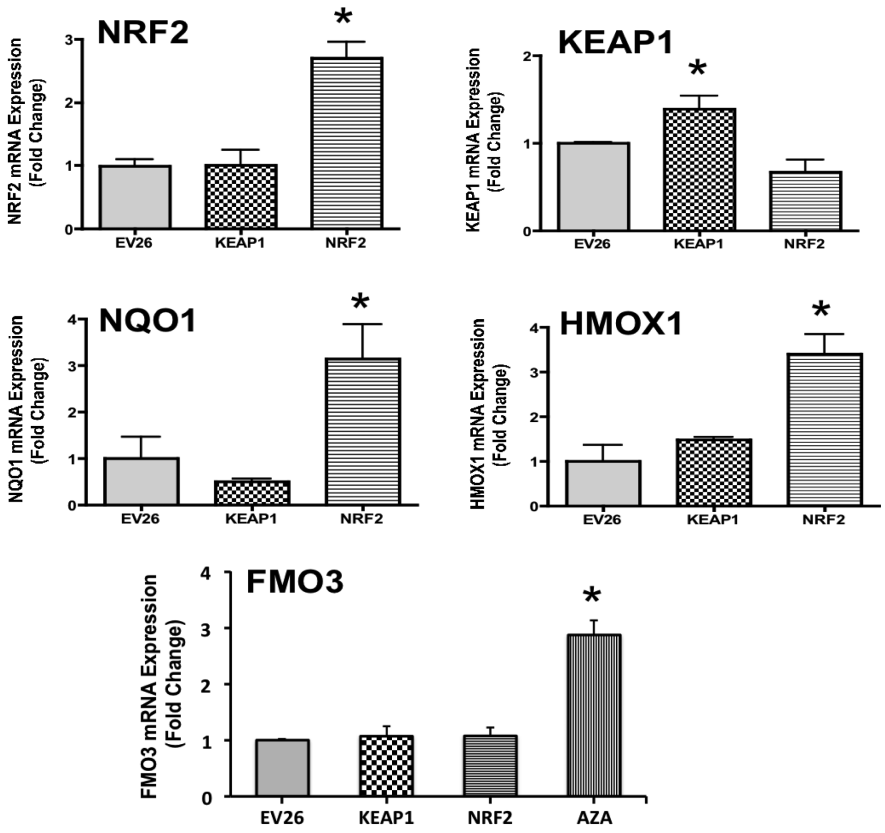

Figure 1. Effect of NRF2 and KEAP1 over-expression on FMO3 mRNA levels in HepG2 cells

HepG2 cells were transiently transfected with the oxidative stress responsive transcription factor, NRF2, or its cytosolic regulatory protein, KEAP1, or treated with 2.5 μM 5-aza-2′-deoxycytidine (AZA). RNA was isolated from cells 48 h after transfection or treatment. cDNA was made using a commercial MMLV-RT kit and the cDNA samples were analyzed by quantitative RT-PCR using human NRF2, KEAP1, NQO1, HMOX1 and FMO3 specific primers. mRNA levels were normalized to the housekeeping gene, β-Actin. NRF2, KEAP1, HMOX1, NQO1 and FMO3 mRNA expression are presented as mean fold change ± SE. Each experiment was done in triplicate and repeated at least three times, and representative experimental results are shown. One-way ANOVA followed by Dunnett's post-hoc test was used for data analysis. Asterisks (*) represent a statistical difference ($p < 0.05$) from empty vector (EV26) transfected group.

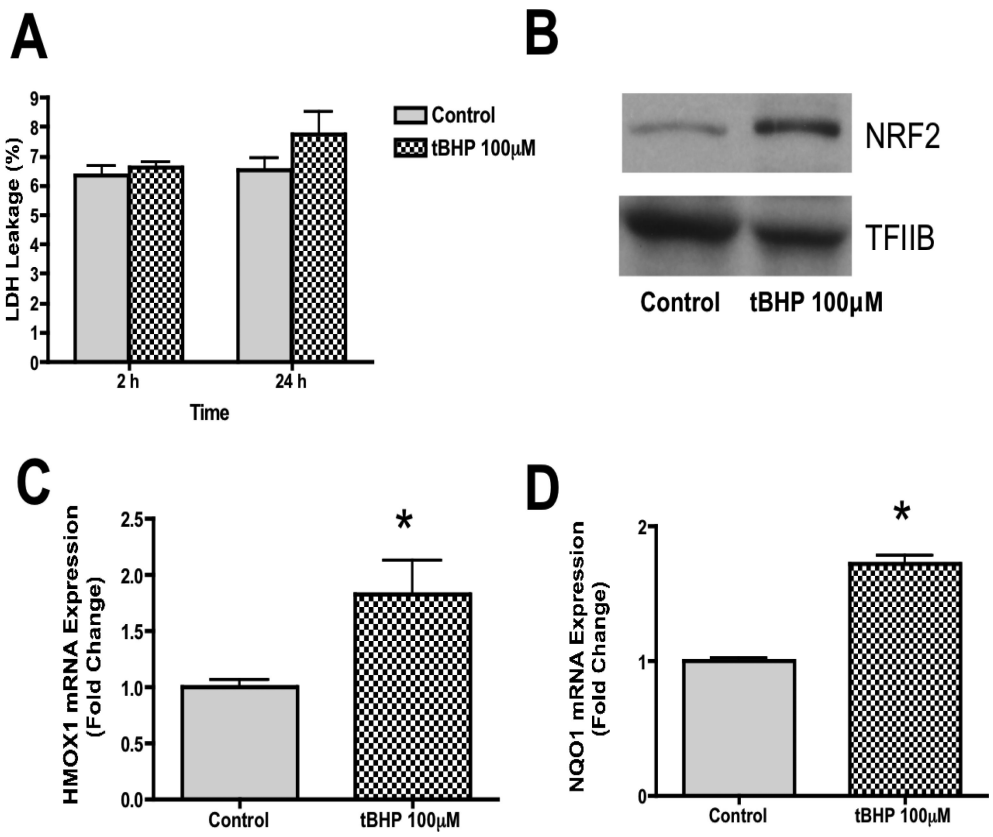

Figure 2. Effect of tBHP treatment on cytotoxicity and NRF2-KEAP1 regulatory pathway in HepG2 cells

(A) HepG2 cells were treated with tBHP (100 μM) or vehicle for 2 h. Cytotoxicity with 100 μM tBHP was determined by measuring LDH activity in the medium 2 and 24 h after treatment. The data are presented as LDH leakage (%) ± SE. **(B)** Western blots for NRF2 were performed using nuclear fractions from tBHP- and vehicle-treated HepG2 cells. RNA was isolated from HepG2 cells 24 h after treatment with tBHP. cDNA samples were analyzed by quantitative RT-PCR for NRF2 target genes HMOX1 **(C)** and NQO1 **(D)**. Gene expression was normalized to the housekeeping gene β-Actin. NQO1 and HMOX1 mRNA expression are presented as mean fold change ± SE. Each experiment was done in triplicate and repeated at least three times, and representative experimental results are shown. Student's t-test was used for data analysis. Asterisks (*) represent a statistical difference ($p < 0.05$) from control treated group.

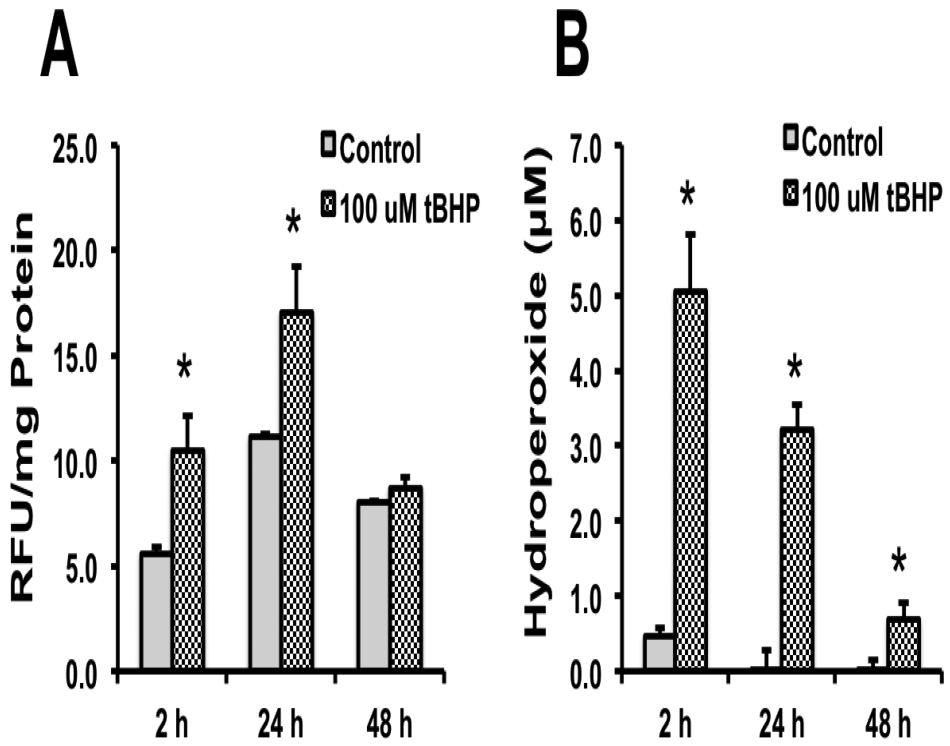

Figure 3. Effect of tBHP treatment on reactive oxygen species (ROS) generation in HepG2 cells

HepG2 cells were treated with tBHP (100 µM) or vehicle for 2, 24 and 48 h. **(A)** ROS activity with in the cell was measured using dichlorofluorescin diacetate (DCFDA) 2, 24 and 48 h after treatment. The data are presented as RFU/mg Protein ± SE. **(B)** Hydroperoxide generation was measured using lipid hydroperoxide assay after 2, 24 and 48 h tBHP treatment. The data are presented as Hydroperoxide (µM) ± SE. Each experiment was done in triplicate and repeated at least three times, and representative experimental results are shown. Two-way ANOVA was performed followed by the Bonferroni's post-test. Asterisks (*) represent a statistical difference ($p < 0.05$) from control treated group.

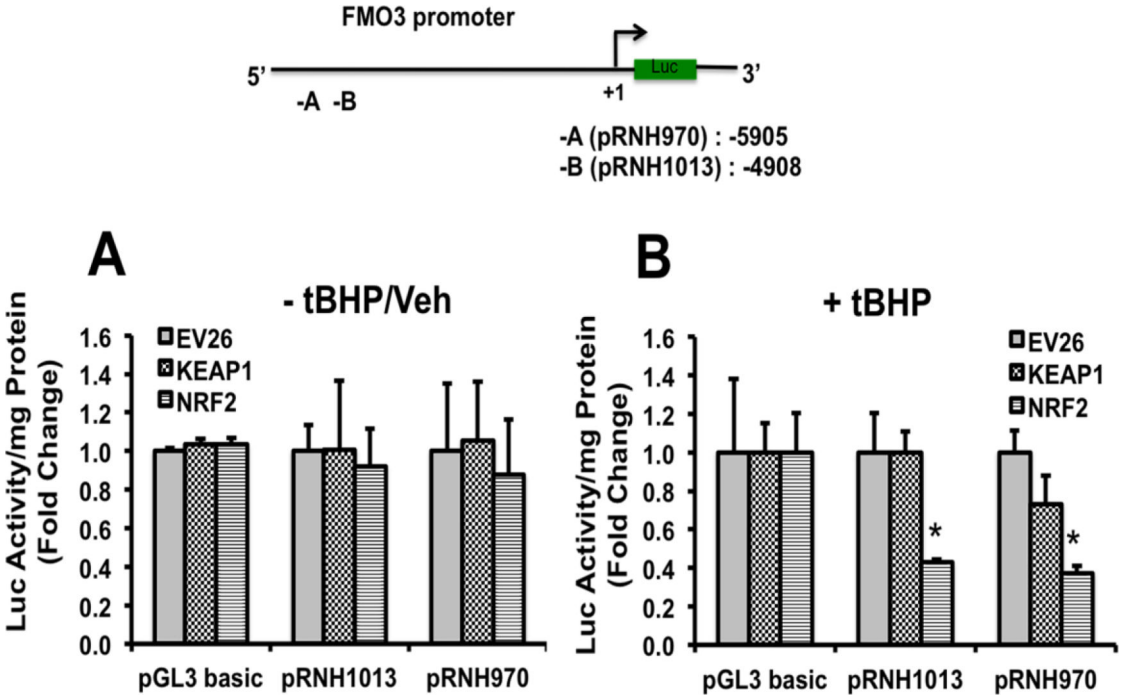

Figure 4. Effect of NRF2 or KEAP1 over-expression on FMO3-directed reporter gene activity

Transcription factor, NRF2, or its cytosolic regulatory protein, KEAP1, were transiently co-expressed with the FMO3 promoter luciferase reporter constructs 5kb-length (pRNH1013), 6kb-length (pRNH970) or the parent plasmid, pGL3Basic, in HepG2 cells. **(A)** Luciferase activity was measured 48 h after transfection, using a commercial kit. Luciferase activity was normalized to total protein and data are presented as mean fold change ± SE. **(B)** HepG2 cells were treated with the pro-oxidant, tBHP (100 μM), or vehicle 24 h after transfection for 6 h. Luciferase activity was measured 42 h after tBHP treatment. Luciferase activity was normalized to total protein and presented as mean fold change ± SE. Each experiment was done in triplicate and repeated at least three times, and representative experimental results are shown. Two-way ANOVA was performed followed by the Bonferroni's post-test. Asterisks (*) represent a statistical difference ($p < 0.05$) from the respective empty vector (EV26) transfected group.

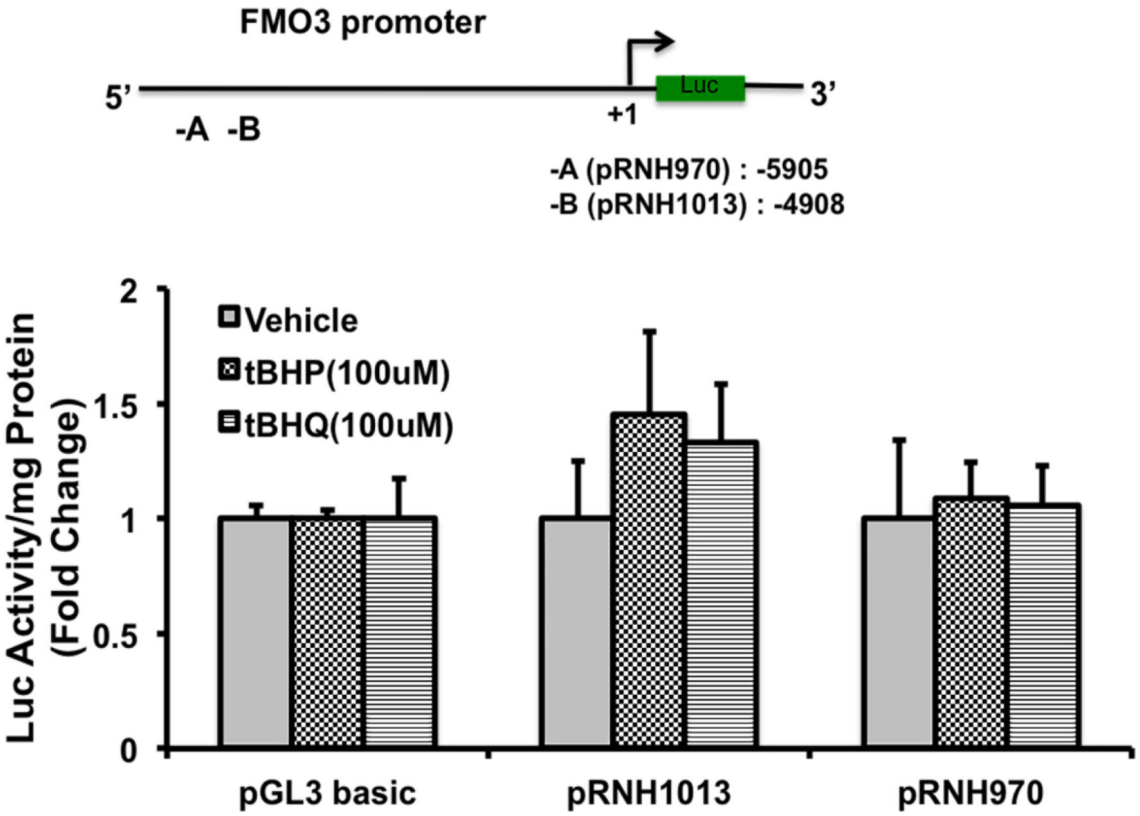

Figure 5. Effect of tBHP and tBHQ on FMO3-directed reporter gene activity

FMO3 promoter luciferase reporter constructs (pRNH1013 & pRNH970) or the parent plasmid, pGL3basic were transiently transfected into HepG2 cells. The transfected cells were treated with the pro-oxidant tBHP (100 μM), tBHQ (100 μM) or vehicle 48 h after transfection for 6 h. Luciferase activity was measured 42 h following treatment with tBHP or tBHQ. Luciferase activity was normalized to total protein and presented as mean fold change ± SE. Each experiment was done in triplicate and repeated at least three times, and representative experimental results are shown. Two-way ANOVA was performed followed by the Bonferroni's post-test. Statistical comparisons revealed no significant differences among the data sets.

Table 1

Primer Sequences for Quantitative RT-PCR

Gene	Primer Sequences
FMO3	Forward: 5′- TTG TAA ATG CTA GCC CTG CC -3′
	Reverse: 5′- CTG TCT GGA AGA GGG GCT G -3′
HMOX1	Forward: 5′- GCC AGC AAC AAA GTG CAA G -3′
	Reverse: 5′- GAG TGT AAG GAC CCA TCG GA -3′
KEAP1	Forward: 5′- TGA CAA GCT TAT GCA GCC AGA TCC CAG G -3′
	Reverse: 5′- GTG AGG ATC CTC AAC AGG TAC AGT TCT GCT GGT -3′
NQO1	Forward: 5′- GGA CTG CAC CAG AGC CAT -3′
	Reverse: 5′- TCC TTT CTT CTT CAA AGC CG -3′
NRF2	Forward: 5′- GCT CAT ACT CTT TCC GTC GC -3′
	Reverse: 5′- ATC ATG ATG GAC TTG GAG CTG -3′