Increased Protein Stability as a Mechanism That Enhances Nrf2-mediated Transcriptional Activation of the Antioxidant Response Element

DEGRADATION Of Nrf2 By the 26 S Proteasome*

Received for publication, July 19, 2002, and in revised form, November 15, 2002
Published, JBC Papers in Press, November 22, 2002, DOI 10.1074/jbc.M207293200

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Nrf2 (NF-E2-related factor 2) is a central transcription factor involved in the transcriptional activation of many genes encoding phase II drug-metabolizing enzymes via the antioxidant response element. Nrf2 has previously been found to undergo nuclear translocation by a phosphorylation-dependent mechanism mediated by protein kinase C in HepG2 cells treated with tert-butylhydroquinone, β-naphthoflavone, or 12-O-tetradecanoylphorbol-13-acetate. In the present report, we have found that the levels of Nrf2 were increased in cells treated with tert-butylhydroquinone or β-naphthoflavone by a post-transcriptional mechanism. Treatment of HepG2 cells with cycloheximide resulted in the loss of Nrf2 within 30 min. By contrast, treatment with the proteasome inhibitors lactacystin or MG-132 caused an accumulation of Nrf2, whereas the reverse effects were observed with PD 98059 and U 0126, two compounds that block the activation of the MAPK/ERK signaling cascade. These data suggest that Nrf2 is degraded by the ubiquitin-dependent pathway and that phosphorylation of Nrf2 leads to an increase in its stability and subsequent transactivation activity.

The antioxidant response element (ARE) is a cis-acting enhancer that mediates the transcriptional activation of genes encoding antioxidant and Phase II drug-metabolizing enzymes in response to electrophilic compounds and phenolic antioxidants. Among genes that contain a functional ARE, which has a core sequence 5′-TGACNNNGC-3′ (1), are those encoding the mouse and rat glutathione S-transferase (GST) A1 and A2 subunits, respectively (2–4), the rat and human NAD(P)H:quinone reductases (NQO1) (5–7), the human γ-glutamylcysteine synthetase heavy (γ-GCSH) and light (γ-GCSL) subunits (8, 9), heme oxygenase 1 (HO-1) (10), and others. Transcriptional activation through the ARE is largely dependent upon the transcription factor NF-E2-related factor 2 (Nrf2) (11), a member of the Cap’n’Collar (CNC) family of bZIP proteins, as demonstrated by in vitro electrophoretic mobility shift assays and transient transfection experiments (12–15). These data are further supported by in vivo studies using Nrf2-deficient mice. The expression of GstA1 and Nqo1 has been shown to be markedly reduced in Nrf2−/− mice as compared with wild-type animals (16). Both the basal and/or the inducible expression of these enzymes and the γ-GCS subunits by known ARE inducers were also found to be impaired in these null mice (16, 17). Furthermore, the decreased expression of many antioxidant and Phase II enzymes in Nrf2−/− mice has been linked to the increased sensitivity of these animals to the toxic effects of acetaminophen and carcinogens (18–20). These data provide strong evidence that Nrf2 is critical in the regulation of ARE-responsive genes.

A number of recent studies have focused on identifying the signaling pathways that may be involved in transducing the ARE-mediated response. These include the mitogen-activated protein kinase cascades (21–23), the phosphatidylinositol 3-kinase (PI3K)-dependent pathways (24–26), and the protein kinase C (PKC)-dependent pathway (27). We have previously reported that activation of Nrf2 in HepG2 cells treated with tBHQ, β-NF, or 12-O-tetradecanoylphorbol-13-acetate involves in part a phosphorylation-dependent mechanism mediated by PKC, which appears to promote translocation of the transcription factor into the nucleus (27). These findings support the notion that Nrf2 may be activated by a process that involves a disruption of its interaction with the cytoskeleton-associated protein Keap1 that retains Nrf2 in the cytoplasm (28). Indeed, we have recently obtained evidence showing that phosphorylation of Nrf2 at Ser-40 by PKC interferes with the association between the two proteins (29).

In the present study, we have further explored the mechanisms involved in the activation of this transcription factor. Nrf2 was found to undergo rapid degradation mediated by the 26 S proteasome but became stabilized in cells exposed to tBHQ and β-NF, leading to its accumulation and enhanced transcriptional activity. The stabilization of Nrf2 appears to depend upon its phosphorylation by a protein kinase(s) associated with the MAPK/ERK signaling cascade. Thus regulation of Nrf2 stability may represent an important mechanism in the activation of ARE-dependent gene expression in response to oxidative stress.
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EXPERIMENTAL PROCEDURES

Chemicals and Antibodies—Cycloheximide (CHX), tBHQ, and β-NF were obtained from Sigma. Lactacystin (LC), MG-132, okadaic acid (OA), staurosporine (stauro), PD 98059, and U 0126 were obtained from Calbiochem. Antibodies against ubiquitin, and a C-terminal peptide of NRF2 were obtained from Santa Cruz Biotechnology. Anti-GAPDH antibodies were obtained from Research Diagnostics Inc. (Flanders, NJ).

In addition, antibodies against a peptide derived from the N-terminal region of NRF2 were raised in rabbits by BIOSOURCE International (Camarillo, CA) for the present study. This antibody specifically recognizes both human and rat NRF2.

Cell Culture—HepG2 and H4IIEC3 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, non-essential amino acids, penicillin, and streptomycin, all of which were obtained from Invitrogen. Cells were seeded in 6-well plates at 40–50% confluency and incubated for 20 h prior to treatment with chemicals or for use in transfection experiments. For time-course experiments, cells were treated with chemicals following a schedule such that they would all be harvested simultaneously. Transient transfections were performed using LipofectAMINE Plus reagents according to the manufacturer’s instructions (Invitrogen). Transfection procedures, plasmid DNA, CAT activity assays, and quantitation methods have been described previously (15).

Immunoblot Analysis and Immunoprecipitation—HepG2 or H4IIEC3 cells were cultured and treated with chemicals as described above. In the CHX experiments, cells were treated with CHX (5 μg/ml) or pretreated with tBHQ (50 μM) for 2 h before addition of CHX in the time-course assays. In experiments where cells were exposed to OA (20 nM) and staurosporine (stauro) reagents were added simultaneously. Following the treatment period, cells were washed twice with ice-cold phosphate-buffered saline buffer and lysed in immunoprecipitation buffer as described previously (27). Lysates were incubated with anti-NRF2 antibodies at 4 °C for 16 h, and the immune complexes were precipitated with protein A-Sepharose beads at 4 °C for an additional 2 h. The precipitates were then washed extensively with immunoprecipitation buffer before fractionation by SDS-PAGE and subsequent immunoblotting with an anti-ubiquitin antibody.

Quantification of the results was performed by exposing the immunoblots directly to a Fuji phosphorimaging device or by analyzing the autoradiogram with a densitometer. For immunoprecipitation, cells were treated with tBHQ (50 μM), LC (5 μM), and MG-132 (10 μM) for 4 h, washed twice with ice-cold phosphate-buffered saline buffer, and lyzed in immunoprecipitation buffer as described previously (27). Lysates were incubated with anti-NRF2 antibodies at 4 °C for 16 h, and the immune complexes were precipitated with protein A-Sepharose beads at 4 °C for an additional 2 h. The precipitates were then washed extensively with immunoprecipitation buffer before fractionation by SDS-PAGE and subsequent immunoblotting with an anti-ubiquitin antibody.

Determination of Human NRF2 and γ-GCS mRNA Levels in HepG2 Cells—The relative levels of mRNA encoding NRF2 and γ-GCS in HepG2 cells were compared by TaqMan® real-time PCR. Total RNA was isolated using Trizol reagent (Invitrogen) and then treated with RNase-free DNase (Promega). The cDNA was synthesized from 500 ng of total RNA using TaqMan® Reverse Transcriptase (Applied Biosystems). The cDNA was quantified by real-time PCR. Total RNA was isolated using Trizol reagent (Invitrogen) and then treated with RNase-free DNase (Promega). The cDNA was synthesized from 500 ng of total RNA using TaqMan® Reverse Transcriptase (Applied Biosystems). The cDNA was quantified by real-time PCR. To ensure that the level of NRF2 protein is affected during induc-
of CHX exposure and 5% at the 1-h time point.

Nrf2 Degradation by the Proteasome—The decreased turnover rate in tBHQ-treated HepG2 cells implies that Nrf2 may become more resistant to degradation. Proteins with regulatory functions, such as the cyclins and transcription activators (30, 31), are commonly degraded by the proteasome through the ubiquitin-dependent pathway. Therefore we sought to determine if Nrf2 might be proteolytically degraded by this pathway. In a time-course experiment, HepG2 cells were treated with the proteasome inhibitor LC for 0 min, 30 min, 1 h, 1.5 h, 2 h, 4 h, 8 h, and 24 h. The cells were lysed, and total cell extracts were analyzed by immunoblotting with anti-Nrf2 and anti-GAPDH antibodies. The results of these experiments show that intracellular Nrf2 protein started to accumulate within 1.5 h of incubation with LC (a 2-fold increase), and this continued with a 5.5-fold increase by 8 h. By 24 h, the Nrf2 protein decreased to a lower level than that of untreated cells (basal level), presumably because LC was no longer effective in the cells or became toxic (Fig. 4A). To support these data a parallel experiment using a structurally different proteasome inhibitor, MG-132, was performed. As with LC, MG-132 caused a similar pattern of Nrf2 accumulation in the cells (data not shown; Fig. 5A, lane 5). To further confirm the involvement of the 26 S proteasome, immunoprecipitation experiments were performed to detect ubiquitinated forms of Nrf2. Following treatment with LC or MG-132 in the presence or absence of tBHQ, HepG2 cell lysates were immunoprecipitated with anti-Nrf2 antibodies and then analyzed by immunoblotting with an anti-ubiquitin monoclonal antibody. As shown in Fig. 4B, protein bands with slower mobility than Nrf2 were detected only in the lysates of cells treated with either LC or MG-132 (Fig. 4C, lanes 3 and 5). Similar observations have been made in recent studies on the ubiquitination of the aryl hydrocarbon receptor (32) and the β2-adrenergic receptor and β-arrestin protein (33).

Transient transfection experiments were also performed to determine if the proteasome inhibitors can mimic ARE inducers such as tBHQ to elicit a transcriptional response through the ARE. As shown in Fig. 6, LC or MG-132 treatment of HepG2 cells
transfected with the GSTA2 ARE-CAT reporter construct induced CAT activity 3.5-fold over the basal level. Further treatment with tBHQ did not lead to higher CAT activity.

Stabilization of Nrf2 Is Dependent on Phosphorylation by the MAPK Pathway—Phosphorylation of Nrf2 by PKC caused by ARE inducers appears to promote its nuclear translocation (27). To determine if phosphorylation of Nrf2 also contributes to its stability, we employed OA, a protein phosphatase inhibitor that induces intracellular hyperphosphorylation (34). Treatment of HepG2 cells with OA resulted in an increase in the Nrf2 protein by 2-fold as determined by immunoblot analysis (Fig. 5A) as well as a 2.5-fold induction of CAT activity from the GSTA2 ARE-CAT construct (Fig. 6). The level of Nrf2 (9.5-fold) as well as the CAT activity (4.5-fold) was increased further in cells co-treated with OA and tBHQ (Fig. 5A, and Fig. 6) as compared with those treated with tBHQ alone (6.2-fold and 3-fold, respectively). In contrast, treatment with the broad spectrum PKC inhibitor staurosporine did not affect the level of Nrf2 (Fig. 5A), although this inhibitor did attenuate the induction of CAT activity by tBHQ (Fig. 6). These data indicate that phosphorylation of Nrf2 by PKC may not be essential for its stability. However, cellular hyperphosphorylation induced by OA appears to have a positive effect on Nrf2 stability and transactivation activity.

It has been previously reported that the MAPK/ERK signaling pathway may be involved in the regulation of the ARE response (21, 22). We sought to determine if phosphorylation events mediated by this pathway may have a role in promoting Nrf2 stability. In this experiment, HepG2 cells were pretreated with PD 98059 or U 0126 for 1 h followed by tBHQ treatment for 4 h. The lysates were analyzed by immunoblotting with both anti-Nrf2 and anti-GAPDH antibodies. The results were quantitated using a densitometer, and the Nrf2 values were plotted after normalization with those of GAPDH. The blot shown is a representative of three independent experiments with similar results. B, HepG2 cells were exposed to Me_2SO (lane 1), tBHQ (50 μM, lane 2), LC (5 μM, lane 3), LC and tBHQ (lane 4), MG-132 (10 μM, lane 5), or MG-132 and tBHQ (lane 6) for 4 h, and total cell lysates were subject to immunoprecipitation with anti-Nrf2 (α-Nrf2) antibodies. The immunoprecipitated complexes were fractionated by SDS-PAGE and immunoblotted with anti-ubiquitin (α-Ub) antibodies. The blot shown is a representative of two independent experiments.

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Fig. 3. Stabilization of Nrf2 in HepG2 cells exposed to tBHQ. HepG2 cells were treated with 5 μg/ml CHX (A) or pretreated with tBHQ (50 μM) for 2 h followed by CHX (B) over a 2-h time-course period. Cells were lysed, and total lysates were immunoblotted with anti-Nrf2 antibodies followed by autoradiography. The times indicated above each panel refers to CHX exposure times. The asterisk (*) indicates a non-specific, cross-reacting protein band. C, the immunoblots were exposed and scanned using a Fuji phosphorimaging device, and the intensity of the protein band was quantitated and plotted on a semi-log graph with the value obtained for cells not treated with CHX set as 100%. The values were normalized with those of GAPDH. The blots shown are representatives of four independent experiments.

Fig. 4. Accumulation of Nrf2 induced by inhibitors of the ubiquitin-dependent proteasome. A, HepG2 cells were exposed to the proteasome inhibitor LC (5 μM) over a 24-h time-course period (as indicated), and total lysates were analyzed by immunoblotting with anti-Nrf2 and anti-GAPDH antibodies. The results were quantitated using a densitometer, and the Nrf2 values were plotted after normalization with those of GAPDH. The blot shown is a representative of three independent experiments with similar results. B, HepG2 cells were exposed to Me_2SO (lane 1), tBHQ (50 μM, lane 2), LC (5 μM, lane 3), LC and tBHQ (lane 4), MG-132 (10 μM, lane 5), or MG-132 and tBHQ (lane 6) for 4 h, and total cell lysates were subject to immunoprecipitation with anti-Nrf2 (α-Nrf2) antibodies. The immunoprecipitated complexes were fractionated by SDS-PAGE and immunoblotted with anti-ubiquitin (α-Ub) antibodies. The blot shown is a representative of two independent experiments.
DISCUSSION

The expression of many antioxidant and Phase II drug-metabolizing enzymes is increased in cells during oxidative stress (35), and the induction process is primarily mediated by the transcription factor Nrf2 acting on the ARE present in the promoter of genes encoding these enzymes. The established mechanism of activation of the ARE-mediated pathway has been demonstrated to lead to the dissociation of Nrf2 from the cytoskeleton-anchored Keap1 protein, which results in its translocation into the nucleus to increase gene transcription (28, 36). Several recent studies have examined the association between these two proteins at the molecular levels, including potential sites involved in the interaction (28), the necessity for dimerization of Keap1 to associate with Nrf2 (37), and critical cysteine residues of Keap1 that become modified by electrophilic agents to induce the release of Nrf2 (38). Separately, phosphorylation of a serine residue in Nrf2 by PKC has been shown to interfere with its binding to Keap1 (29).

In the present study, we have examined other mechanisms involving Nrf2 that are important for ARE-driven transcription. We observed that the level of cellular Nrf2 increased in HepG2 cells treated with either tBHQ or \( \text{H}_{2}\text{O}_{2} \), two structurally diverse compounds that activate gene expression via the ARE (1, 2). The increase of cellular Nrf2 by \( \text{H}_{2}\text{O}_{2} \) occurred at a later time point as compared with tBHQ. This delay is consistent with the observation that \( \text{H}_{2}\text{O}_{2} \) must first be metabolized to a reactive intermediate by CYP1A1 to become an active inducer of the ARE response (2).

Our results suggest that the increase in the level of Nrf2 in response to tBHQ is mediated by a post-transcriptional mechanism, rather than an increase in \( \text{Nrf2} \) mRNA levels. These findings are consistent with other studies that have demonstrated that \( \text{Nrf2} \) mRNA levels are unaffected by various inducers of ARE activity (13, 39). A more recent study, however, reported that the expression of Nrf2 was increased in murine keratinocytes by \( \text{H}_{3} \text{H}_{2} \)-1,2-dithiole-3-thione (D3T), but this effect was attributed to an increase in the rate of \( \text{Nrf2} \) transcription (40). These results are contradictory to those observed in this study.

In the present study, we have examined other mechanisms involving Nrf2 that are important for ARE-driven transcription. We observed that the level of cellular Nrf2 increased in HepG2 cells treated with either tBHQ or \( \beta \)-NF, two structurally diverse compounds that activate gene expression via the ARE (1, 2). The increase of cellular Nrf2 by \( \beta \)-NF occurred at a later time point as compared with tBHQ. This delay is consistent with the observation that \( \beta \)-NF must first be metabolized to a reactive intermediate by CYP1A1 to become an active inducer of the ARE response (2).

Our results suggest that the increase in the level of Nrf2 in response to tBHQ is mediated by a post-transcriptional mechanism, rather than an increase in \( \text{Nrf2} \) mRNA levels. These findings are consistent with other studies that have demonstrated that \( \text{Nrf2} \) mRNA levels are unaffected by various inducers of ARE activity (13, 39). A more recent study, however, reported that the expression of Nrf2 was increased in murine keratinocytes by \( \text{H}_{3} \text{H}_{2} \)-1,2-dithiole-3-thione (D3T), but this effect was attributed to an increase in the rate of \( \text{Nrf2} \) transcription (40). These results are contradictory to those observed in this study.
and other studies and are possibly due to differences in the experimental procedures, cell types, and compounds being used. Our data also suggest that increased Nrf2 stability, rather than an increase in the rate of protein translation, is responsible for the higher level of Nrf2 observed in tBHQ- or β-NP-treated cells. We have also obtained evidence demonstrating that the 26 S proteasome plays an important role in the regulation of the Nrf2 protein. Thus it appears that the steady-state level of Nrf2 is maintained by a precise balance between the rates of its synthesis and its degradation by the proteasome. We hypothesize that in response to oxidative stress Nrf2 would continue to be synthesized at a normal rate, but the rate of its degradation decreases such that the balance would now tip toward accumulation of the protein, ultimately leading to an enhanced transcriptional activity.

These data raise an important question as to the mechanism(s) that regulate Nrf2 stability in the cell. Since degradation by the 26 S proteasome requires prior ubiquitination of the substrate molecule, recognition and targeting of the Nrf2 protein by the ubiquitin ligases would now tip toward accumulation of the protein, ultimately leading to an enhanced transcriptional activity.

Thus the induction of ARE-dependent enzymes appears to be mediated by induction of the increase in the stability of Nrf2 will require the identification of the specific protein kinase(s) involved as well as the site(s) of phosphorylation. Equally important is the identifica-

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