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Translational Control of Nrf2 Protein in Activation of Antioxidant Response by Oxidants

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ABSTRACT

Nf-E2 related factor-2 (Nrf2) is a basic leucine zipper transcription factor that binds and activates the antioxidant response element (ARE) in the promoters of many antioxidant and detoxification genes. We found that H_2O_2 treatment caused a rapid increase in endogenous Nrf2 protein level in rat cardiomyocytes. Semiquantitative or real-time reverse transcription-polymerase chain reaction failed to show an increase of Nrf2 mRNA level by H_2O_2 treatment. Measurements of Nrf2 protein stability excluded the possibility of Nrf2 protein stabilization. Although inhibiting protein synthesis with cycloheximide prevented H_2O_2 from elevating Nrf2 protein level, RNA synthesis inhibition with actinomycin D failed to do so. Measurements of new protein synthesis with [^{35}S]methionine incorporation confirmed that H_2O_2 increased the translation of Nrf2 protein. Inhibitors of phosphoinositide 3-kinase were able to abolish the induction of Nrf2 protein by H_2O_2. Although H_2O_2 increased phosphorylation of p70 S6 kinase, rapamycin failed to inhibit H_2O_2 from elevating Nrf2 protein. H_2O_2 also induced phosphorylation of eukaryotic translation initiation factor (eIF) 4E and eIF2α within 30 and 10 min, respectively. Inhibiting eIF4E with small interfering siRNA or increasing eIF2α phosphorylation with salubrinal did not affect Nrf2 elevation by H_2O_2. Our data present a novel phenomenon of quick onset of the antioxidant/detoxification response via increased translation of Nrf2 by oxidants. The mechanism underlying such stress-induced de novo protein translation may involve multiple components of translational machinery.

Heart failure is a leading cause of mortality worldwide. An initial event, such as hypertension or cardiac ischemia, can lead to an intermediate hypertrophic phenotype of the heart, which may eventually deteriorate into failure. An increased expression of several antioxidant genes has been detected in the early stage of heart failure. Hypertrophic hearts initially contain higher levels of superoxide dismutase, glutathione peroxidase, and total glutathione (Gupta and Singal, 1989; Singh et al., 1995; Dhalla et al., 1996; Hill and Singal, 1996). Patients with dilated or hypertrophic cardiomyopathy show an increased expression of NAD(P)H:quinone oxidoreductase 1 (Hwang et al., 2000). Levels of superoxide dismutase and the reduced glutathione-to-oxidized glutathione ratio both decrease when hearts transition from hypertrophy to failure (Gupta and Singal, 1989; Singh et al., 1995; Dhalla et al., 1996). The loss of antioxidant reservoir is observed in the late stage of heart failure. The molecules controlling the adaptive response associated with the early stage of cardiac hypertrophy remain unidentified.

NF-E2 related factor-2 (Nrf2) is a basic leucine zipper transcription factor that binds and activates the antioxidant response element (ARE) after heterodimerizing with a binding partner. Many antioxidant and detoxification-related genes contain the ARE in the promoters, such as glutathione transferases, hemeoxygenase-1, superoxide dismutase 1, and thioredoxin (Lee et al., 2003). Activation of Nrf2 transcription factor has been linked to cytoprotection (Nguyen et al., 2003; Jaiswal, 2004; Lee et al., 2005). The inducers of Nrf2

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ABBREVIATIONS: Nrf2, NF-E2 related factor-2; ARE, antioxidant response element; PI3, phosphatidylinositol 3; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; siRNA, small interfering RNA; eIF, eukaryotic translation initiation factor; PAGE, polyacrylamide gel electrophoresis; RT, reverse transcription; LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; MG132, *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal; PCR, polymerase chain reaction; CXM, cycloheximide; UTR, untranslated region; IRES, internal ribosomal entry site; ER, endoplasmic reticulum.

activation come in a variety of forms, from natural product isothiocyanates and coumarin to toxicants such as metals and quinones (Nguyen et al., 2003). Although Nrf2-mediated ARE activation has been studied in multiple tissues, this pathway has not been well characterized in the heart.

As a transcription factor, Nrf2 level and activity are regulated at several levels: transcription, degradation, translocation, and post-translational modifications such as phosphorylation (Huang et al., 2000, 2002; Kong et al., 2001; Kwak et al., 2003; Zhang and Hannink, 2003; Nioi and Hayes, 2004). A positive feedback loop through an ARE-like element in the promoter has been shown to regulate *Nrf2* gene transcription in the cellular response to the anticancer agent 3*H*-1,2-dithiole-3-thione (Kwak et al., 2002). Nrf2 can be phosphorylated at Ser40 in vitro by protein kinase C (Huang et al., 2002). There is evidence that PI3-kinase and extracellular signal-regulated kinases regulate the phosphorylation and therefore the activity of Nrf2 (Kang et al., 2000; Zhang et al., 2006). An important mechanism controlling the increase of Nrf2 protein level is through a decreased rate of Nrf2 protein degradation. Keap1, an inhibitor of Nrf2, is known to bind Nrf2 and hold it in the cytoplasm, where Keap1 recruits an E3 ubiquitin ligase, resulting in Nrf2 ubiquitination and therefore degradation by the proteasome (Zhang and Hannink, 2003). Disrupting the interaction with Keap1 causes stabilization of Nrf2 (Nguyen et al., 2003; Jaiswal, 2004; Lee and Johnson, 2004; Motohashi and Yamamoto, 2004). However many of the studies examining the interaction between Nrf2 and Keap1 use gene overexpression approaches. How endogenous *Nrf2* gene responds to chemical stress is less well understood.

Recent evidence suggests that chemical stress can cause selective protein translation. We found that low to mild doses of oxidants activate the ARE in cardiomyocytes in an Nrf2-dependent manner (Purdom-Dickinson et al., 2007). A rapid increase of Nrf2 protein level has been observed with oxidant exposure in cardiomyocytes. We address here the critical regulatory mechanism controlling the rapid increase of Nrf2 protein.

Materials and Methods

Cell Culture and H_2O_2 or Pharmacological Inhibitor Treatment. Neonatal rat cardiomyocytes were prepared as described previously (Coronella-Wood et al., 2004; Purdom and Chen, 2005). Cells were seeded at 0.3×10^6 cells per well for six-well plates or 2.5×10^6 per 100-mm dish. Freshly isolated cells were cultured in low glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) for 3 days before 24-h serum starvation (0.5% FBS/DMEM) and 10-min treatment with 100 μM H_2O_2. Pharmacological inhibitors were dissolved in dimethyl sulfoxide at 1000× concentration. Vehicle or pharmacological inhibitors were added to cells 1 h beforeH_2O_2 treatment. After H_2O_2, the media were changed to fresh media (0.5% FBS/DMEM) with vehicle or inhibitors added back.

Transfection. ARE-luciferase plasmid (0.2 μg, kindly provided by Dr. Jeffery Johnson) was cotransfected with *Renilla reniformis*-luciferase plasmid (0.04 μg) using Fugene 6 transfection reagent

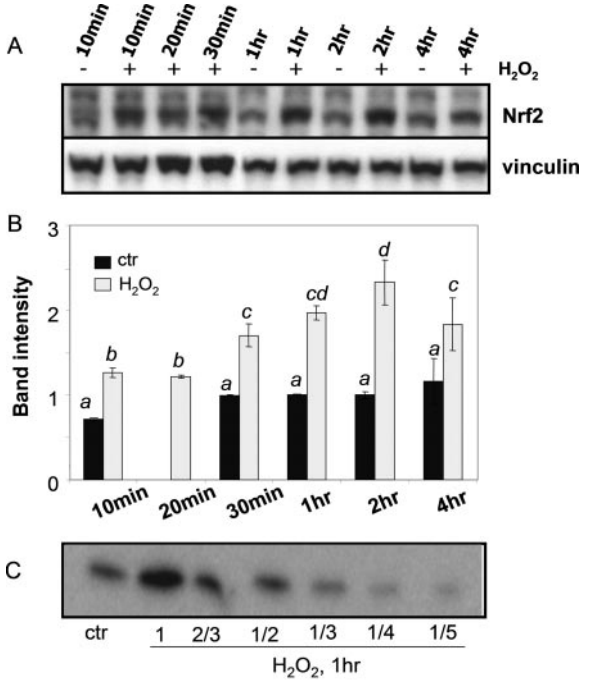

Fig. 1. H_2O_2 treatment induces a rapid accumulation of Nrf2 protein. Cardiomyocytes were treated with 100 μM H_2O_2 for 10 min, at which time the media was changed and cells were harvested for Western blot analysis (30 μg of protein/lane) at the indicated time points (A). A representative gel is shown (A). The intensity of bands was quantified using Image J software (http://rsb.info.nih.gov/ij/), and the data represent the averages and standard deviations from three independent experiments (B). H_2O_2 sample harvested at 1 h after treatment was diluted in series for Western blot to verify the -fold induction (C). Each group of means that is not significantly different from the others is indicated by a common letter symbol. Therefore, means in the *a* group are significantly different from means in the *b* group, and so on.

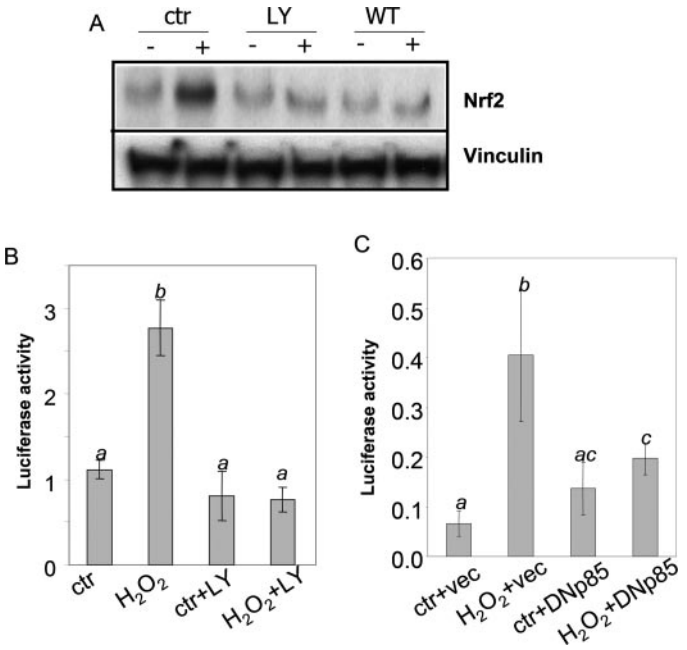

Fig. 2. Inhibition of PI3 kinase prevents Nrf2 induction or ARE activation. ARE-luciferase reporter construct (0.2 μg) was transfected along with TK-*R. reniformis* luciferase (0.04 μg), which corrects for the transfection efficiency (B). Additional 0.2 μg of control or DNp85 plasmid was included for cotransfection (C). Cardiomyocytes without (A) or with plasmid transfection (B and C) were pretreated with LY294002 (LY, 20 μM, A and B) or Wortmannin (WT, 2 μM, A) for 1 h before exposure to 100 μM H_2O_2 for 10 min. The inhibitor was added back to the corresponding group for 1-h incubation before harvesting for Western blot analyses (30 μg of protein per lane, A) or were harvested 4 h later for dual luciferase assay (B, C). Each group of means that is not significantly different from the others is indicated by a common letter symbol. Therefore, means in the *a* group are significantly different from means in the *b* group, and so on.

(Roche) as described previously (Purdom-Dickinson et al., 2007). To test the effect of PI3-Kinase inhibition, dominant-negative p85 (0.2 μg) or corresponding empty vector (generous gifts from Dr. Wataru Ogawa) was included in the transfection. Firefly luciferase and *R. reniformis* luciferase were measured using a Dual Luciferase Assay System (Promega) and a Luminometer (Turner Designs).

Cardiomyocytes were transfected with two different siRNA sequences against eukaryotic initiation factor 4E (eIF4E; Ambion, Austin, TX) using oligofectamine (Invitrogen, Carlsbad, CA). siRNAs (100 nM each; total, 200 nM) were mixed with oligofectamine in the Opti-MEM media and incubated with cells in DMEM for 6 h before FBS was added to a final concentration of 10% for overnight recovery. At 48 h after transfection, cells were treated for 10 min with 100 μM H_2O_2 before harvesting. The level of eIF4E was verified by Western blot, and the approach results in approximately 50% knockdown of eIF4E expression.

Western Blot Protocol. Cytosolic lysates were obtained using extraction buffer (1% Triton X-100, 10 mM Tris, pH 7.4, 5 mM EDTA, 50 mM NaCl, 50 mM NaF, and freshly added 2 mM dithiothreitol, 1 mM Na_2VO_3, 1 mM phenylmethylsulfonyl fluoride, 100 μg/ml leupeptin, and 10 μg/ml aprotinin) and retaining the supernatant after 10 min centrifugation at 13,000*g*. Total cell lysates or nuclear enriched fractions obtained from pellets after centrifugation of the lysates were prepared in Laemmli lysis buffer [125 mM Tris, pH 6.8, 50% (v/v) glycerol, 2.4% (w/v) SDS, and freshly added 100 μM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin]. Samples were diluted by addition of 0.5 volume of Laemmli sample buffer [65 mM Tris, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, with 5% freshly added β-mercaptoethanol] before 10 min of boiling. After SDS-PAGE, Western Blot was performed using antibodies against Nrf2 (Santa Cruz Biotechnology, Santa Cruz, CA), Thr389 phospho-p70S6 kinase, total p70S6 kinase, Ser209 phospho-eIF4E, total eIF4E), Ser51 phospho-eIF2α, or total eIF2α (Cell Signaling Technology, Danvers, MA). Secondary antibodies conjugated with the horseradish peroxidase (Invitrogen) were used for Enhanced Chemiluminescence Reaction.

Reverse-Transcription and PCR. Cells were harvested in TRIzol (Invitrogen, Carlsbad, CA) for extracting RNA. Total RNA (2 μg) was used for reverse transcription (RT), and a tenth of the resultant cDNAs were used for each PCR reaction using Nrf2 primer pair: forward, 5′-GCCAGCTGAACTCCTTAGAC-3′; reverse, 5′-GATTCGTGCACAGCAGCA-3′. For real-time RT-PCR, total RNA samples were used with probe and primer sets purchased from ABI (Nrf2, Rn00477784_m1; β-glucuronidase, Rn00566655_m1).

[^{35}S]Methionine Labeling and Immunoprecipitation. Serum-starved cells in 100-mm dishes were incubated for 20 min in labeling medium (DMEM without methionine, cysteine, and L-glutamine; Invitrogen) to reduce the intracellular pools of methionine. Inhibitors (LY294002 and cycloheximide) were added 5 min beforeH_2O_2 treatment (100 μM, 10 min). After H_2O_2, the media was replaced with fresh labeling medium containing 200 μCi of [^{35}S]methionine per dish and the corresponding inhibitors. Labeled cells were harvested 1 h later in 200 μl of extraction buffer containing 50 μM MG132. For immunoprecipitation, 500 μg of cellular protein were incubated with Nrf2 antibody and Protein G beads in radioimmunoprecipitation buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, with freshly added 1 mM dithiothreitol, 1 mM Na_3VO_4, 2 μM leupeptin, 10 units/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). The beads were then washed eight times with the buffer before boiling in Laemmli sample buffer for SDS-PAGE. Proteins were stained using Silver Stain Plus reagents (Bio-Rad Laboratories, Hercules, CA), and the gel was dried for autoradiography to visualize the newly synthesized Nrf2 using a PhosphorImager.

Statistics. Statistical analyses were performed using one-way analysis of variance followed by Bonferroni analysis for cross-comparisons with Stata 8.2 software (Stata Corporation, College Station, TX).

Result

An increase in the level of Nrf2 protein has been observed within 10 min after a pulse treatment of H_2O_2 (Fig. 1). The increase reached a peak of 2-fold at 1 to 2 h after H_2O_2 treatment (Fig. 1, A and B). Sample loading titration experiments confirmed approximately 2-fold induction of Nrf2 protein at 1 h after H_2O_2 treatment (Fig. 1C), consistent with band intensity quantifications (Fig. 1B). Within the 10-min time frame, H_2O_2 activates the PI3 kinase pathway in cardiomyocytes (Tu et al., 2002). Pharmacological inhibition of PI3 kinase using LY294002 or wortmannin blocked H_2O_2 from inducing Nrf2 elevation (Fig. 2A). A luciferase reporter assay, which measures the activation of ARE, was used to further verify the effect of PI3 kinase inhibition (Purdom-Dickinson et al., 2007). Treatment with LY294002 or cotransfection with dominant-negative p85, the regulatory subunit of PI3 kinase, showed an inhibition of H_2O_2-induced ARE

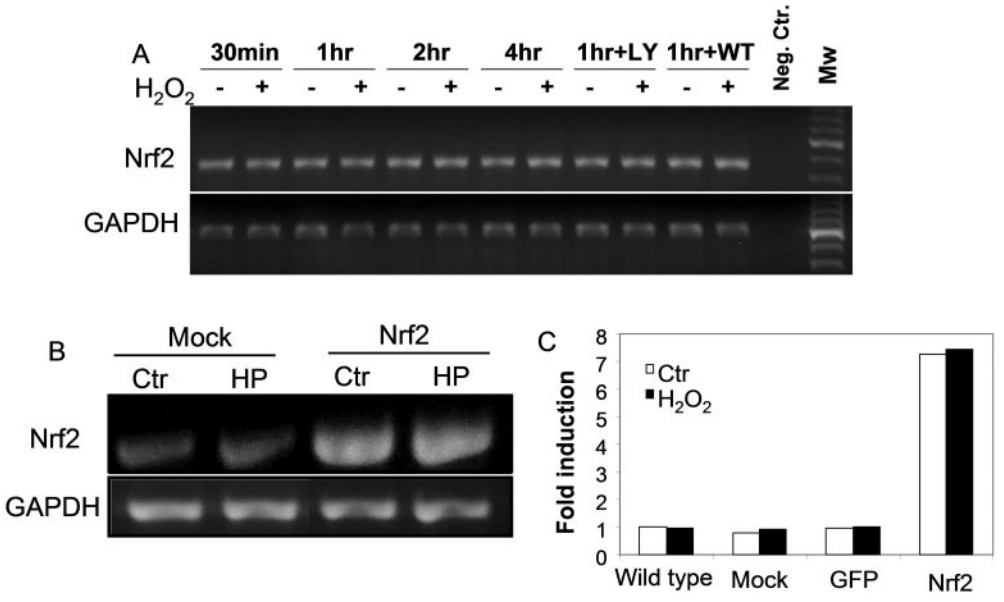

Fig. 3. H_2O_2 treatment does not induce Nrf2 mRNA. Cardiomyocytes were treated with 100 μM H_2O_2 for 10 min and were harvested at indicated time points for RNA preparation. Semiquantitative RT-PCR (A and B) or real-time RT-PCR (C) was performed as described under *Materials and Methods*. Cells were pretreated with 20 μM LY294002 (LY) or 2 μM wortmannin (WT) before H_2O_2 treatment (A). Replication-deficient adenovirus-infected CMCs were used as a positive control (B and C).

activation (Fig. 2, B and C). These data indicate a role of PI3 kinase in H_2O_2-induced rapid Nrf2 accumulation and activation.

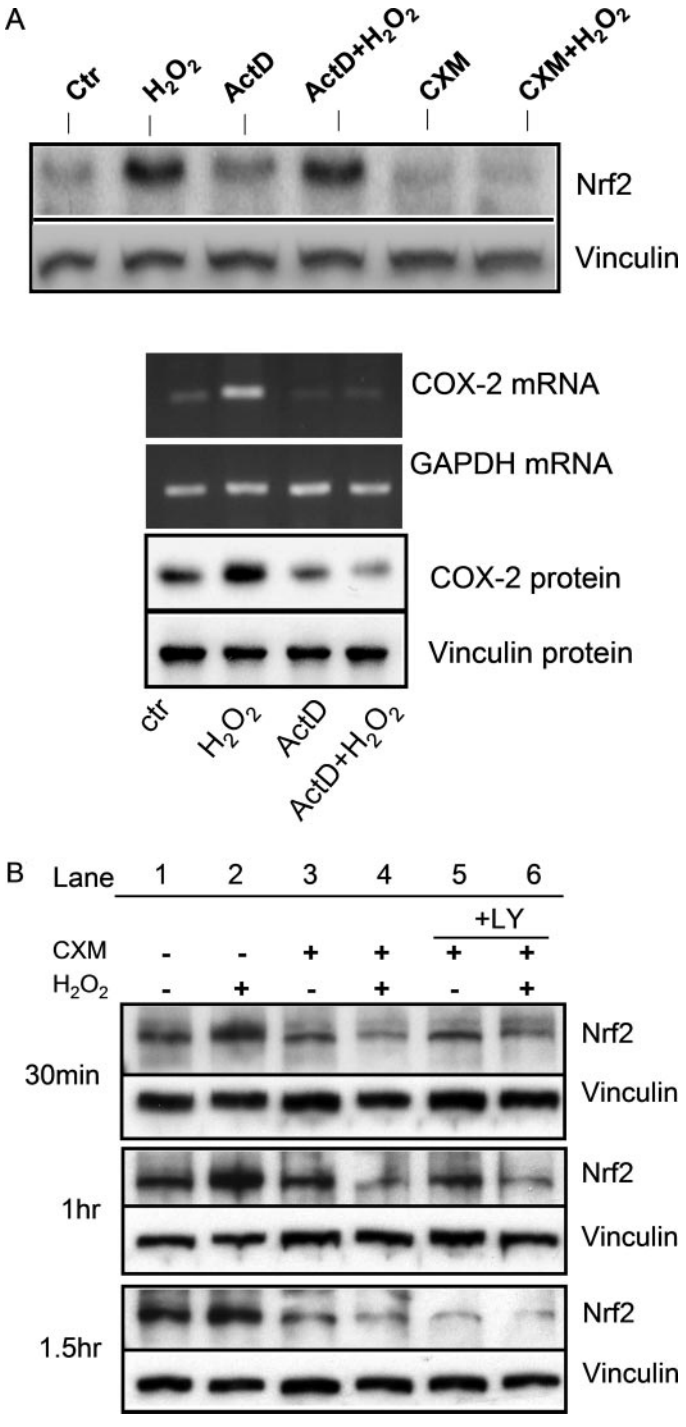

Fig. 4. Cycloheximide but not actinomycin D blocks H_2O_2-induced Nrf2 protein increase. Cardiomyocytes were pretreated with cycloheximide (CXM, 0.5 μg/ml) or actinomycin D (ActD, 0.5 μM) for 1 h before treatment with 100 μM H_2O_2 for 10 min (A). CXM or ActD was added back when the media was changed, and cells were harvested at 1 h after H_2O_2 treatment for Western blot to detect Nrf2 or cyclooxygenase-2 (COX-2) protein (30 μg of protein/lane) or RT-PCR to detect COX-2 mRNA (A). Cardiomyocytes were treated with 100 μM H_2O_2 for 10 min and were allowed to recover for 1 h, when CXM (0.5 μg/ml) or CXM plus LY294002 (LY, 20 μM) were added to the cells (B). The cells were then harvested at 30-min intervals for Western blot (30 μg of protein/lane) to measure Nrf2 protein (B).

An increase in the level of Nrf-2 protein suggests three possibilities: 1) increased transcription, 2) protein stabilization, or 3) an increase in Nrf-2 protein translation. To determine whether H_2O_2 causes an increase in Nrf2 transcription, we performed semiquantitative RT-PCR at various time points after H_2O_2 treatment (Fig. 3A). The results failed to show elevated Nrf2 mRNA with H_2O_2 treatment (Fig. 3A). By comparison, the positive control (i.e., cardiomyocytes infected with replication-deficient adenoviral constructs encoding Nrf2) showed higher levels of Nrf2 mRNA (Fig. 3B). Real-time RT-PCR also confirmed no elevation of Nrf2 mRNA by H_2O_2 treatment (Fig. 3C). To further indicate that Nrf2 was not regulated at the transcriptional level, we included RNA synthesis inhibitor actinomycin D. Actinomycin D at the dose used is capable of inhibiting transcription as shown by its effect in preventing H_2O_2 from inducting the *cyclooxygenase-2* gene at mRNA and protein levels (Fig. 4A, bottom). However, actinomycin D failed to block H_2O_2 from inducing Nrf2 protein (Fig. 4A, top). In contrast, when cells were treated with the protein synthesis inhibitor cycloheximide (CXM), H_2O_2 could no longer increase the level of Nrf2 (Fig. 4A).

Nrf2 protein is known to be degraded by the proteasome. If H_2O_2 inhibited Nrf2 degradation and LY294002 reversed the inhibition, a sustained elevation of Nrf2 protein should be observed in H_2O_2-treated cells even when new protein synthesis is blocked, and LY294002 would somehow prevent such an increase. We compared the stability of Nrf2 protein and the effect of LY294002 in a time course study. Cardiomyocytes were given time (1 h) to increase the level of Nrf2 protein after H_2O_2 treatment before addition of CXM to inhibit further protein synthesis (lane 2 compared with lane 1, Fig. 4B). Within 30 min of CXM addition, H_2O_2-treated cells showed a reduction of Nrf2 protein level compared with untreated cells (lane 4 compared with lane 3; Fig. 4B). The addition of LY294002 did not enhance the rate of Nrf2 degradation (lane 6 compared with lane 4; Fig. 4B). These data argue against the possibilities that H_2O_2 increases Nrf2 level by preventing Nrf2 degradation and LY294002 accelerates Nrf2 protein degradation.

To demonstrate that H_2O_2 causes an increase in Nrf2 translation, we used [^{35}S]methionine incorporation assay to measure newly synthesized Nrf2. Nrf2 antibody-immunoprecipitated samples contain a band corresponding to Nrf2 molecular weight (Fig. 5). An increase in the intensity of the band with H_2O_2 treatment indicates an increase in newly translated Nrf2 protein (Fig. 5). Quantification of band intensities indicates 2.0 $\pm$ 0.4-fold induction of newly synthesized Nrf2 protein by H_2O_2 treatment from three independent experiments. As expected, CXM efficiently blocked Nrf2 as well as background protein synthesis (Fig. 5A). The increase in Nrf2 was weakened by the presence of LY294002 (Fig. 5A), showing an average 50% inhibition by LY294002 based on the intensities of the bands compared with that of H_2O_2 treatment alone (Fig. 5B). These data support the hypothesis that PI3 kinase is involved in regulating Nrf2 translation after oxidative stress. Measurements of the overall protein synthesis in 6 h after H_2O_2 treatment by [^{3}H]leucine incorporation show that H_2O_2 treatment did not cause significant increase or loss of overall protein synthesis (Table 1). LY294002 alone did not significantly inhibit the overall protein synthesis but decreased protein synthesis in

H_2O_2-treated cells (Table 1). The data suggest that H_2O_2 treatment selectively enhances Nrf2 protein synthesis.

PI3 kinase generally activates several intermediates that eventually funnel through p70S6 kinase, which then phosphorylates the ribosomal protein S6 important for the assembly of 43S preinitiation complex (Gingras et al., 2001; Fingar and Blenis, 2004). H_2O_2 treatment caused p70S6 kinase activation (Tu et al., 2002). Inhibiting PI3 kinase with LY294002 or wortmannin blocked p70S6 kinase phosphorylation (Tu et al., 2002). Rapamycin, an inhibitor of the immediate upstream regulator of p70S6 kinase mTOR, can block p70S6 kinase phosphorylation effectively but failed to prevent H_2O_2 from inducing Nrf2 elevation (data not shown).

Phosphorylation of eIF4E or eIF2α represents two mechanisms of translational initiation (Dever, 2002; Preiss and Hentze, 2003; Gebauer and Hentze, 2004; Holcik and Sonenberg, 2005). We found that H_2O_2 treatment induced phosphorylation of eIF4E at 20 min (Fig. 6). This phosphorylation remained detectable 2 h after H_2O_2 treatment (Fig. 6). By comparison, phosphorylation of eIF2α appeared within 10 min of H_2O_2 treatment (Fig. 6). The level of eIF2α phosphorylation returned to baseline 2 h after H_2O_2 treatment (Fig. 6). It seems that eIF2α phosphorylation occurred earlier than eIF4E phosphorylation and the time course of eIF2α phosphorylation is consistent with Nrf2 induction.

When inhibitors of PI3 kinase were tested for their effect on phosphorylation of eIF4E or eIF2α, LY294002 seemed to inhibit phosphorylation of eIF4E and eIF2α (Fig. 7). For reasons unknown, wortmannin induced the level and phosphorylation of eIF4E and eIF2α in the controls (Fig. 7). To test which translational pathway mediates Nrf2 protein synthesis, we used siRNA against eIF4E. Because siRNA against eIF2α was not available, we used a specific inhibitor of eIF2α dephosphorylation, salubrinal, which enhances eIF2α activity (Boyce et al., 2005). Although levels of eIF4E protein were reduced approximately 50% using siRNA, Nrf2 accumulation was unaffected, as shown by the intensities of the bands (Fig. 8A). Salubrinal alone induced eIF2α phosphorylation similar to the level of H_2O_2 treatment at 30 to 60 min, and seemed to enhance H_2O_2-induced eIF2α phosphorylation (Fig. 8B). However, salubrinal failed to increase Nrf2 by itself or to enhance H_2O_2-induced Nrf2 (Fig. 8B). These data point to a complex mechanism of translational control of Nrf2 protein.

Discussion

This study found that H_2O_2 causes a rapid increase of Nrf2 protein in cardiomyocytes. Elimination of transcription and protein stabilization as contributors to oxidant-induced Nrf2 increase led us to explore protein translation as a primary means regulating this occurrence. We have provided evidence that H_2O_2 treatment indeed results in an increase in newly synthesized Nrf2 protein in cardiomyocytes. Our study focuses on the endogenous *Nrf2* gene, unlike the experimental systems using transfected *Nrf2* gene. Although the literature argues for increased Nrf2 protein stability as a means of protein accumulation (Kong et al., 2001; Nguyen et al., 2003; Jaiswal, 2004; Motohashi and Yamamoto, 2004), a key piece of experimental evidence used overexpression approaches with a Nrf2 transgene that does not contain 5′ untranslated region (UTR) (Zhang and Hannink, 2003). Endogenous *Nrf2* gene may differ from Nrf2 transgenes in protein translational control.

The process of protein translation is divided into three stages: initiation, elongation, and termination. The rate-limiting step of translation relies on the process of initiation predominantly. For 95 to 97% of mRNA species in mammalian cells, initiation of translation requires 5′ m^7GpppN cap structure in front of the start codon, 3′ poly(A) RNA tail, at least 12 eIFs, and poly(A) tail binding proteins (Dever, 2002; Preiss and Hentze, 2003; Holcik and Sonenberg, 2005). The process of initiation contains four physical steps: 1) formation of a 43S preinitiation complex from the small (40S) ribosomal subunit, eIFs, and Met-$tRNA^{Met}$; 2) binding of the 43S complex to the vicinity of the 5′ m^7GpppN cap structure on mRNA; 3) scanning of the 5′UTR of the mRNA and start codon AUG. The consensus sequence around AUG (i.e., the Kozak sequence) is GCC(A/G)CCAUGG in most genes; and 4) joining of a large 60S subunit to assemble a complete (80S)

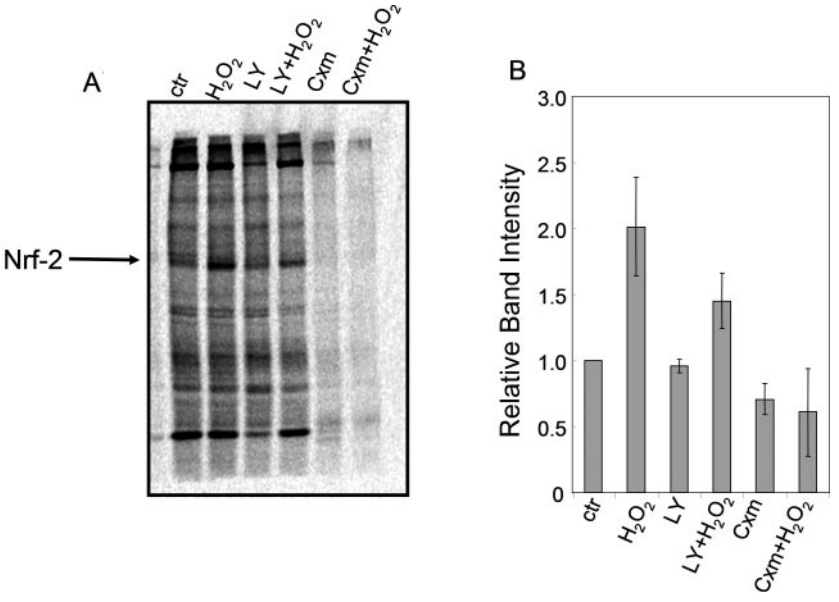

Fig. 5. H_2O_2 induces newly synthesized Nrf2 protein. Cardiomyocytes were treated with 100 μM H_2O_2 for 10 min in the absence or presence of LY294002 (LY, 20 μM) or CXM (0.5 μg/ml). Cells were placed in [^{35}S]methionine labeling medium immediately after H_2O_2 treatment and were harvested 1 h later for immunoprecipitation of Nrf2 protein followed by SDS-PAGE. The resultant bands were visualized using a PhosphoImager (A). The intensity of bands was quantified using NIH Image J software. The band intensity is set to 1 for the control, and all others are relative to the control. The data represent the averages and standard deviations from three independent experiments (B).

ribosome. The eIF4E acts as a recruiter that first locates and sits on the 5′ cap structure to attract the binding of the 43S preinitiation complex (Gingras et al., 1999; Preiss and Hentze, 2003; Holcik and Sonenberg, 2005).

An internal ribosomal entry site (IRES) promotes the binding of 40S ribosome to an internal portion of the mRNA to initiate translation in 3 to 5% of mRNA species. Although IRES-mediated protein translation was first discovered with viral proteins, approximately 50 cellular proteins have been found to exhibit IRES-mediated translation (http://www.iresite.org; http://ifr31w3.toulouse.inserm.fr/iresdatabase). Examples of these proteins include c-Myc, c-Jun, hypoxia-inducible factor-1α, $p27^{Kip1}$, activated protein C, apoptotic peptidase activating factor 1 (Apaf-1), X-chromosome-linked inhibitor of apoptosis protein (XIAP), and GRP78. The mRNA species of these cellular proteins contain 5′UTRs varying in length from 83 nucleotides (activated protein C) to 152 nucleotides ($p27^{Kip1}$) to 407 nucleotides (c-myc) or 577 nucleotides (apoptotic peptidase activating factor 1) (http://ifr31w3.toulouse.inserm.fr/iresdatabase). The IRES sequences usually have high GC content, a feature essential for formation of secondary structures containing "stems and loops." Because the IRES sequences are heterogeneous and apparently each gene contains a distinct IRES sequence that forms a unique secondary structure (Merrick, 2004), it prohibits quick identification of an IRES in Nrf2 mRNA. According to GenBank sequence information, mouse, rat, or human Nrf2 gene encodes mRNA containing 5′UTRs extending 82 (rat) to 114 (human) or 233 (mouse) nucleotides upstream of the start codon. Rat Nrf2 5′UTR is 94% homologous with the 3′ portion of mouse Nrf2 5′UTR, whereas human Nrf2 5′UTR does not share significant sequence homology with rat or mouse Nrf2 5′UTR. The common feature between these genes is the lack of well-defined Kozak sequence. Zuker's MFOLD software predicts stable secondary structure with "stems and loops" among these 5′UTR sequences. These features support the hypothesis of an IRES-mediated protein translation in H_2O_2-induced Nrf2 elevation.

Increasing evidence suggests that a rapid onset of selective protein translation serves as an important mechanism for cells to deal with stress (Sheikh and Fornace, 1999; Holcik and Sonenberg, 2005). IRES-mediated protein translation enables cells to express a small number of proteins, whereas the overall protein synthesis through 5′ m^7GpppN cap-dependent mechanism has been shut down to conserve energy.

TABLE 1

LY294002 inhibits overall protein synthesis in H_2O_2-treated cells

Cardiomyocytes in 24-well plates were treated with 100 μM H_2O_2 for 10 min in the absence or presence of LY294002 (20 μM) or CXM (0.5 μg/ml). Cells were placed in DMEM containing 0.2 μCi/well [^{3}H]leucine immediately after H_2O_2 treatment and were harvested 6 h later to measure incorporated [^{3}H]leucine by trichloroacetic acid precipitation.

	Ctr	LY294002	CXM
		relative cpm	
Control	1.00 + 0.08	1.07 + 0.22	0.36 + 0.05
H_2O_2	1.03 + 0.27	0.54 + 0.13	0.34 + 0.11

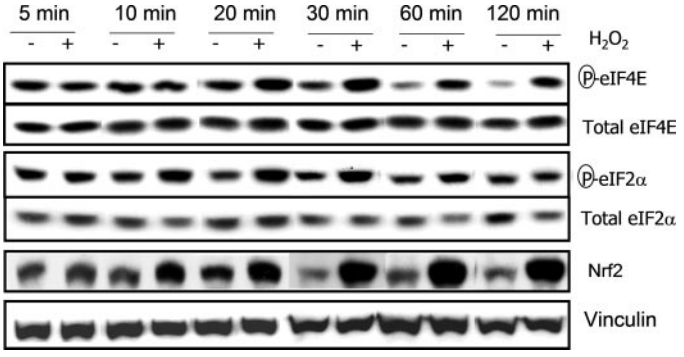

Fig. 6. H_2O_2 induces time-dependent phosphorylation of eIF4E and eIF2α. Cardiomyocytes were treated with 100 μM H_2O_2 for 10 min, and the cells were harvested at the indicated time points for measurement of phosphorylated or total eIF4E and eIF2α by Western blot analyses (30 μg of protein/lane).

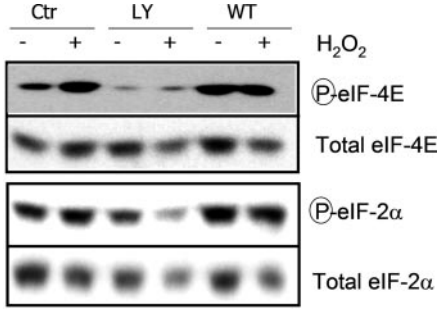

Fig. 7. Inhibition of PI3-kinase blocks phosphorylation of both eIF4E and eIF2α. Cardiomyocytes were pretreated with 20 μM LY294002 (LY) or 2 μM wortmannin (WT) for 1 h before treatment with 100 μM H_2O_2 for 10 min. The inhibitors were added back, and the cells were harvested 1 h later for measurement of phosphorylated or total eIF4E or eIF2α by Western blot (30 μg of protein/lane).

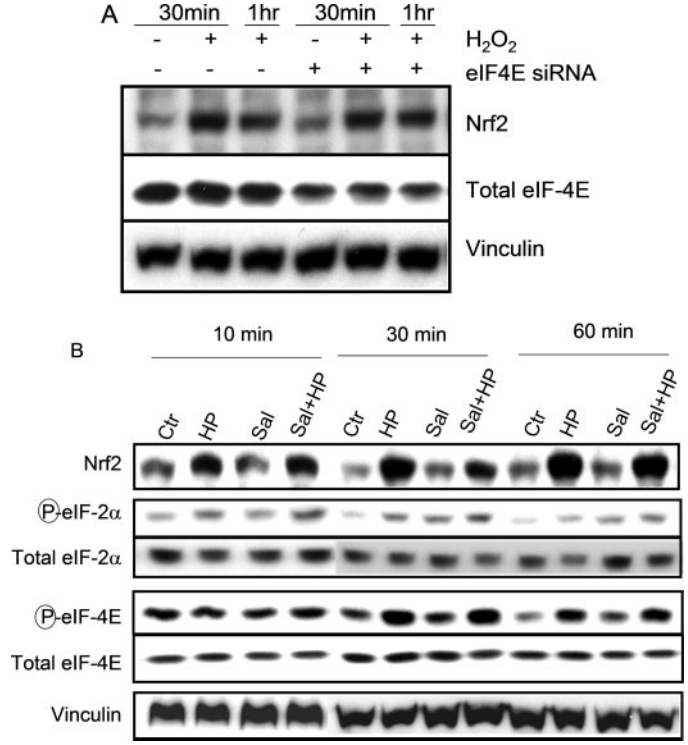

Fig. 8. Reducing eIF4E or increasing eIF2α phosphorylation does not affect Nrf2 induction. Cardiomyocytes were transfected with siRNA against eIF4E as described under *Materials and Methods* and were treated with 100 μM H_2O_2 for 10 min at 48 h after siRNA transfection (A). Cardiomyocytes were pretreated with salubrinal (Sal, 50 μM) for 30 min before 10-min treatment with 100 μM H_2O_2 (B). Cells were harvested at the indicated time points after H_2O_2 treatment for measurements of Nrf2, phosphorylated or total eIF4E, and phosphorylated or total eIF2a using Western blot analyses (30 μg of protein/lane).

There is evidence that in certain experimental systems, eIF2α phosphorylation is critical for IRES-dependent translation. Amino acid depletion, UV irradiation, viral infection, heat shock, hypoxia, and endoplasmic reticulum (ER) stress have been shown to induce eIF2α phosphorylation and selective protein translation through IRES (Holcik and Sonenberg, 2005). The kinases that phosphorylate eIF2α include the general control nonderepressible-2 (GCN2), protein kinase RNA (PKR), heme-regulated inhibitor kinase (HRI), and protein kinase RNA-like ER kinase (PERK) (Holcik and Sonenberg, 2005). There is evidence that PERK participates in Nrf2 expression under an ER stress condition (Cullinan and Diehl, 2004).

In our study, inhibitors of PI3 kinase prevent H_2O_2 from elevating the steady-state Nrf2 protein levels and newly synthesized Nrf2 protein. Modulating individual components of the usual pathways of translational initiation (i.e., p70S6 kinase, eIF4E, or eIF2α), does not seem to affect H_2O_2 from inducing Nrf2 protein. This suggests that a combination of several pathways or a p70S6 kinase-, eIF4E- or eIF2a-independent pathway downstream of PI3 kinase regulates Nrf2 protein translation. Based on the data from [^{35}S]methionine labeling experiments (Fig. 5), LY294002 inhibited H_2O_2 from inducing Nrf2 protein. The overall protein synthesis as measured by [^{3}H]leucine incorporation was reduced when LY294002 was added to H_2O_2-treated cells (Table 1). This suggests that LY294002 may inhibit stress-induced protein synthesis in general. Despite the negative data with rapamycin, eIF4E siRNA, or an activator of eIF2α in H_2O_2-induced Nrf2 protein elevation, it is possible that multiple components of the translational machinery collaborate to turn on Nrf2 translation. Therefore, inhibiting one component may not be sufficient to block Nrf2 induction. Along this line, although Salubrinal caused phosphorylation of eIF2α, additional signals such as those induced by H_2O_2 seem to be required to cooperate with eIF2α for permitting efficient translation of Nrf2 protein. In other words, eIF2α activity may be necessary but not sufficient in regulating stress induced protein translation. One caveat of eIF4E siRNA experiments is the lack of complete elimination of eIF4E protein. This is probably related to the fact that cardiomyocytes are difficult to transfect. Although eIF4E protein level is significantly reduced, the remaining eIF4E may be sufficient to work in concert with other eIFs in initiating protein translation. With 5′ m^{7}GpppN cap-dependent protein translation, 12 eIFs carry out the process in multiple steps. Therefore, although one or a few eIFs are important in initiating the coordination of the translational machinery, other eIFs may play a role in stress-induced protein translation once the initiation complex is assembled. The fact that LY294002 inhibits Nrf2 induction and three key components of protein translation shown here (i.e., phosphorylation of p70S6K, eIF4E, and eIF2α) supports this "multicomponents" argument of stress-induced protein translation.

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