Nrf2 Dependent Induction of Proteasome and Pa28αβ Regulator is Required for Adaptation to Oxidative Stress

Andrew. M. Pickering1,2, Robert. A. Linder1,2, Hongqiao. Zhang1,3, Henry. J. Forman1,3, and Kelvin. J. A. Davies1,2*

1 Ethel Percy Andrus Gerontology Center of the Davis School of Gerontology, the University of Southern California, Los Angeles, CA 90089, U.S.A.

2 Division of Molecular & Computational Biology, Department of Biological Sciences of the Dornsife College of Letters, Arts & Sciences, the University of Southern California, Los Angeles, CA 90089, USA

3 University of California at Merced, Merced, CA 95343, USA

*Running Title: Nrf2, Proteasome, and oxidative stress adaptation

*Address correspondence to: Prof. Kelvin. J. A. Davies, Andrus Gerontology Center, University of Southern California, 3715 McClintock Ave, Los Angeles, CA 90089-0191, U.S.A.
Fax: 213-740-6462; E-mail: kelvin@usc.edu

Keywords: Nrf2, Proteasome, Oxidative Stress, Immunoproteasome, Pa28αβ

Background: Adaptation to oxidative stress involves increased expression of 20S Proteasome, Pa28αβ, and Immunoproteasome.

Results: Blocking Nrf2 prevents proteasome and Pa28αβ induction, and Nrf2 is required for full adaptation.

Conclusion: Adaptation occurs through Nrf2-dependent induction of 20S proteasome and Pa28αβ while immunoproteasome is induced independently.

Significance: The Nrf2 signal transduction pathway controls 20S Proteasome/ Pa28αβ contributions to stress-adaptation, but not immunoproteasome contributions.

SUMMARY

The ability to adapt to acute oxidative stress (e.g. H2O2, peroxynitrite, menadione, paraquat) through transient alterations in gene expression is an important component of cellular defense mechanisms. We show that such adaptation includes Nrf2-dependent increases in cellular capacity to degrade oxidized proteins that are attributable to increased expression of the 20S Proteasome and the Pa28αβ (11S) Proteasome regulator. Increased cellular levels of Nrf2, translocation of Nrf2 from the cytoplasm to the nucleus, and increased binding of Nrf2 to antioxidant response elements (ARE’s) or electrophile response elements (EpRE’s) in the 5’-untranslated region of the Proteasome β5 subunit gene [demonstrated by chromatin immunoprecipitation (or ChIP) assay] are shown to be necessary requirements for increased Proteasome/Pa28αβ levels, and for maximal increases in proteolytic capacity and stress resistance; Nrf2 siRNA and the Nrf2 inhibitor retinoic acid both block these adaptive changes and the Nrf2 inducers DL-sulforaphane, lipoic acid, and curcumin all replicate them without oxidant exposure. The Immunoproteasome is also induced during oxidative stress adaptation, contributing to overall capacity to degrade oxidized proteins and to stress resistance. Two of the three Immunoproteasome subunit genes, however, contain no ARE/EpRE elements, and Nrf2 inducers, inhibitors, and siRNA all have minimal effects on Immunoproteasome expression during adaptation to oxidative stress. Thus, Immunoproteasome appears to be (at most) minimally regulated by the Nrf2 signal transduction pathway.
Despite antioxidant defenses, such as superoxide dismutases and glutathione peroxidases, oxidative stress represents a constant danger to cell and organismal viability. Reactive oxygen and nitrogen species can cause protein, lipid, sugar, DNA, and RNA modification. Oxidative modifications to proteins are common, and a major cellular defense strategy is to rapidly degrade mildly oxidized proteins before they can aggregate and cross-link to form insoluble cell inclusion bodies (1-16). Insufficient proteolytic capacity or increased oxidant generation, or both, can result in compromised cell function or even cell death (1,12,17-24). Over a period of many years, we (1-6,15) and others (11-14,16,25) have demonstrated that the bulk of oxidatively damaged proteins in the cytoplasm, endoplasmic reticulum, and nucleus are degraded by the Proteasome. More recently, we have also shown that the Immunoproteasome plays a significant role (26,27). In mitochondria, oxidized proteins are preferentially degraded by the Lon protease (7-10,28).

In previous studies, we have demonstrated that mammalian cells, as well as bacteria and yeast, can transiently adapt to oxidative stress (19,26,29-31). This is an adaptive process (sometimes called hormesis) in which cells treated with a mild dose of an oxidant will, for a period of time (=24-48 h), become more resistant to a higher dose of the same (or related) oxidant that would normally be toxic. Recently, we have demonstrated that this adaptive response includes an increased abundance of 20S Proteasomes, Immunoproteasomes, and PA28αβ (or 11S) Proteasome regulators (26); all these proteins were shown to play key roles in the oxidative stress response, and each was required for full adaptation. Other groups have also reported induction of various forms of the Proteasome and Proteasome regulators, by oxidative stress (32-36).

The Nrf2 [Nuclear factor (erythroid-derived 2)-like 2] transcription factor is an important component of responses to oxidative stress (37-44). Under non-stressful conditions, Nrf2 is maintained at low levels through rapid degradation by Keap1-dependent ubiquitin conjugation (44-46), followed by targeted degradation by the 26S Proteasome. As a product of this rapid turnover, newly translated Nrf2 is found predominantly in the cytoplasm. With Keap1 inactivation, as a product of factors such as oxidative stress, Nrf2 levels increase due to diminished proteasomal degradation, and Nrf2 is phosphorylated and translocated to the nucleus in mechanisms mediated by PKCδ and Akt (47). Once in the nucleus, Nrf2 binds to a cis-acting enhancer sequence, upstream of numerous antioxidant genes, known as the antioxidant response element (ARE) or electrophile responsive element (EpRE), and promotes the synthesis of several antioxidants, and enzymes responsible for repairing/removing oxidative damage and restoring cell viability (37).

It has been shown that Nrf2 knock-out in mice results in decreased tolerance to oxidative stress (48,49). Additionally, results by Kwak et al (44) showed that the phenolic antioxidant 3H-1,2-dithiole-3-thione (D3T), which induces many cellular antioxidants and phase 2 enzymes, can also enhance mammalian Proteasome expression through the Keap1-Nrf2 signaling pathway. These results led us to hypothesize that the transient stress-adaptation, involving Proteasome and Proteasome regulators, that we described previously (26), might be primarily under the control of the Nrf2 transcription factor. In the present study we have, therefore, tested whether oxidative stress-induced increases in 20S Proteasome, Immunoproteasome and the Pa28αβ regulator, as well as increased stress-resistance, are actually under the control of Nrf2, and whether Nrf2 is necessary and/or sufficient for their induction and for adaptation to various forms of oxidative stress.

**EXPERIMENTAL PROCEDURES**

*Materials* - All materials were purchased from VWR unless otherwise stated. Murine Embryonic Fibroblasts (MEF), catalog #CRL-2214, were purchased from ATCC (Manassas, VA, USA). Cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM), catalog #10-013-CV, from Mediatech.
(Manassas, VA) and supplemented with 10% Fetal Bovine Serum (catalog #SH30070.03) from Hyclone (Logan, UT, USA): henceforth referred to as ‘complete media.’ Cells were typically incubated at 37°C under 5% CO₂ and ambient oxygen.

**Adaptation to Oxidants** - MEF cells were grown to 10% confluence (≈250,000 cells per ml) then pre-treated with 100 nM - 100 µM H₂O₂ (catalog # H1009-100ml) from Sigma-Aldrich (St Louis, MO, USA), 1 nM - 1 µM peroxynitrite (catalog # 516620) from Merck (Darmstadt, Germany), 0.2 nM – 100 nM menadione (catalog # ME105) from Spectrum Chemicals (Gardena, CA, USA), or 10pM – 100 nM paraquat (catalog # PST-740AS) from Ultra Scientific (Kingstown, RI, USA), for 1 h at 37°C to induce adaptation to oxidative stress. Cells were then washed once with phosphate-buffered saline (PBS), which was finally replaced with fresh complete media.

**Induction or inhibition of Nrf2** - MEF cells were grown to 5% confluence and treated with varying concentrations of Nrf2 inducers. DL-sulforaphane (catalog # S2441-5mg) or curcumin (catalog # C1386-5G) from Sigma Aldrich (St Louis, MO, USA). Lipoic Acid (catalog #L1089) was purchased from Spectrum Chemicals (Gardena, CA, USA), dissolved in N,N,Dimethylformaldehyde, and combined with complete media at a final concentration of 0.1%; and a comparable concentration of N,N,Dimethylformaldehyde was added to control cells. Curcumin was dissolved in ethanol and combined with complete media at a final concentration of 0.1%; and a comparable concentration of ethanol was added to control cells. In some assays cells were treated with the Nrf2 inhibitor all-Trans-retinoic acid (catalog # R2625-100MG) purchased from Sigma Aldrich (St Louis, MO, USA). Trans-retinoic acid was dissolved in ethanol and combined with complete media at a final concentration of 0.1%; a comparable concentration of ethanol was added to control cells.

**Western blot analysis** - MEF cells were harvested from 25-75 cm² flasks by trypsinization. Cells were washed twice with PBS to remove trypsin and then lysed in RIPA buffer, (catalog # 89901) from Thermo Fisher (Waltham, MA, USA), supplemented with protease inhibitor cocktail (catalog #11836170001) from Roche (Nutley, NJ, USA). Protein content was quantified with the BCA Protein Assay Kit (Pierce, Rockford, IL, USA) according to the manufacturer’s instructions. For Western analysis, 5 - 20 µg of protein was run on SDS–PAGE and transferred to PVDF membranes. Using standard Western blot techniques, membranes were treated with Proteasome regulator subunit PA28α antibodies (catalog # PW8185-0100) from Enzo Life sciences (Plymouth Meeting, PA, USA), Immunoproteasome subunit anti-LMP2/β1i antibody (catalog # ab3328) purchased from Abcam (Cambridge, MA, USA), 20S Proteasome anti-β1 antibody (catalog # sc-67345) or anti-Nrf2 antibody (catalog # sc-722) both from SantaCruz Biotechnology (Santa Cruz, CA, USA). The blocking buffer employed for Western blotting was Startingblock™ buffer (catalog # 37539) from Thermo Fisher (Waltham, MA, USA) and the wash buffer was 1x PBS containing 0.1% Tween 20. An enhanced chemiluminescence kit (Pierce, Rockford, IL) was used for chemiluminescent detection and membranes were analyzed using the biospectrum imaging system (UVP, Upland, CA, USA).

**siRNA ‘knock-down’ of Nrf2 or Proteasome** - Nrf2 (catalog # sc-37049), β1 (catalog # sc-62865), β1i (Lmp2) (catalog # sc-35821), PA28α (catalog # sc-151977) and Scrambled Control (catalog # sc-37007) siRNA were purchased from Santa Cruz biotechnology (Santa Cruz, CA). For experiments with these siRNAs, MEF were seeded at a density of 100,000 cells per well in 6 or 48 well plates and grown to 10% confluence. siRNA treatment was then performed as described in the Santa Cruz Biotechnology product manual.

**Fluorpeptide proteolytic assays** - MEF were harvested by cell scraping in phosphate buffer. Cells were then re-suspended in 50 mM Tris, 25 mM KCl, 10 mM NaCl, 1 mM MgCl₂, 1 mM DTT (pH 7.5) and lysed by 3 freeze-thaw cycles. Protein was quantified using a BCA Protein Assay Kit. Then 5.0 µg of cell lysate per sample were transferred in triplicate to 96 well plates, and 2 µM of N-succinyl-Leu-Leu-Val-
Tyr-AMC (catalog # 80053-860) purchased from VWR (Chester, PA, USA) was then added to the plates. Plates were incubated at 37°C and mixed at 300 rpm for 4 h. Fluorescence readings were taken at 10 minute intervals using an excitation wavelength of 355 nm and an emission of 444 nm. Following subtraction of background fluorescence, fluorescence units were converted to moles of free AMC, with reference to an AMC standard curve of known amounts of AMC (catalog # 164545) purchased from Merck (Whitehouse Station, NJ, USA). In some experiments, cells were treated with 1 μM of the Proteasome inhibitor MG132 (catalog # 474790) from Merck (Whitehouse Station, NJ) or 5 μM of the Proteasome inhibitor Lactacystin (catalog # 80052-806) from VWR (Chester, PA, USA), 30 minutes prior to incubation and addition of substrates. MG132 was dissolved in DMSO at a 100X concentration and combined with samples at a concentration of 1%. In these experiments, control cells were treated with an equivalent concentration of plain DMSO.

**Proteolytic assay of radiolabeled proteins** - Tritium-labeled Hemoglobin [³H]Hb was generated in vitro as described previously (4,6,15,26) using the [³H]formaldehyde and sodium cyanoborohydride method of Jentoft and Dearborn (50), and then extensively dialyzed. Before dialysis, some purified radiolabelled proteins were oxidatively modified by exposure to 1.0 mM H₂O₂ for 1 h in order to generate oxidized substrates. All substrates were then incubated with cell lysates to measure proteolysis. Percentage protein degraded for both Hb and oxidized Hb was calculated by release of acid-soluble (supernatant) counts, by liquid scintillation after addition of 20% TCA (trichloroacetic acid) and 3% BSA (as carrier) to precipitate remaining intact proteins (5,12,15,26), in which % degradation = 100 x (acid-soluble counts – background counts) / total counts.

**Cell counting assay** - Cells were seeded in 100 μl samples at a density of 100,000 cells per ml in 48 well plates. Twenty four hours after seeding, some cells were pre-treated with an oxidant or an Nrf2 inducer. At 48 h after seeding, cells were challenged with a toxic dose of 100 μM – 1 mM hydrogen peroxide for 1 h followed by addition of fresh complete media. Cells were harvested 24 h after challenge, using trypsinization. The cell density of 100μl samples of cell suspensions was then obtained using a Cell Counter purchased from Beckman Coulter (Fullerton, CA, USA).

**Chromatin Immunoprecipitation (ChIP) Assay** – Four million cells were prepared at 10% confluence, the cells were exposed to either 0 or 1 μM H₂O₂ for 1h. ChIP analysis was performed using the reagents and methods provided in a Chromatin Immunoprecipitation Assay Kit (catalogue# 17-295) purchased from Millipore (Temecula, CA, USA). Briefly cells were cross-linked with 1% formaldehyde for 10 minutes, washed twice with PBS, dislodged through scraping and re-suspended in 1 ml of 1% SDS lysis buffer containing protease inhibitor. Samples were sonicated using 10 bursts of 5 seconds, output of 50 watts (Branson Sonifier 140, Branson Ultrasonic, Danbury, CT), and then centrifuged at 13,000g for 10 minutes. The supernatant was removed and diluted in a 10 fold excess of ChIP dilution buffer. (1% of samples were removed at this point to later form the input samples). Samples were pre-cleared using a 30 minute incubation with 30 μl/ml of Salmon Sperm DNA/Protein A Agarose Slurry. Samples were then incubated for 1 h with 8 μg/ml of Nrf2 antibody (catalog # sc-13032) purchased from Santacruz Biotechnology (Santa Cruz, CA, USA) then 30 μl/ml of Salmon Sperm DNA/Protein A Agarose slurry was added and samples were incubated overnight at 4°C under gentle agitation. After this, the bead slurry was subjected to sequential 10 minute washes with Low Salt Immune Complex, High Salt Immune Complex, LiCl Immune complex and TE buffer. Samples were detached from the bead slurry with two washes of 250 μl of 1% SDS 0.1 M NaHCO₃, then reverse cross-linked by incubation with 200 μl of NaCl for 4 h at 65°C, 10 μM EDTA, 40 μM Tris-HCl, and 20 μg of Proteinase K was then added to the samples and samples were incubated for 1 h at 45°C. DNA was isolated and purified from the samples using phenol-chloroform-isoacyl alcohol. PCR was then performed on samples as described below. 5 μl of DNA from each sample was combined with 15 μl of the PCR SyBr Green Master Mix and
Nrf2 Regulates Proteasome Adaptation Revised 01.18.2012

Real-time PCR assay of mRNA levels -Total RNA was extracted using TRIzol reagent and treated with DNasefree reagent according to the manufacturer’s (Invitrogen, Carlsbad, CA, USA, catalog# 1908) protocol to remove DNA. RNA samples were then reverse-transcribed using the TaqMan random hexamers (catalog# N808–0234) purchased from Applied Biosystems (Branchburg, NJ, USA) and the mRNA levels were measured by real-time PCR polymerase chain reaction (RT-PCR) using a 7500 real-time PCR system purchased from Applied Biosystems. In brief, 5μl of reverse transcription reaction product was added to a reaction tube containing 12.5μl of SYBR green PCR Master Mix (catalog# 4367659), 5.5μl of sterile water and 1μl of a 5μM working solution of each primer (forward and reverse) for the proteasome subunit β5 subunit or GAPDH mRNA. The total PCR sample reaction was 25μl. The primer sequences used were as follows: 20S proteasome subunit β5: 5′-GCTGGGCTAACATGGTGTATCAT-3 and 5′-AAGTCACTCATTTGACTTG-3′ as used previously (44). GAPDH: 5′-GATGCAGGGATGATGTTC-3′ and 5′-TGCACCACCAACTGCTTAG-3′.

RESULTS

H2O2, peroxynitrite, paraquat, and menadione pretreatment all increase proteolytic capacity. We have previously reported that adaptation to H2O2 includes large increases in proteasomal proteolytic capacity (26). We now needed to determine if the increase in Proteasome is specific to H2O2, or if it is a more general response to oxidants. We first pretreated MEF cells with various concentrations of H2O2, peroxynitrite, or the redox cycling agents paraquat and menadione for 1 h. Then, 24 h later, we harvested and lysed the cells and measured proteolytic capacity by degradation of the fluorogenic peptide, suc-LLVY-AMC which is widely used to estimate the chymotrypsin-like activity of the Proteasome (5,26,51). We saw a 2-fold increase in proteolytic capacity with H2O2 or paraquat pre-treatment, a 2.5-fold increase with peroxynitrite pre-treatment and a 2-3 fold increase with menadione pre-treatment (Fig. 1A-D). In lysates of untreated cells, the selective Proteasome inhibitor lactacystin caused an 80-90% inhibition of proteolysis. In lysates of oxidant pre-treated cells, lactacystin inhibited degradation by 90-95%, indicating that Proteasome is largely responsible for most of the oxidant-induced adaptive increase in proteolytic capacity (Fig. 1A-D). This experiment was repeated using another Proteasome-selective inhibitor, MG132 which blocked 50% of activity in untreated cells, and 60% of activity following under oxidative stress adaptation (Fig. 1E-F).

H2O2 adaptation increases Nrf2 protein levels and Nrf2 nuclear translocation.

ARE/EpRE sequences are present in the upstream untranslated region of all 20S Proteasome subunit genes examined. If Nrf2 is involved in our model of adaptation to oxidative stress, we would expect to see an increase in total Nrf2 protein levels as a product of enhanced stability following detachment from the Keap1 complex, as well as translocation of Nrf2 from the cytosol to the nucleus: indicative of Nrf2 functioning as a nuclear transcription factor (39,40,45). For initial experiments we used H2O2 as our adaptive oxidant and found that a mild dose of H2O2 caused a two-fold increase in cellular Nrf2 levels (Fig. 2A); this is consistent with previous reports of stress-related induction of Nrf2 (38,40,42,44,45). When we blocked Nrf2 synthesis, using Nrf2 siRNA, we lost the increase in Nrf2 protein (Fig. 2B). We
next examined Nrf2 localization using immunocytochemistry, and saw a notably stronger nuclear-localized staining of Nrf2 in H$_2$O$_2$ treated cells compared to a more widespread staining of all cell compartments in untreated cells (Fig. 2C).

*Nrf2 is an important regulator for the H$_2$O$_2$ induced increase in proteolytic capacity.* Having determined that Nrf2 levels were increased, and that Nrf2 translocated to the nucleus under the conditions of our cellular H$_2$O$_2$ adaptation model, we next wanted to determine if Nrf2 is actually required for the increased proteolytic capacity reported in Fig 1. To examine this we blocked Nrf2 expression by two distinct methods: siRNA and retinoic acid. First, we explored for an Nrf2 siRNA treatment level and time period that would not diminish basal Nrf2 levels, as Nrf2 is maintained at extremely low levels in unstressed cells, but would block adaptive increases in Nrf2. As shown in both Fig. 2B and the inset to Fig. 3A, we were successful in blocking the oxidative stress-induced increase in Nrf2 levels, without reducing the basal levels of Nrf2. Cells pre-treated with Nrf2 siRNA and then exposed to an adaptive dose of H$_2$O$_2$ did not exhibit an H$_2$O$_2$ induced increase in proteolytic capacity, but cells treated with a scrambled siRNA vector showed a normal induction of proteolytic capacity (Fig. 3A). As a further test of Nrf2 involvement, we repeated the experiment of Fig 3A, using retinoic acid treatment, which has been shown to prevent Nrf2 expression in cells (52), as a different means of blocking Nrf2. When we pre-treated cells with retinoic acid and then attempted to adapt the cells to H$_2$O$_2$ as in Fig 3A, we saw no significant increase in Nrf2 levels, and no increase in proteolytic capacity (Fig. 3B).

*Nrf2 is an important regulator of the H$_2$O$_2$ induced increase in proteolytic capacity to degrade oxidized proteins.* While the degradation of suc-LLVY-AMC provides a good approximation of the chymotrypsin-like activity of the Proteasome, what really counts is proteasomal capacity to degrade oxidized proteins. To examine this question, we incubated cell lysates with tritium-labeled hemoglobin ([$^3$H]Hb) and oxidized [$^3$H]hemoglobin ([$^3$H]Hb$_{ox}$). Adaptation to H$_2$O$_2$ pre-treatment caused a two-fold increase in capacity to degrade [$^3$H]Hb, but an almost four-fold increase in selectivity for [$^3$H]Hb$_{ox}$ (Fig. 3C). In contrast, cells pre-treated with siRNA against Nrf2, prior to H$_2$O$_2$ treatment, exhibited no increase in [$^3$H]Hb degradation and less than a 25% increase in capacity to degrade [$^3$H]Hb$_{ox}$ (Fig. 3C). The results of Fig 3 provide strong evidence that Nrf2 has an important role in the increase in proteolytic capacity induced during adaptation to oxidative stress.

*Nrf2 regulates the H$_2$O$_2$ induced expression of 20S Proteasome and PA28$\alpha$$\beta$ but not Immunoproteasome.* Since oxidative stress can increase the levels of 20S Proteasome, Immunoproteasome and the Proteasome regulator Pa28$\alpha$$\beta$ (26,32,33,35,53,54), all of which have been shown to have critical roles in adaptation to oxidative stress (26,32,33,35,53,54), we wanted to determine whether the increases in proteolytic capacity reported in Fig 3 are explained by changes in proteasome, and to determine if Nrf2 plays a critical upstream role. For these studies, we used Western blot analyses of control and H$_2$O$_2$ adapted cells, pre-treated with either Nrf2 siRNA or a scrambled siRNA vector. With scrambled (control) siRNA treatment we saw a two- to three-fold H$_2$O$_2$ induced increase in 20S Proteasome, Immunoproteasome, and Pa28$\alpha$$\beta$ protein levels (Fig. 4A-C). With Nrf2 siRNA pre-treatment, however, the H$_2$O$_2$ induced increase in 20S Proteasome (Fig. 4A) and PA28$\alpha$$\beta$ (Fig. 4B) was lost, indicating that 20S Proteasome and PA28$\alpha$$\beta$ are regulated by Nrf2 during adaptation to stress. In contrast to 20S Proteasome and PA28$\alpha$$\beta$, Nrf2 siRNA treatment had only a weak effect on the H$_2$O$_2$ induced increase in Immunoproteasome levels (Fig. 4C). Nevertheless, H$_2$O$_2$ mediated increases in 20S Proteasome, Immunoproteasome, and Pa28$\alpha$$\beta$ were clearly all important for adaptive increases in cell tolerance (survival) of H$_2$O$_2$ challenge treatments (Fig. 5A). Thus, we must conclude that Immunoproteasome regulation during oxidative stress is either wholly or partially independent of Nrf2, and other factor(s) must be involved. In support of this idea, we find that, although 20S Proteasome subunits contain a few
ARE/EpRE sequences in their promoter regions, ARE/EpRE sequences are completely absent in two of the three Immunoproteasome-specific subunits (Fig. 5B). While such analyses are not conclusive, the results are certainly suggestive. We confirmed that at least some of the EpRE elements upstream of 20S Proteasome subunits are not only present, but H₂O₂ induces binding of Nrf2 to these sequences. To test this we performed a chromatin immunoprecipitation assay (ChIP) on an EpRE element in the 5’-untranslated region (5’-UTR) of the Proteasome β5 subunit gene, which has previously been shown to have functional EpRE elements (44). This EpRE element showed a strong increase in Nrf2 binding under H₂O₂ exposure (Fig. 5C), thus demonstrating that 20S Proteasome induction under our H₂O₂ adaptation conditions is mediated by the Nrf2 signal transduction pathway. Using RT-PCR, we were also able to demonstrate a corresponding, hydrogen peroxide-induced, two-fold, increase in cellular mRNA levels of the 20S proteasome β5 subunit during the same time period (Fig. 5D).

Pre-treatment with Nrf2 ‘inducers’ causes increased tolerance to oxidative stress.

We have developed a transient oxidative stress-adaptive model in which pre-treatment of cells with a low concentration of H₂O₂ causes changes in gene expression that permit survival of a much higher, normally toxic, challenge dose of H₂O₂ delivered 24 h later (26,30). Without pre-treatment with a mild dose of H₂O₂, the challenge dose causes protein oxidation, growth arrest, diminished DNA and protein synthesis, and some degree of apoptosis; all these measures of toxicity are avoided or minimized if cells are adapted by pre-treatment with a mild dose of H₂O₂ before being exposed to the challenge dose (26,29-31,55). We now wanted to test if adaptive resistance to H₂O₂ toxicity could be achieved by pre-treatment with a wide range of Nrf2 inducers (both oxidative and non-oxidative). In other words, we wanted to test whether adaptive increases in oxidative stress resistance, via increased proteasomal capacity, is a general feature of the Nrf2 signal transduction pathway. As shown in Fig 6A, 1.0 mM H₂O₂ challenge caused a 65% decrease in cell counts in non-adapted, naïve, cells; this was mostly due to prolonged growth arrest, as previously shown (26,29-31,55). In contrast, cells that had been pre-treated with (low concentrations of) a range of oxidants exhibited substantially less toxicity: only a 29% growth arrest with H₂O₂ pre-treatment, 37% with paraquat, 42% with menadione, and 50% with peroxynitrite (Fig. 6A). We also tested other inducers of Nrf2, including DL-sulforaphane (56-58), curcumin (59-62), and lipoic acid (63-65). Growth-arrest induced by H₂O₂ challenge was decreased (from 65%) to 39% with DL-sulforaphane pre-treatment, to 31% with curcumin pre-treatment, and to only 35% with lipoic acid pre-treatment (Fig. 6A). While it is important to note that these agents are not exclusive inducers Nrf2, the fact that all produced protective effects provides additional support for an important role for Nrf2 in oxidative stress adaptation.

Nrf2, 20S Proteasome, PA28αβ and Immunoproteasome all play important roles in the H₂O₂ induced adaptive increase in oxidative stress tolerance.

The 20S Proteasome, the Immunoproteasome and the PA28αβ regulator all seem to play important roles in adaptation (26). We now confirmed this conclusion, using siRNA directed against the 20S Proteasome, Immunoproteasome and PA28αβ regulator. With H₂O₂ challenge there was a 55% decrease in cell growth; this was reduced to only a 30% decrease with H₂O₂ pre-treatment and adaptation (Fig. 5A). However, if cells were first pre-treated with siRNA against 20S Proteasome, Immunoproteasome or PA28αβ, the adaptive response was severely blunted and H₂O₂ challenge induced growth-arrest returned to 50-60% (Fig. 5A). Having shown that Nrf2 plays a key regulatory role in H₂O₂ induced increases in 20S Proteasome and PA28αβ (Fig. 4) we were interested in testing if the adaptive role of these proteins in increasing tolerance to H₂O₂ challenge is also Nrf2 dependent. Using the ‘pre-treatment & challenge model’ there was a shift from 65% growth arrest to only 35% growth arrest with H₂O₂ pre-treatment; this returned to 67% growth arrest if cells were pre-treated with Nrf2 siRNA (Fig. 6B), indicating a significant role for Nrf2 in H₂O₂ induced tolerance to oxidative stress.
Nrf2 and Proteasome are key factors in the adaptive increase in tolerance to oxidative stress produced by Nrf2 ‘inducers’. Having observed an adaptive response with the use of multiple Nrf2 ‘inducers’ we wanted to determine if Proteasome and the Pa28αβ regulator are always involved in Nrf2-dependent adaptation. To test this we performed western blots on cells 24 h after pre-treatment with a range of concentrations of various Nrf2 inducers. We observed modest increases (≈40%) in 20S Proteasome with lipoic acid and curcumin treatment, and more than a two-fold increase with DL-sulforaphane (Fig. 7A). To test the role of both Nrf2 and Proteasome in the adaptive response to Nrf2 ‘inducers’ we used the pre-treatment & challenge model of Fig 6, with a background of scrambled siRNA, Nrf2 siRNA or 20S Proteasome siRNA (Fig. 7B). With H\textsubscript{2}O\textsubscript{2} challenge of non-adapted cells there was a 68% growth-arrest. Lipoic acid pre-treatment reduced growth arrest to ≈50%; however, growth arrest was returned to ≈85% with either Nrf2 or 20S Proteasome siRNA treatment. Similarly, DL-sulforaphane treatment reduced growth arrest to ≈40%, which was returned to ≈85% with either Nrf2 or 20S Proteasome siRNA treatment. Curcumin treatment reduced growth arrest to ≈40% which was restored to ≈85% with 20S Proteasome siRNA and to 70% with Nrf2 siRNA (Fig. 7B).

DISCUSSION

Our studies reveal a mechanistic link between Nrf2, the 20S Proteasome, the PA28αβ (11S) Proteasome regulator, and transient adaptation to oxidative stress. It now appears clear that the Nrf2 signal transduction pathway plays a major role in both the increased proteasomal capacity to degrade oxidized proteins, and the increased cellular tolerance to oxidative stress that are induced by pre-treatment with a mild dose of oxidant.

We find that cellular capacity to degrade oxidized proteins, and intracellular levels of the 20S Proteasome, Immunoproteasome, and the PA28αβ (11S) regulator are all increased two- to three-fold during adaptation to oxidative stress. Similar results were obtained with the oxidants H\textsubscript{2}O\textsubscript{2} and peroxynitrite, and the redox-cycling agents menadione and paraquat. Proteasome inhibitors, and siRNA directed against the 20S Proteasome β1 subunit, the Immunoproteasome β1i (LMP2) subunit, or the PA28α (11S) regulator subunit, all significantly limited the increase in cellular proteolytic capacity and partially prevented the increased resistance to oxidative stress (cell growth).

Cellular levels of Nrf2 were significantly increased by adaptation to oxidative stress, and Nrf2 was seen to translocate to the nucleus, and to bind to ARE/EpRE sequence(s) upstream of the Proteasome β5 subunit gene. Blocking the induction of Nrf2, with siRNA or with retinoic acid, significantly limited the adaptive increases in cellular proteolytic capacity, 20S Proteasome and the Pa28αβ regulator. Increases in the Immunoproteasome, however, were only partially blocked by Nrf2 siRNA. Blocking Nrf2 induction also limited the increase in oxidative stress resistance (cell growth). When, instead of using oxidant exposure, we pre-treated cells with the Nrf2 inducers lipoic acid, curcumin, or sulforaphane, we observed increased cellular proteolytic capacity, increased 20S Proteasome, and increased cellular resistance to oxidative stress (cell growth); both Nrf2 siRNA and 20S Proteasome β1 subunit siRNA effectively blocked these increases.

These results suggest that oxidants, redox cycling agents, and other Nrf2 ‘inducers’ cause adaptation through the upregulation of Nrf2 and its translocation to the nucleus. This, in turn, induces expression of the 20S Proteasome and the PA28αβ regulator. In contrast, the Immunoproteasome, whose levels were also increased by adaptation to oxidative stress, appears to be only partially regulated by Nrf2, if at all.

The Nrf2 signal transduction pathway is known to respond to stressful conditions (37-46,66). Under non-stress conditions Nrf2 is retained in the cytoplasm through the formation of a complex with several proteins, including Keap1. In this state it is constantly turned over through ubiquitin-dependant 26S Proteasome degradation. This permits a high expression rate, enabling rapid accumulation of Nrf2 when
degradation is blocked, while ensuring low Nrf2 steady-state levels under normal conditions. Pre-
treatment with an oxidant, or other Nrf2 inducer, liberates Nrf2 from the Keap1 complex. This also prevents further Nrf2 degradation resulting in a dramatic rise in Nrf2 cellular levels as well as its translocation to the nucleus. Once there, it can bind to anti-oxidant response elements (ARE’s) that have also been called electrophile response elements (EpRE’s), in a range of genes.

We find that genes encoding many 20S Proteasome subunits contain at least one if not multiple ARE/EpRE sequences in their upstream, untranslated regions (Fig. 5B) and have shown that at least some of these ARE/EpRE sequences have a strong increase in Nrf2 binding under H₂O₂ exposure. In contrast, we find only a single subunit of the three Immunoproteasome subunits contains the ARE/EpRE sequence. It is tempting to suggest that this difference in density of ARE/EpRE sequences may explain the differential sensitivity of the 20S Proteasome and the Immunoproteasome to Nrf2 siRNA and retinoic acid, and to propose that Immunoproteasome may be regulated by another mechanism.

Nrf2 is not the only protein that can bind to ARE/EpRE sequences, and it is certainly possible that other signal transduction proteins may bind to proteasomal and Pa28αβ (11S) regulator ARE/EpRE elements, and/or to Immunoproteasome. We are also searching for other potential pathways for Immunoproteasome induction, of which the Interferon Regulatory Factor 1 (67-69) appears to be a good candidate. Finally, there may well be overlapping pathways of signal transduction that act synergistically, or antagonistically, to dynamically adjust Proteasome/Immunoproteasome levels during adaptation to oxidative stress.

In conclusion, we find that increases in 20S Proteasome and Pa28αβ (11S) regulator expression are largely mediated by the Nrf2 signal transduction pathway during adaptation to oxidative stress. These Nrf2-dependent increases in 20S Proteasome and Pa28αβ (11S) are shown to be important for fully effective adaptive increases in cellular stress resistance. In contrast, the Immunoproteasome, which also contributes to oxidative stress adaptation, is shown to be minimally responsive to Nrf2 control.
REFERENCES


**FOOTNOTES**

*This research was supported by grant #RO1-ES003598, and by ARRA Supplement 3RO1-ES 003598-22S2, both from the NIH/NIEHS to KJAD.

The abbreviations used are: *H<sub>2</sub>O<sub>2</sub>* , hydrogen peroxide; MEF, murine embryonic fibroblasts; Nrf2, Nuclear factor (erythroid-derived 2)-like 2; PA28αβ, Proteasome Activator 28 αβ, ARE, Anti-oxidant response element; EpRE, electrophile response element; AMC, 7-Amino-4-Methylcoumarin; Suc-LLVY-AMC, *N*-Succinyl-Leucine-Leucine-Valine-Tyrosine-7-Amino-4-Methylcoumarin.

**FIGURE LEGENDS**

**Fig. 1:** Oxidant Pre-treatment Increases Proteolytic Capacity in a Proteasome Dependent Manner. Cells treated with a mild dose of a range of oxidants exhibit increased proteolytic capacity, the majority of which (80-95%) is blocked by the Proteasome selective inhibitor lactacystin. MEF cells were grown to 10% confluence (~250,000 cells per ml) and treated with **A:** 0μM - 100μM *H<sub>2</sub>O<sub>2</sub>* , **B:** 0μM - 1μM Peroxynitrite, **C:** 0 nM – 100 nM Paraquat or **D:** 0 nM – 100 nM Menadione. All treatments were for one h in complete media, following which the media was removed and replaced with fresh complete media (see Experimental Procedures). After 24 h, cells were lysed and diluted to a protein concentration of 50μg per ml. Proteolytic capacity was determined by cleavage of the Proteasome chymotrypsin-like substrate suc-LLVY-AMC (see Experimental Procedures). Where used, 5μM Lactacystin was added to samples, 30 minutes prior to incubation with suc-LLVY-AMC. Values are Means ± SE, n = 3. **E:** MEF cells were prepared as described in A and pre-treated with 100nM peroxynitrite, 1μM *H<sub>2</sub>O<sub>2</sub>* , 1nM menadione, or 1nM paraquat for one hour in complete media; following this, the media was removed and replaced with fresh complete media. In some samples, 1μM MG132 was added 30 minutes prior to incubation with suc-LLVY-AMC. Cells were incubated, harvested lysed, diluted and analyzed for proteolytic capacity by lysis of the fluorogenic peptide suc-LLVY-AMC, as in panels A-D, and Experimental Procedures. Values are Means ± SE, n = 3. **F:** Results from E were re-plotted with the decrease in activity resulting from addition of MG132 plotted as a percent of the proteolytic capacity of cells not treated with the inhibitor.

**Fig. 2:** Nrf2 Protein Levels and Nuclear Translocation During Oxidative Stress Adaptation. **A:** *H<sub>2</sub>O<sub>2</sub>* treatment causes an increase in whole-cell levels of Nrf2. MEF cells were grown to 10% confluence, treated for 1 h with 0 - 100μM *H<sub>2</sub>O<sub>2</sub>* , and then washed and resuspended (see Experimental Procedures). Cells were harvested and lysed 24 h following oxidant pre-treatment as described in the Western blot section of Experimental Procedures. Cell lysates (20μg) were run on SDS-PAGE and transferred to PDVF membranes. The membranes were screened with antibodies directed against Nrf2 and β-tubulin and results were quantified by densitometry. All experiments were repeated in triplicate and densitometric band intensities for Nrf2 were normalized to those of β-tubulin. Values are Means ± SE, n = 3. **B:** The increase in Nrf2 band intensity is lost with Nrf2 siRNA pre-treatment. Samples were prepared as in Panel A except that, 4 h prior to *H<sub>2</sub>O<sub>2</sub>* treatment, cells were pre-treated with siRNA against Nrf2, or with a
scrambled vector, and gels were then run as in Panel A. C: Treatment of cells with H$_2$O$_2$ causes Nrf2 to shift from a broad cytoplasmic distribution to a nuclear localization. MEF cells were grown to 50% confluence and treated with 100 µM H$_2$O$_2$ for 1 h then fixed and stained with an antibody directed against Nrf2, as described in the immunocytochemistry section of Experimental Procedures. Representative photographs are shown, but the experiment was repeated several times with similar results.

Fig. 3: Increased Proteolytic Capacity is Blocked by Inhibition of Nrf2. A: The increase in proteolytic capacity caused by H$_2$O$_2$ treatment was blocked by inhibition of Nrf2 expression through pre-treatment with Nrf2 siRNA. MEF cells were grown to 10% confluence and treated with either control or Nrf2 siRNA for 24 h as described in Experimental Procedures. Then, 4 h after initiation of siRNA treatment, half the cells were exposed to 1µM H$_2$O$_2$ for 1 h, washed, and resuspended (see Experimental Procedures). Capacity to degrade the fluorogenic peptide suc-LLVY-AMC was determined 24 h after initiation of siRNA treatment (see Experimental Procedures). Values are Means ± SE, n = 3. The inset to Panel A shows a representative Western blot. B: Treatment of cells with the Nrf2 inhibitor retinoic acid also blocked the H$_2$O$_2$ induced increase in proteolytic capacity. MEF cells were seeded at 5% confluence and treated with 3 µM Retinoic acid. When cells reached 10% confluence half were exposed to 1µM H$_2$O$_2$ and proteolytic capacity (suc-LLVY-AMC lysis) was determined 24 h after treatment as in Panel A. Values are Means ± SE, n = 3. The inset to Panel B shows a representative Western blot. C: The H$_2$O$_2$ induced increase in selective capacity to degrade oxidized proteins is also blocked by inhibition of Nrf2 expression. MEF cells were prepared and lysed as described in Panels A and B. Lysates were incubated for 4 h with $[^3]$H]Hb or $[^3]$H]Hb$_{ox}$. Percent protein degraded was calculated after addition of 20% TCA and 3% BSA, and centrifugation to precipitate remaining intact proteins (5,12,15,26). Percent protein degradation was determined by release of acid soluble counts in TCA supernatants, by liquid scintillation as follows: % degradation = (acid soluble counts – background counts) / total counts x 100. Results are Means ± SE, n = 3.

Fig. 4: H$_2$O$_2$ Induced Expression of Proteasome and Proteasome Regulators is Nrf2 Dependent. H$_2$O$_2$ treatment causes an increase in A: 20S Proteasome and B: the Proteasome regulator Pa28, both of which appear to depend upon Nrf2 expression. The increase in Immunoproteasome C: with H$_2$O$_2$ treatment may be only partly Nrf2 dependent, at best. MEF cells were prepared, treated and harvested as described in Fig 3. The cells were then lysed, and samples were run on SDS-PAGE gels and transferred to PDVF membranes as in Fig 2. Membranes were treated with antibodies directed against 20S Proteasome subunit β1, Immunoproteasome subunit β1i (LMP2), Proteasome regulator subunit PA28α, Nrf2 and β-tubulin. Graphs A, B, and C show the levels of 20S Proteasome β1 subunit (Panel A), Pa28α regulator (Panel B), and Immunoproteasome β1i or LMP2 (Panel C) each divided by β-tubulin levels for each well and then plotted as a percent of control. Values are Means ± SE, n = 3. D: Representative Western blots for β1, β-tubulin, β1i (LMP2), and Pa28α (all ± Nrf2 siRNA) for graphs A-C.

Fig. 5: 20S Proteasome is Required for H$_2$O$_2$ Adaptation, and Contains Active EpRE Elements A: MEF cells were grown, and incubated with siRNA directed against, 20S Proteasome subunit β1, Immunoproteasome subunit β1i (LMP2), Proteasome regulator subunit PA28α, or a scrambled vector, then pre-treated with 1µM H$_2$O$_2$ as described in Fig 3. After 24 hours, cells were challenged with a dose of 1mM H$_2$O$_2$ for 1 hour, washed, and resuspended in fresh complete media. After another 24 hours, cells were harvested and cell counts were taken. Values are plotted as a percent of unchallenged samples treated with scrambled siRNA which had an average cell density of 110,000 cells per ml at the point of counting. Values are Means ± SE, n = 4. B: ARE/EpRE Consensus Sequences (TGANNNGC / GCNNNNTCA) are present upstream of all 20S Proteasome and Immunoproteasome subunit genes Data represent sequences 5kb upstream of promoters, based on NCBI data base of Mus musculus. ARE/EpRE sequences are highlighted. C: H$_2$O$_2$ treatment causes increased binding of Nrf2 to one of the EpRE
elements upstream of the promoter of the Proteasome β5 subunit gene. Cells were grown to 10% confluence then exposed to 1μM H$_2$O$_2$ for 1 h. ChIP analysis was then performed as described in Experimental Procedures. Non-specific binding was measured through performing a ChIP assay in the absence of the Nrf2 antibody and input was as an internal control by representing 1% of the sample prior immunoprecipitation. D. H$_2$O$_2$ treatment causes increased mRNA expression of the 20S proteasome β5 subunit. Cells were grown to 10% confluence then exposed to 1μM H$_2$O$_2$ for 1 h. After this cells were harvested, the mRNA levels of the 20S proteasome subunit β5 and the loading control GAPDH were then determined through reverse transcriptase PCR followed by qPCR. Values are plotted, in arbitrary units, adjusted by levels of GAPDH. Values are Means ± SE where n = 3

Fig. 6: Pre-treatment with Nrf2 Inducers Causes Increased Tolerance to Oxidative Stress
A: Pretreatment of cells with a mild, non-toxic, dose of a range of oxidants, and other inducers of Nrf2, causes increased tolerance to a subsequent toxic H$_2$O$_2$ challenge. MEF cells were grown to 10% confluence and treated with 1 nM peroxynitrite, 1 nM menadione, 10μM DL-sulforaphane, 1μM paraquat, 500μM curcumin, 100 nM H$_2$O$_2$ or 500μM lipoic acid, for 1 h, then washed and resuspended (see Experimental Procedures). After 24 h, cells were challenged with 1 mM H$_2$O$_2$ for 1 h, then washed and resuspended. After another 24 h, cells were harvested and counted. Values are Means ± SE, n = 3. B: The increase in tolerance to H$_2$O$_2$ challenge induced by mild oxidant pretreatment is lost by blocking Nrf2 expression. MEF cells were grown, and treated with siRNA directed against Nrf2 or a scrambled vector, then pre-treated (or not) with 1μM H$_2$O$_2$ as described in Fig 3. After 24 h, cells were challenged with 1 mM H$_2$O$_2$ for 1 h, then washed and resuspended in fresh complete media. After another 24 h, cells were harvested and cell counts were taken. Values are plotted as a percent of unchallenged samples treated with scrambled siRNA, and values are Means ± SE, n = 4.

Fig. 7: Lipoic Acid, DL-Sulforaphane and Curcumin Promote Adaptation in an Nrf2 and Proteasome Dependent Manner.
A: MEF cells were grown to 10% confluence and treated with 1 nM-100 nM Lipoic acid, 1 nM-100 nM DL-Sulforaphane, or 1 nM-100 nM Curcumin. After 24 h, cells were harvested, lysed, run on SDS-PAGE gels and transferred to PDVF membranes as in Fig 2. Membranes were treated with antibodies directed against 20S Proteasome subunit β1, Immunoproteasome subunit β1i (LMP2), Proteasome regulator subunit PA28α, and β-tubulin. B: The adaptive response produced by Nrf2 inducers is lost or blunted by blocking either Nrf2 or Proteasome expression. MEF cells were grown, and pretreated with siRNA directed against Nrf2, 20S Proteasome subunit β1, or a scrambled vector then, 4 h later, treated with 500μM lipoic acid, 10μM DL-sulforaphane, or 500μM curcumin, as described in Fig 6. After 24 h, cells were challenged with 1 mM H$_2$O$_2$ for 1 h, then washed and resuspended in fresh complete media. After another 24 h, cells were harvested and cell counts were taken. Values are Means ± SE, n = 4, plotted as a percent of unchallenged samples treated with scrambled siRNA.
Figure 1: Oxidant Pre-treatment Increases Proteolytic Capacity in a Proteasome Dependent Manner

- Lactacystin
+ Lactacystin

H₂O₂ (µM)

Peroxynitrite (nM)

Paraquat (nM)

Menadione (nM)

Proteolytic Activity (100 x nMols AMC released min⁻¹ mg protein⁻¹)

- MG132
+ MG132

Control
Peroxynitrite
Menadione
Paraquat
H₂O₂

% MG132 Inhibition

25
50
75

Downloaded from http://www.jbc.org/ by guest on August 29, 2017
Figure 2: Nrf2 Protein Levels and Nuclear Translocation During Oxidative Stress Adaptation

A

B

C

Control

H_2O_2 Treatment

250X

1000X
Figure 3: Increased Proteolytic Capacity is Blocked by Inhibition of Nrf2

A

B

C

Proteolytic Capacity (nmol AMC Released min⁻¹ mg Lysate⁻¹)

Protein Degraded (% [³H]Hb or [³H]Hb₆₅)

Control siRNA Nrf2 siRNA

Control 1 μM H₂O₂

Control siRNA Nrf2 siRNA

Control 1 μM H₂O₂

0 1 0 1

0 1 0 1

Control Retinoic acid

Control H₂O₂ Retinoic acid

Control Nrf2 siRNA

Control H₂O₂ Nrf2 siRNA

[³H]Hb [³H]Hb₆₅
Figure 4: \( H_2O_2 \) Induced Expression of Proteasome and Proteasome Regulators is Nrf2 Dependent

A. 20S Proteasome

B. Pa28αβ

C. Immunoproteasome

D. Western Blot Analysis

Scrambled siRNA

Nrf2 siRNA
Figure 5: 20S Proteasome is Required for Adaptation and Contains Active EpRE Elements
Figure 6: Pre-treatment with Nrf2 Inducers Causes Increased Tolerance to Oxidative Stress

A

Cell Count (% of Unchallenged Control)

B

Cell Count (% of Unchallenged Control)

siRNA pre-treatment

Control
Nrf2

1mM H2O2 Challenge

Control
H2O2 Pre-treated
Figure 7: Lipoic Acid, DL-Sulforaphane, and Curcumin Promote Adaptation in an Nrf2 and Proteasome Dependent Manner

A

Relative 20S Proteasome level (% of Control)

B

Cell Count (% of Unchallenged Control)

- 20S Proteasome β1 siRNA
- Nrf2 siRNA
- Control siRNA

Lipoic Acid
DL-Sulforaphane
Curcumin

Control
Lipoic Acid
DL-Sulforaphane
Curcumin

0 1 10 100

0 50 100 150 200 250

0 10 20 30 40 50 60 70

DI-Sulforaphane/Lipoic acid
Curcumin (nM)
Nrf2 dependent induction of proteasome and Pa28αβ regulator is required for adaptation to oxidative stress
Andrew M. Pickering, Robert A. Linder, Hongqiao Zhang, Henry J. Forman and Kelvin J. A. Davies

J. Biol. Chem. published online February 3, 2012

Access the most updated version of this article at doi: 10.1074/jbc.M111.277145

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/early/2012/02/03/jbc.M111.277145.full.html#ref-list-1