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Conditioned Media Downregulates Nuclear Expression of Nrf2

Saheli Sarkar^{1,2}, Christine K. Payne^{2,3}, and Melissa L. Kemp^{1,3,*}

¹The Wallace H. Coulter Department of Biomedical Engineering, Georgia Institute of Technology and Emory University, Atlanta, Georgia 30332

²School of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, Georgia 30332

³Parker H. Petit Institute for Bioengineering and Biosciences, Georgia Institute of Technology, Atlanta, Georgia 30332

Abstract

Nuclear factor erythroid 2-related factor-2 (Nrf2) is a redox-sensitive transcription factor that activates several antioxidant and cytoprotective genes in response to oxidative stress. The role of Nrf2 activators and the intracellular regulation of Nrf2 have been studied extensively. In comparison, little is known about the self-regulation of Nrf2 due to experimental techniques commonly used to synchronize cellular signaling. Here we report that endogenous Nrf2 was downregulated in the nucleus of HeLa and MDA-MB-231 cells serum starved for 24hrs. Nrf2 expression was rescued by the addition of unconditioned media irrespective of its serum content. No concomitant change was observed in the expression of the primary inhibitor of Nrf2, Kelch-like ECH-associated protein-1 (Keap1). Nrf2 was upregulated by *tert*-butyl hydroquinone, although there was limited increase in Nrf2 in conditioned media-treated cells as compared to unconditioned media-treated cells. Decreasing the fraction of conditioned media in culture resulted in a dose-dependent increase in Nrf2 protein level. Taken together, our data suggests the existence of a complex self-regulatory mechanism for endogenous Nrf2 signaling.

Keywords

Nrf2; Keap1; conditioned media; starvation; serum-free media; cell synchronization

INTRODUCTION

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a Cap 'n' Collar basic leucine zipper transcription factor crucial for defense against oxidative stress¹⁴. Nrf2 is widely expressed in most tissues and cell lines²⁵ and extensively regulated (reviewed in^{34, 37}). In the basal state, Nrf2 degradation by the ubiquitin pathway is expedited by Kelch-like ECH-associated protein-1 (Keap1), which serves as an adapter to the Cul3-Rbx ubiquitin ligase complex. The Nrf2-Keap1 interaction is generally thought to occur in the cytoplasm, although Pickett and colleagues have argued that Nrf2 exists primarily in the nucleus in a constitutively active form²⁷. Regardless of the location for this protein-protein regulation, Nrf2 activation is known to be induced by oxidative stress. Accumulation of reactive oxygen species (ROS) or electrophilic compounds leads to thiol modifications of Keap1 and phosphorylation of Nrf2, disrupting the Nrf2-Keap1 complex. Free Nrf2 binds to the Antioxidant Response Element (ARE) DNA sequence in conjunction with small Maf or Jun proteins and cofactors such as CREB-binding protein (CBP). ARE is found in the promoters of a multitude of

* Corresponding author Melissa Kemp Associate Professor Georgia Institute of Technology 315 Ferst Drive, Room #1314 Atlanta, GA 30332-0363. Tel: 404-385-6341 Fax: 404-385-8109 melissa.kemp@bme.gatech.edu.

genes that serve as cytoprotective antioxidants, phase I and II detoxification enzymes and transporters¹⁰. The Nrf2-ARE interaction activates a large-scale transcription program that ultimately promotes adaptation to oxidative stress and cell survival. However, Nrf2 activation can also be detrimental during tumor progression as constitutive expression of Nrf2 has been implicated in chemoresistance and tumorigenesis^{7, 40}. Dysregulation of this pathway due to mutation or epigenetic modification of Nrf2 or Keap1 has been observed in various types of cancers, pulmonary inflammatory diseases, neurodegenerative diseases and hepatic toxicity¹⁵.

In two different studies, authors have shown that Nrf2 disruption interferes with cell cycle progression^{11, 31}. Primary alveolar epithelial cells from Nrf2^{-/-} mice were shown to have undergone G₂/M checkpoint arrest, which was reversed by glutathione³¹. Human lung carcinoma cell lines A549 and NCI-H292 were arrested in G₀/G₁ phase after Nrf2 knockdown¹¹. These data suggest that Nrf2 is differentially regulated during cell cycle. From a technical perspective, the practice of cell synchronization at the G₀/G₁ phase via serum starvation could have a strong impact on Nrf2 analysis, yet it is a well-established experimental method of inducing quiescence in *in vitro* cultures by lowering serum content to 0.1-0.5% FBS for various periods extending from minutes to days²⁸.

The lack of a standardized protocol causes an inherent variability in experimental systems. Comprehensive studies have suggested that serum starvation can lead to broad spectrum changes in intracellular as well as secreted proteins; thus the interpretation of Nrf2 results from different laboratories may be problematic. For example, cells have been starved in media containing 0-1% FBS from 2hrs to 24hrs as part of culture conditions in Nrf2 signaling studies^{1, 12, 23, 30, 45, 46}. Serum starvation (0.5% FBS, 24hrs) led to significantly different protein and phosphoprotein expression in gliomas and adenocarcinomas²¹. While gliomas showed upregulated Akt, PI-3K and PKC and anti-apoptotic pathway components, adenocarcinomas downregulated Akt, Gab2 and survivin and increased p53. Eichelbaum *et al.* found that 3hrs of complete serum deprivation could alter protein secretion slightly (5-34 proteins), while 24hrs of serum deprivation resulted in the modified secretion of >160 proteins in two cell lines⁸. These proteins include growth factors, cytokines and regulators of proliferation, signaling and cholesterol homeostasis. Thus, serum starvation evidently results in altered cellular dynamics and consequently, the outcome of an experiment.

Several reports have examined secreted proteins present in conditioned media from a variety of cell^{9, 19, 43}. The efforts of these researchers show that conditioned medium is a rich source of proteins including, but not limited to, metabolic, differentiation, motility, adhesion, transcription, translation and signal transduction factors. Both *in vitro* and *in vivo*, these factors are used by cells for autocrine and paracrine regulation of various cellular processes^{16, 29, 44}. However, very little is known about the impact of conditioned media on Nrf2 signaling. Astrocyte-conditioned media has been shown to promote nuclear accumulation of Nrf2 and activate transcription of heme oxygenase-1 in microglia²⁴. Depending on the type of stimuli used to generate conditioned media, neuronal viability was differentially mediated by the Nrf2 pathway¹⁸. Conditioned media obtained from LPS-stimulated microglia altered Nrf2 activation in astrocytes⁴. These studies suggest that paracrine soluble factors regulate Nrf2 signaling mechanisms in brain cell cultures.

In this study, we report that conditioned media from serum starved HeLa cells down-regulates endogenous Nrf2 expression in the nucleus. In contrast, unconditioned low-serum media increases Nrf2 expression within 3hrs. A second cell line, MDA-MB-231 breast cancer cells, showed similar trends. Decreasing the quantity of conditioned media in culture resulted in a dose-dependent increase in Nrf2 expression. No concomitant change was observed in Keap1 levels. A potent Nrf2 activator, *tert*-butyl hydroquinone (tBHQ),

upregulated Nrf2; however, the increase in Nrf2 expression in the presence of conditioned media was not as strong as in unconditioned media. In summary, our findings imply that self-conditioning of cell culture media is an important factor in the regulation of endogenous Nrf2 expression.

MATERIALS AND METHODS

Reagents and Antibodies

Dulbecco's Modified Eagle Medium (DMEM) containing 4.5g/L glucose and Fetal Bovine Serum (FBS) were purchased from Sigma-Aldrich (St. Louis, MO). Penicillin/streptomycin was obtained from Mediatech (Manassa, VA). Hank's Balanced Salt Solution (HBSS) containing 1g/L glucose was from Thermo Scientific (Rockford, IL). *Tert*-butyl hydroquinone (*t*BHQ) was obtained from Acros Organics (Fair Lawn, NJ). The primary antibodies used in this study were Nrf2 (C-20, Santa Cruz Biotechnology, Santa Cruz, CA), Keap1 (Cell Signaling Technology, Danvers, MA), TATA-binding protein (TBP, Abcam, Cambridge, MA) and Actin (Sigma). Secondary antibodies (IRDye 680CW donkey anti-mouse and IR dye 800CW donkey anti-rabbit) were purchased from LI-COR Biosciences (Lincoln, NE).

Cell Culture

The human cervical adenocarcinoma cell line HeLa was purchased from American Type Culture Collection (Manassas, VA). The human breast adenocarcinoma cell line MDA-MB-231/RFP, originally purchased from Cell Biolabs (San Diego, CA), was a gift from Dr. Manu O. Platt (Georgia Institute of Technology, Atlanta, GA). Both cell lines were regularly cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin in a humidified atmosphere of 37°C and 5% CO₂. Culture media was replaced every 2-3 days. All experiments were performed at 60-70% confluency.

1×10⁶ HeLa or MDA-MB-231 cells were seeded in 175cm² flasks and allowed to grow for 48hrs, following which they were serum starved for 24hrs in DMEM supplemented with 0.5% FBS and 1% penicillin/streptomycin (serum starvation media). The starved cells were then treated with either conditioned or unconditioned media for 3hrs unless otherwise mentioned. In specific instances, cells were serum-deprived in DMEM containing 1% penicillin/streptomycin only (serum-free media). For consistency, conditioned media was freshly prepared for each experiment following the same procedure, *i.e.*, incubating cells with serum starvation media for 24hrs or serum-free media for 12hrs. Whenever HBSS was used, it was supplemented with 1% penicillin/streptomycin. Nrf2 was activated by adding 100μM *t*BHQ to conditioned or unconditioned media for 3hrs.

Cell Lysis and Fractionation

Cytoplasmic and nuclear lysates were obtained using Nuclear Extraction Kit (EMD Millipore, Billerica, MA). Briefly, cells were washed and scraped into ice-cold PBS, centrifuged and lysed with 1X cytoplasmic lysis buffer containing protease inhibitor cocktail and dithiothreitol (DTT) diluted 1:1000 and 1:2000 respectively according to the manufacturer's instructions. After 15min on ice, the suspension was centrifuged at 250×g for 5min. The pellet was re-suspended in lysis buffer and sheared by passing through a 25 gauge needle five times. A second centrifugation was done at 8000×g for 20min at 4°C. The supernatant was collected and saved as cytoplasmic lysate. The nuclear pellet was suspended in nuclear extraction buffer containing protease inhibitors and DTT, sonicated five times for 3min each followed by 3min on ice. The suspension was placed in a shaker for 1hr at 4°C. Nuclear lysates were obtained by centrifugation at 14000×g for 5min at 4°C. All lysates

were stored at -80°C. Protein content was assayed using Micro BCA™ Assay Kit (Thermo Scientific).

Western Blotting

Western blots were carried out with 60µg of cytoplasmic or nuclear protein using 12% Mini-PROTEAN® TGX™ gels (Bio-Rad Laboratories, Hercules, CA). After transferring proteins to PVDF membranes, they were blocked with Near Infra-Red Blocking Buffer (Rockland Immunochemicals, Gilbertsville, PA) at room temperature for 1hr. Each PVDF membrane was divided into two parts, the top part containing proteins of molecular weight >50kDa and the bottom part containing proteins of molecular weight <50kDa. The top parts were incubated with either Nrf2 (detected at 100kDa) or Keap1 (60kDa), while the bottom parts were incubated with Actin (42kDa) for cytoplasmic lysates or TBP (38kDa) for nuclear lysates. All primary antibodies except TBP were diluted 1:1000 in blocking buffer and incubated overnight at 4°C. TBP was diluted 1:2000. Secondary antibodies were diluted 1:10000 in blocking buffer and incubated for 1hr at room temperature. Membranes were imaged with a Li-Cor Odyssey Infrared Imaging System.

Data Analysis

Western blots were analyzed with Image Studio software. Protein expression was quantified by normalizing sample intensity with the loading controls. The values are represented in arbitrary units (A.U). All experiments were repeated at least three times and data reported as mean +/- standard error. Statistical significance was obtained using Student's t-test. P-values < 0.05 were considered significant.

RESULTS

Conditioned Media Downregulates Nrf2 Expression

To study the effect of conditioning on Nrf2 expression, HeLa cells were serum starved in media containing 0.5% FBS for 24hrs and then treated with conditioned media or control (unconditioned 0.5% FBS) media for 4hrs. Endogenous Nrf2 expression was evident in control media-treated cells, whereas conditioned media-treated cells showed significantly lower expression (Fig. 1A, top panel and Fig. 1B). Further experiments demonstrated that 3hrs of treatment with control media resulted in Nrf2 upregulation at similar levels (data not shown). Since there are conflicting reports in literature regarding the subcellular localization of Nrf2^{13, 27}, we tested both cytoplasmic and nuclear fractions. We were unable to detect Nrf2 in the cytoplasmic fraction of either sample (Fig. 1A, bottom panel).

To determine whether the effect of conditioning on Nrf2 was HeLa-specific, a second cell line, MDA-MB-231, was similarly treated. MDA-MB-231 cells are considered to have low levels of Nrf2 in non-stressed conditions but the Nrf2-Keap1 pathway can be strongly activated by various stimuli³². Serum-starved MDA-MB-231 cells also showed conditioned media induced-downregulation of Nrf2, although the decrease was not as drastic as in HeLa cells (Fig. 1C, D). Treatment of starved MDA-MB-231 cells with unconditioned media containing 10% FBS did not result in greater expression of Nrf2 (Fig. 1C).

Increasing Proportion of Conditioned Media Concomitantly Decreases Nrf2 Expression

To investigate whether soluble factors present in the conditioned media were responsible for downregulating Nrf2, conditioned media was mixed with unconditioned media in increasing ratios (0:100, 25:75, 50:50, 75:25, 100:0). The combined media were used to treat HeLa cells for 3hrs. Nrf2 expression decreased with increase in proportion of conditioned media (Fig. 2A, B). Since Keap1 promotes Nrf2 ubiquitination and degradation, increase in Keap1

levels could explain lowered Nrf2 expression in conditioned cells. However, Keap1 expression was unaltered in both cytoplasmic and nuclear lysates of HeLa cells (Fig. 2C).

tBHQ Overrides the Effect of Conditioned Media

Previous reports have shown that Nrf2 is strongly activated by tBHQ^{17, 27, 38}. Here the effect of tBHQ on conditioned media-treated HeLa cells was examined. HeLa cells were serum-starved as described before and treated with either conditioned or unconditioned media supplemented with 100µM tBHQ for 3hrs. tBHQ increased Nrf2 expression in both types of media treatment (Fig. 3A, B); however, the Nrf2 increase was more pronounced in unconditioned media-treated cells (Fig. 3B). Although tBHQ was able to overcome conditioning-mediated decrease in Nrf2, it did not rescue Nrf2 expression completely.

Addition of Fresh Media, Irrespective of FBS Content, is Necessary for Nrf2 Increase

In order to understand the contribution of media and FBS, cells were either deprived of only serum or both serum and media. This type of treatment has been used previously to study the role of reactive oxygen species and autophagy³³. In this study, complete serum deprivation resulted in Nrf2 decrease within shorter duration. HeLa cells were maintained in serum-free culture media for 12hrs, followed by treatment with serum-free conditioned media. This led to a significant loss of Nrf2 expression, which was reversed by addition of unconditioned media (Fig. 4A). Moreover, cells treated with HBSS showed further decrease in Nrf2 levels (Fig. 4B). Taken together, this suggests that recovery of Nrf2 expression occurs irrespective of the presence of serum in culture.

DISCUSSION

Nrf2 is a key player in the regulation of cellular redox homeostasis as well as in metabolism, proliferation, apoptosis and inflammation, on account of extensive cross-talk with multiple pathways (reviewed in³⁹). In this study, we sought to understand the regulation of Nrf2 in response to conditioned media in serum-starved cells. We observed that serum-free or serum-deprived cells express decreased Nrf2 in the presence of conditioned media, which was reversed by addition of tBHQ. The phenomenon of Nrf2 downregulation was recapitulated in both HeLa cells and MDA-MB-231 cells, suggesting that our observations may indicate a regulatory mechanism that operates independent of basal Nrf2 protein levels (Fig. 1). Decrease in Nrf2 was significant in HeLa but not in MDA-MB-231 cells. While Nrf2 is not mutated in these cell lines³⁵, it is expressed strongly in HeLa cells²⁵ and weakly in MDA-MB-231 cells under basal conditions³². The observed difference in Nrf2 levels in our study could be attributed to differential endogenous Nrf2 expression in these lines.

Nrf2 levels were regulated by the dose of conditioned media added to culture, implicating the possibility of self-regulation by secreted factors in conditioned media under starvation conditions (Fig. 2). Interestingly, there is no significant difference in Nrf2 expression over time during regular culture in 10% FBS-supplemented media (data not shown). This suggests that conditioning of fed and starved cells yields differential regulation of Nrf2. Instead of a putative autocrine mechanism via secretion, an alternative means of downregulation could be mediated by the depletion of nutrients such as pyruvate or amino acids during conditioning, thus leading to the suppression of Nrf2. This depletion mechanism is strongly supported by our finding that HBSS does not promote Nrf2 rescue (Fig. 4). Recovery of Nrf2 expression occurred upon addition of unconditioned media to the cells irrespective of serum content. Previously, glucose deprivation has been shown to cause nuclear accumulation of Nrf2⁵; however, the high glucose content of DMEM in our culture media makes it an unlikely candidate, particularly within the time periods employed (12-24hrs). Furthermore, addition of HBSS containing glucose did not cause an increase in

Nrf2 (Fig. 4). Since the culture media included a large number of amino acids and vitamins, an exhaustive screening of the essential growth components is required to identify the potential molecules depleted over the experimental conditions that elicit control over Nrf2 expression.

Nrf2 is tightly regulated in the intracellular environment and is a short-lived protein in the basal state ($t_{1/2} \sim 15$ min). Since Keap1 functions as the primary inhibitor of Nrf2, we tested the levels of Keap1 in conditioned cells for the possibility that upregulation of Keap1 leads to suppressed expression of Nrf2. Keap1 has been shown to translocate into the nucleus and downregulate Nrf2 expression^{27, 36}. However, conflicting reports have proposed that Keap1 functions primarily as a cytoplasmic protein⁴¹. We observed no changes in response to the treatment regimen (Fig. 2C). In our studies, Keap1 was detected more prominently in cytoplasmic extracts while Nrf2 was detected in nuclear extracts in all samples. Decrease in Nrf2 in conditioned cells was not mirrored by an increase in Keap1 in the nucleus. The loss of nuclear Nrf2 was potentially unrelated to nucleocytoplasmic trafficking, as there was no increase in cytosolic Nrf2. Changes in Nrf2 expression have been reported to be mediated at the level of transcription²², translation³⁰ or protein stability²⁶, all of which could be susceptible to serum starvation-induced regulation. Detailed investigation into the mechanistic aspects of Nrf2 downregulation is beyond the scope of this study.

Our study found that in the absence of oxidative stress or well-characterized activators of Nrf2, stimulation with media alone resulted in a large increase in Nrf2. This suggests that the endogenous Nrf2 signaling pathway was highly sensitized due to starvation and conditioning, which affected basal and induced Nrf2 expression. This also implies that the technique of cell synchronization via serum starvation can alter the availability of Nrf2, which in turn can lead to variability in experimental results depending on the conditions of serum withdrawal. Starvation-induced effects are specific to cell lines and to the status of cell cycle prior to treatment. It has been reported that compared to other cell types such as fibroblasts, cancer cells are relatively immune to the effects of serum starvation-mediated growth arrest²⁰. HeLa S3 cells maintained in serum-free media for 24hrs failed to undergo G₁ arrest or apoptosis⁴². Decreased protein synthesis was observed in HeLa cells grown in 0.5% FBS-containing media for 24hrs⁶. It is worth noting that in our study, the unconditioned media used to stimulate serum-starved cells also contained low serum (0.5% FBS) and in specific instances, no serum at all. We suggest that the resultant downregulation of Nrf2 is not solely due to lack of serum in culture conditions but mediated by self-conditioning.

The underlying mechanism responsible for Nrf2 self-regulation remains to be determined. It should be noted that ROS formation due to serum starvation has been examined previously, and an interesting question raised by our findings is the possible connection between Nrf2 downregulation and ROS formation. Even short duration (3-13hrs) of serum starvation is reported to generate ROS in CHO cells³³. In HeLa cells, lack of glucose, pyruvate, serum and L-glutamine led to superoxide generation at 72hrs while lack of amino acids and serum led to both superoxide and hydrogen peroxide (H₂O₂) formation within 24hrs³. The Nrf2-Keap1 pathway is highly sensitive to ROS-dependent oxidative stress in cells. Thus it seems counterintuitive that in our study Nrf2 would be downregulated in cells undergoing redox imbalance. An increase in ROS levels has been associated with downregulation of Nrf2 in other cell types; for example, in a cystic fibrosis model, Ziady and colleagues have reported that suppression of Nrf2 signaling mediated by cyclic AMP was associated with increased H₂O₂^{2, 47}. Further investigation is required to determine whether conditioning-induced ROS accumulation regulates Nrf2 in HeLa cells.

In conclusion, we have investigated Nrf2 regulation mediated through self-conditioning in this study. These findings have strong implications in understanding the basal regulation of Nrf2 during nutrient withdrawal and quiescence.

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Figure 1.

Conditioning decreases Nrf2 expression in the nucleus. (A) HeLa cells were serum-starved (0.5% FBS) for 24hrs and treated with conditioned or unconditioned media containing 0.5% FBS for 4hrs. *Top Panel:* Nrf2 expression in the nucleus (NE: Nuclear Extracts). *Bottom Panel:* Nrf2 expression in cytoplasm (CE: Cytoplasmic Extracts). (B) Nrf2 levels were quantified by normalizing band intensities against a nuclear loading control (TBP). The data is represented as arbitrary units (A.U) for three independent experiments (Mean $\pm$ SD). Statistical significance ($p < 0.05$) is indicated by the asterisk (*). (C) The same treatment regimen yields similar results in MDA-MB-231 cells. Cells treated with unconditioned media containing 10% FBS do not show significantly different Nrf2 expression. (D) Quantitative representation of Nrf2 in the nucleus of MDA-MB-231 cells.

Figure 2.

Decrease in Nrf2 expression is associated with the presence of conditioned media in culture. (A) HeLa cells were serum-starved (0.5% FBS) for 24hrs then treated with conditioned media containing decreasing proportions of unconditioned media for 3hrs. (B) Quantification of Nrf2 levels obtained from four independent experiments (Mean $\pm$ SD). (C) Keap1 expression did not change in cytoplasmic (CE) or nuclear (NE) extracts of HeLa cells (n=3).

Figure 3. tBHQ overcomes the effect of conditioned media. (A) HeLa cells were serum-starved (0.5% FBS) for 24hrs and treated with conditioned or unconditioned media containing 100 μ M tBHQ for 3hrs. (B) The decrease in Nrf2 is shown for three independent experiments (Mean $\pm$ SD).

Figure 4.

Complete serum deprivation suppresses Nrf2 in shorter periods (12hrs). (A) HeLa cells were maintained in serum-free media for 12hrs followed by treatment with serum-free conditioned and unconditioned media or HBSS for 3hrs. (B) Normalized Nrf2 levels were obtained from three independent experiments (Mean $\pm$ SD), * $p < 0.05$.