Extracellular redox status regulates Nrf2 activation through mitochondrial reactive oxygen species

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The redox status of the extracellular compartment has only just been elucidated as a mechanism controlling intracellular signal transduction and correlates with aging, diabetes, heart disease and lung fibrosis. In the present paper, we describe a mechanism by which oxidizing extracellular environments, as maintained by the cysteine/cystine (Cys/CySS) redox couple, induce mitochondria-derived ROS (reactive oxygen species) generation and cause the activation of Nrf2 (nuclear factor-erythroid 2-related factor 2), inducing an antioxidant response. NIH 3T3 cells were cultured in medium with extracellular Cys/CySS redox potentials (Eᵢₒ), ranging from 0 to −150 mV. Cellular and mitochondrial ROS production significantly increased in cells incubated under more oxidizing extracellular conditions (0 and −46 mV). Trx2 (thioredoxin-2) is a mitochondrial-specific oxidoreductase and antioxidant and became oxidized in cells incubated at 0 or −46 mV. MEFs (mouse embryonic fibroblasts) from Trx2-overexpressing transgenic (Trx2 Tg) mice produced less intracellular ROS compared with WT (wild-type) MEFs at the more oxidizing extracellular conditions. Nrf2 activity was increased in WT MEFs at the 0 or −46 mV conditions, but was inhibited in Trx2 Tg MEFs under the same conditions. Furthermore, Nrf2-regulated gene expression was significantly increased in the WT MEFs, but not in the Trx2 Tg MEFs. These results show that the Cys/CySS redox status in the extracellular compartment regulates intracellular ROS generated primarily in the mitochondria, which play an important role in the activation of Nrf2 and up-regulation of antioxidant and detoxification systems.

Key words: cysteine, nuclear factor-erythroid 2-related factor 2 (Nrf2), reactive oxygen species (ROS), redox, thiol, thioredoxin.

INTRODUCTION

Previous studies have highlighted the independence of different thiol/disulfide redox couples [1–3]. These critical observations provide a framework for mechanisms controlling redox-sensitive elements and further the basic knowledge of how changes in these couples facilitate a specific response. Furthermore, these same redox couples have been shown to be differentially regulated in the various subcellular compartments in response to oxidative stress and physiological stimuli [4–7]. Compartmentation of selected redox couple changes may further the specificity in the redox control of signal transduction. One compartment that has only recently received much attention is the extracellular compartment. Unlike intracellular thiol pools where GSH is the most abundant small non-protein biothiol, extracellular compartments are primarily regulated by Cys/CySS (cysteine/cystine). In humans, extracellular GSH and GSSG concentrations are approximated to be between 1.6 and 2 μM and between 0.05 and 0.17 μM respectively, and yield an Eᵢₒ (extracellular redox potential) of −126 to −138 mV [8–10]. Total cysteine pools as measured by Cys and CySS concentrations are much higher and are measured to be between 10 and 13 μM and 46 and 86 μM respectively, and yield an Eᵢₒ between −71 and −87 mV [8–10]. In mice, total cysteine pools are smaller, where Cys and CySS concentrations are measured at 18.4 and 33 μM respectively [11]. Murine extracellular Cys/CySS Eᵢₒ is more reducing than in humans, determined to be −100 mV ([11] and B.R. Imhoff and J.M. Hansen, unpublished work).

Shifts in the extracellular redox state have been associated with many different environmental exposures, pathologies and disease states, including aging, lung fibrosis, heart disease, diabetes, age-related macular degeneration, smoking and chemotherapy [8–10,12–14]. Additionally, extracellular Eᵢₒ has been associated with changes in proliferation and apoptosis [15–17]. Although it is not entirely clear, extracellular control of cellular events appears to be due, at least in part, to the generation of intracellular ROS (reactive oxygen species). Treatment of cells with non-permeable thiol-reactive reagents, AMS (4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid) and qBBr (monobromotrimethylammoniobimane bromide), blocked the availability of free cell-surface membrane thiols prior to exposure to various extracellular redox conditions [18]. Treatment caused a decrease in intracellular ROS production, suggesting that pathways that cause an increase in intracellular ROS may be influenced by cell-surface proteins, although the exact mechanisms involved are currently unknown. Because the extracellular Eᵢₒ is capable of dictating intracellular ROS production, it may serve as a mechanism to regulate redox-sensitive elements to respond to their environment. Nrf2 (nuclear factor-erythroid 2-related factor 2) is a redox-sensitive transcription factor that regulates the ARE (antioxidant-response element) controlling the expression of many different antioxidant and detoxification enzyme systems. There are many different potential mechanisms by which Nrf2 can be regulated and some of these appear to be more or less active depending on the physiological circumstances [19]. Nrf2 is normally found bound to the ARE of antioxidant and detoxification genes, and the redox state of the extracellular compartment can change the availability and activation of the ARE, thus regulating the expression of these genes. In this study, we investigate the role of extracellular redox status on the activation of Nrf2.

Abbreviations used: AMS, 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid; ARE, antioxidant-response element; BrdU, bromodeoxyuridine; Cys, cysteine; CySS, cystine; DCF, 2',7'-dichlorodihydrofluorescein diacetate; DMEM, Dulbecco’s modified Eagle’s medium; Eᵢₒ, extracellular redox potential; EMSA, electrophoretic mobility-shift assay; FBS, foetal bovine serum; GCLc, glutamate-cysteine ligase catalytic subunit; Keap1, Kelch-like ECH (erythroid cell-derived protein with cap’n’collar homology)-associated protein 1; KO, knockout; MEF, mouse embryonic fibroblast; NOQ1, NADPH quinone oxidoreductase-1; Nrf2, nuclear factor-erythroid 2-related factor 2; Pₚₓ, peroxiredoxin; RFU, relative fluorescence unit(s); ROS, reactive oxygen species; SFN, sulforaphane; Tg, transgenic; TNF, tumour necrosis factor α; TR, thioredoxin reductase; Trx, thioredoxin; WT, wild-type; Xc⁻, cysteine/glutamate transporter.

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to Keap1 [Kelch-like ECH (erythroid cell-derived protein with cap’n’collar homology)-associated protein 1] in the cytoplasm, rendering it inactive. Nrf2 inactivation is believed to be not only due to Keap1 sequestration, but also due to being constitutively degraded by the Keap1-associated proteins, Cul3 (Cullin-3) and Rbx1 (ring-box 1), which together form a core E3 ubiquitin ligase complex and are involved in the ubiquitination and subsequent degradation by the 26S proteasome [20]. Electrophiles and ROS are believed to modify specific Cys residues on Keap1 that result in the release of Nrf2, inhibiting constitutive degradation of Nrf2 and allowing it to translocate to the nucleus facilitating its binding to the ARE. Genes that are known to be up-regulated through Nrf2 activation include GSH synthesis enzymes [GCLc (glutamate–Cys ligase catalytic subunit) and GCLm (glutamate–Cys ligase modifier subunit)], Trx1 (thioredoxin-1), Prxs (peroxiredoxins), TR1 (thioredoxin reductase 1), X− (CySS/glutamate transporter), NQO1 (NADPH:quinone oxidoreductase-1), GSH regulatory enzymes [GPx-1 (GSH peroxidase-1), GSTs (glutathione transferases) and GSSG-Rd1 (GSSG reductase 1)], HO-1 (haem-oxygenase 1) [21–28]. Nrf2 activation provides a critical first response to oxidant stress and promotes the regulation of the intracellular redox environment.

It is currently unknown how extracellular redox states affect Nrf2 activity. In the present study, we show that oxidizing extracellular redox states promote ROS production in the mitochondria, which then promotes Nrf2 activation. Interestingly, this process can be inhibited by the mitochondrial-specific antioxidant, Trx2 (thioredoxin-2). Our results illustrate a novel mechanism by which Nrf2 can be activated, and suggest that cells have evolved a mechanism by which they can respond to redox changes in their extracellular environment.

**MATERIALS AND METHODS**

**Cell culture and MEF (mouse embryonic fibroblast) isolation**

NIH 3T3 MEFs were purchased from A.T.C.C. and were grown in DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 10% (v/v) FBS (foetal bovine serum) and antibiotics. Primary WT (wild-type), Nrf2-knockout (termed Nrf2 KO) and Trx2-transgenic (termed Trx2 Tg) MEFs [29] were collected from time-mated primagravia dams on gestational day 13.5. Embryos were removed and dissected to isolate the trunk from other portions of the body. The remaining sample was washed with PBS multiple times, finely minced with a razor blade and trypsinized (0.25%) for 10 min at 37°C with some agitation. All procedures using animals were approved via Emory’s Institutional Animal Care and Use Committee (IUCAC). Animal care was under the supervision of Emory’s Department of Animal Resources and their veterinary staff. Isolated cells were cultured in DMEM supplemented with 10% (v/v) FBS and antibiotics. Primary MEFs were not used beyond four passages. Verification of WT and Trx2 Tg genotypes was performed using PCR as described previously [29] (results not shown).

Different extracellular thiol/disulfide redox potentials were achieved by using various concentrations of Cys and CySS in Cys-free DMEM supplemented with antibiotics (final pH 7.4). The amounts of Cys/CySS added to the redox media for the various redox potential states are listed in Table 1 [15]. Verification of Cys and CySS concentrations were determined by HPLC with fluorescence detection [9]. Ranges chosen for these studies were based on ranges that are reported in human studies that evaluated plasma Cys/CySS $E_h$ [8,9].

In experiments where Cys/CySS $E_h$ was followed over time, NIH 3T3 cells were cultured until confluent and then placed in new medium (see above) at the Cys/CySS $E_h$ of either 0 or $−150$ mV. An aliquot (70 μl) was collected during the first 24 h and derivatized by HPLC as previously described [9]. To evaluate the role of Nrf2 in the regulation of extracellular Cys/CySS $E_h$, cells were pretreated overnight (14 h) with the Nrf2 stimulant SFN (sulforaphane; 5 μM).

**BrdU (bromodeoxyuridine)-incorporation assay**

Assessment of cellular proliferation was performed using a fluorimetric BrdU Assay Kit (Calbiochem) according to the manufacturer’s instructions. Cells were plated in a 96-well plate at a density of $5 \times 10^4$ cells/well. Following attachment, cells were incubated in Cys/CySS redox medium containing BrdU as described above at redox potentials ranging from 0 to $−150$ mV. Cultures were incubated overnight (16 h). Fluorescence measured in RFU (relative fluorescence units) is indicative of BrdU incorporation and denotes cellular proliferation.

**Extracellular protein modification by extracellular redox status**

NIH 3T3 cells were grown until confluent in normal growth medium, which was removed and replaced with Cys/CySS redox medium at, $−80$ or $−150$ mV for 20 min. Oregon Green–maleimide (Invitrogen, 300 μMfinal concentration) was added to the medium for an additional 20 min. Cells were washed briefly and then fixed in 4% (w/v) paraformaldehyde in PBS. Cells were then viewed by fluorescence microscopy. Green fluorescence is representative of reduced extracellular protein thiols.

**Fluorescence-based detection of cellular and mitochondrial ROS**

Cells were grown until confluent in 96-well plates and then loaded with DCF (2′,7′-dichlorodihydrofluorescein diacetate) in loading medium [DMEM with 1% (v/v) FBS] for 30 min. Cells were then placed in Cys/CySS redox medium with potentials ranging from 0 to $−150$ mV. Fluorescence (488 nm excitation and 530 nm emission) was measured after 20 min in a M2 microplate fluorimeter (Molecular Devices). For mitochondrial-derived ROS (superoxide anion), cells were incubated as described above and then washed and loaded with 5 μM MitoSox Red (Invitrogen) for 10 min at 37°C. Cells were washed and then fluorescence was measured in a fluorimeter at 510 nm excitation and 580 nm emission.

**Trx2 redox Western blot analysis**

The redox state of Trx2 was determined by redox Western blot analysis with AMS as described previously by Halvey et al. [4] and based upon the original method of Damdimopoulos et al. [30]. Briefly, proteins were precipitated with ice-cold 10% TCA (trichloroacetic acid) for 30 min on ice, washed briefly with acetone and then labelled with AMS for 30 min. Proteins

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<th>$E_h$ (mV)</th>
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were separated by non-reducing SDS/PAGE (15 % acrylamide), electroblotted on to a nitrocellulose membrane and then probed with a rabbit primary antibody against human Trx2. Secondary detection was performed with an anti-rabbit Alexa Fluor® 680-labelled secondary antibody (Invitrogen). Densitometric analysis of membranes was performed with the Odyssey System software (Li-Cor). Band densitometric values were used with the Nernst equation to estimate the steady-state redox potential of Trx2 [6].

**Subcellular localization of Nrf2**

NIH 3T3 cells were incubated under different redox conditions (see above) for 2 h. Cytosolic and nuclear fractions were collected with the Nu-Per fractionation kit (Pierce Chemical) according to the manufacturer’s instructions. Detection of Nrf2 was carried out via SDS/PAGE with immunoblotting using an anti-Nrf2 antibody (Santa Cruz Biotechnology) and fluorescent secondary antibodies (Alexa Fluor® 680-labelled; Invitrogen). The Odyssey Scanner system (Li-Cor) was used to detect Nrf2 via the Odyssey scanning software.

**EMSA (electrophoretic mobility-shift assay)**

Oligonucleotides were purchased from Li-Cor and were 5′-labelled with IRDye 700. The oligonucleotide sequences for the NQO1-ARE sequences were 5′-CGCGTCTCGA ACTTTCAG-TCTAGGTCA CAGTGTCGGCAAAATT-3′ and 5′-CGGCTCTGAACTTTTCA GTCAGTGTCGGCAAAATT-3′ [31]. Binding reactions were performed with 5 μg of nuclear isolates from WT and Trx2 Tg MEFs after incubation in Cys/CySS redox medium (0 to −150 mV) for 30 min. Separation of protein–DNA complexes was achieved by loading reactions on to a native TBE [Tris/borate/EDTA (45 mM Tris/borate and 1 mM EDTA)] 5 % polyacrylamide gel, which was run for 2 h at 70 V. Visualization was performed with the IR Li-Cor Odyssey scanner. Supershift assays were performed to verify the shifted band as the Nrf2/oligonucleotide band from (Figure 2). More reducing extracellular conditions (−109 to −150 mV) showed no significant difference from DCF fluorescence measured under the −80 mV condition will be compared with the −80 mV condition.

**RESULTS**

**Oxidizing extracellular Cys/CySS Eh decreases BrdU incorporation and cellular proliferation**

Since most normal extracellular Cys/CySS Eh values have been reported to be approx. −80 mV, the −80 mV condition will be used as the primary comparison with other redox conditions, unless otherwise stated. NIH 3T3 cells were incubated in the various extracellular Cys/CySS redox media overnight and then assessed for their ability to incorporate BrdU as a measure of cellular proliferation. BrdU incorporation was significantly less (P < 0.05) in cells that were incubated at either 0 or −46 mV (Figure 1), indicating a decreased rate of proliferation under oxidizing extracellular conditions.

**Extracellular oxidizing conditions induce ROS production**

DCF fluorescence was significantly higher (P < 0.05) in NIH 3T3 cells that were maintained in more oxidizing Cys/CySS redox media, where 0 and −46 mV conditions caused an increase of approx. 65 % and 35 % respectively, compared with DCF fluorescence measured under the −80 mV extracellular conditions (Figure 2). More reducing extracellular conditions (−109 to −150 mV) showed no significant difference from −80 mV.

The precise origin of the ROS produced as a result of an oxidizing extracellular environment is currently unknown. Since a major source of ROS is derived from mitochondria, we used the superoxide anion indicator, MitoSox Red, which fluoresces upon oxidation by superoxide anion, to determine whether ROS production in mitochondria is enhanced under conditions of oxidizing extracellular redox status. Similar to DCF fluorescence, MitoSox Red fluorescence was substantially increased (P < 0.05) in NIH 3T3 cells incubated in oxidizing conditions (Figure 3), demonstrating the induction of mitochondria-derived ROS by oxidizing extracellular Cys/CySS conditions.

**Oxidizing extracellular Cys/CySS Eh oxidizes thiol residues on extracellular surface proteins**

Previous work has demonstrated that oxidizing extracellular conditions cause oxidation of surface membrane thiols in bovine
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Figure 2 Cellular ROS production increases at more oxidizing extracellular Cys/CySS Eh

DCF was used to determine ROS generation in cells incubated at various Cys/CySS Eh. More oxidizing extracellular conditions demonstrated an increase in DCF fluorescence and are indicative of an increase in intracellular ROS generation. Values are means ± S.E.M. and are from three independently performed experiments. *P < 0.05 compared with the −80 mV conditions.

Figure 3 Mitochondrial ROS production increases at more oxidizing extracellular Cys/CySS Eh

MitoSox Red fluorescence was used a specific measure of mitochondrial ROS, primarily superoxide anion, in cells incubated at various Cys/CySS Eh. More oxidizing extracellular conditions produced an increase in mitochondrial ROS, as observed with relative increase in MitoSox Red fluorescence. Values are means ± S.E.M. and are from three independently performed experiments. *P < 0.05 compared with the −80 mV conditions.

Oxidizing extracellular Cys/CySS Eh induces Trx2 oxidation

The present study has demonstrated the increase in intracellular ROS production as a consequence of extracellular redox conditions (Figure 2), yet the origins of intracellular ROS generation are not well understood. Since the mitochondria are a major source of ROS in most cells and MitoSox Red fluorescence was increased in cells incubated in oxidizing redox medium, we measured the redox state of Trx2, a major detoxification system found only in the mitochondria. Using redox Western blot methodology, Trx2 redox states were determined to be significantly more oxidized in cells that were maintained under 0 and −46 mV extracellular Eh, where Trx2 Eh was determined to be −324 (± 6.1) and −328 (± 2.2) mV respectively (Figure 5). These values represented an increase in Eh of approx. +18 and +14 mV respectively, from −80 mV [−342 (± 2.8) mV]. Reducing conditions did not affect Trx2 Eh. Multiple ROS-metabolizing systems are present in the mitochondria, including Trx2. Trx2 in conjunction with Prxs, namely Prx-3, actively detoxify ROS. Oxidation of Trx2 is indicative of mitochondrial ROS production, as well as active ROS detoxification. Whole-cell GSH Eh was not altered by extracellular redox states (Figure 5C). Taken together, these results suggest that the mitochondrion is the compartment most affected by extracellular redox conditions and may play the principal role in the regulation of downstream events.

Trx2 overexpression inhibits intracellular ROS production

MEFs were collected from WT and Trx2 Tg mice and were used to assess the amount of cytosolic ROS produced as a consequence of changes in the extracellular redox environment. WT MEFs responded to the extracellular Cys/CySS redox environment in a similar fashion to NIH 3T3 cells, where there was an increase in
Brussels sprouts [32]. SFN has potent anticancer properties that are found in many cruciferous vegetables, such as broccoli and this occurs is not well understood. SFN is a natural product that by cells over time [15], but the cellular mechanism by which

\[ \text{oxidizing extracellular conditions} \]

\[ 0 \text{ mV} \]

\[ -150 \text{ mV} \]

The upper band represents the reduced band (Red) and the lower band represents the oxidized forms of Trx2 was achieved via derivatization with AMS (see the Materials and methods section).

It has been shown that extracellular Cys/CySS \[ E_h \] production and the regulation of the extracellular redox states. In WT MEFs, Trx2 Tg MEFs incubated in oxidizing extracellular Cys/CySS \[ E_h \] conditions. \( (\text{Figure 5}) \) Redox Western blot analysis of the Trx2 redox state. Separation of the oxidized and reduced forms of Trx2 was achieved via derivatization with AMS (see the Materials and methods section). The upper band represents the reduced band (Red) and the lower band represents the oxidized band (Ox). The oxidizing extracellular conditions of both 0 and \(-46 \text{ mV}\) showed extensive oxidation of Trx2, but was relatively reduced under the other more reducing extracellular conditions. \( (\text{Figure 5}) \) Redox potentials measured via densitometric analysis of the bands and the Nernst equation showed a significant oxidation. Values are means \( \pm \) S.E.M. and are from three independently performed experiments. \( * P < 0.05 \) compared with \(-80\), \(-109\), \(-131\) and \(-150 \text{ mV} \), \( E_h \). (C) Glutathione redox potentials were unaffected by extracellular redox conditions.

cytosolic ROS production in cells incubated at 0 and \(-46 \text{ mV}\), but no differences in the \(-109\) to \(-150 \text{ mV}\) conditions (Figure 6). Trx2 Tg MEFs incubated in oxidizing extracellular Cys/CySS redox media did not show an increase in cytosolic ROS production, but rather production remained uniform in all of the cultures, suggesting that Trx2 overexpression is capable of quenching ROS production as a result of the extracellular redox states.

SFN pretreatment results in reduction of extracellular ROS production and the regulation of the extracellular redox environment

It has been shown that extracellular Cys/CySS \[ E_h \] is re-regulated in cells incubated at 0 and \(-46 \text{ mV}\), but no differences in the \(-109\) to \(-150 \text{ mV}\) conditions (Figure 6). Trx2 Tg MEFs incubated in oxidizing extracellular Cys/CySS redox media did not show an increase in cytosolic ROS production, but rather production remained uniform in all of the cultures, suggesting that Trx2 overexpression is capable of quenching ROS production as a result of the extracellular redox states.

Trx2 overexpression inhibits the activation of Nrf2 induced by oxidizing extracellular redox states

Nuclear isolates from WT and Trx2 Tg MEFs incubated under the various redox conditions were analysed for Nrf2–DNA binding (Ox). The oxidizing extracellular conditions of both 0 and \(-46 \text{ mV}\) showed extensive oxidation of Trx2, but was relatively reduced under the other more reducing extracellular conditions. By 8 h, cells that lacked the pretreatment had re-regulated the extracellular compartment to \(-51.9 \text{ mV} \pm 3.3\), but at the same timepoint pretreated cells had re-regulated their extracellular compartment to \(-65.3 \text{ mV} \pm 3.7\). At the 24 h timepoint, extracellular Cys/CySS \[ E_h \] in both SFN-treated and untreated cells was virtually unchanged from the 8 h timepoint. In cells that were initially incubated at 0 mV, cells that were not pretreated with SFN re-regulated their cellular environment to \(-39.3 \text{ mV} \pm 3.3\) by 8 h, but the extracellular redox state in pretreated cells were significantly more reduced, measuring \(-52.6 \text{ mV} \pm 3.4\). By 24 h, re-regulated extracellular redox potentials were even more reduced, measured as \(-52.4 \pm 1.9\) and \(-61.2 \pm 2.8 \text{ mV}\) in untreated and SFN-pretreated cultures respectively. Results show that Nrf2 stimulation can modulate the ability of the cells to regulate their extracellular redox states.

Figure 5 Trx2 is oxidized in cells maintained under oxidizing extracellular Cys/CySS \( E_h \) conditions

(A) Redox Western blot analysis of the Trx2 redox state. Separation of the oxidized and reduced forms of Trx2 was achieved via derivatization with AMS (see the Materials and methods section). The upper band represents the reduced band (Red) and the lower band represents the oxidized band (Ox). The oxidizing extracellular conditions of both 0 and \(-46 \text{ mV}\) showed extensive oxidation of Trx2, but was relatively reduced under the other more reducing extracellular conditions. (B) Redox potentials measured via densitometric analysis of the bands and the Nernst equation showed a significant oxidation. Values are means \( \pm \) S.E.M. and are from three independently performed experiments. *P < 0.05 compared with \(-80\), \(-109\), \(-131\) and \(-150 \text{ mV} \), \( E_h \). (C) Glutathione redox potentials were unaffected by extracellular redox conditions.

Figure 6 Overexpression of Trx2 decreases intracellular ROS generation stimulated by extracellular oxidizing environments

ROS was measured with the DCF indicator fluorophore in primary MEFs from WT and Trx2 Tg mouse embryos. DCF fluorescence was significantly increased in WT MEFs under oxidizing extracellular conditions, but did not increase in Trx2 Tg MEFs. The results suggest that Trx2 overexpression can inhibit ROS production induced by oxidizing extracellular conditions. Values are means \( \pm \) S.E.M. and are from three independently performed experiments. *P < 0.05 compared with \(-80\), \(-109\), \(-131\) and \(-150 \text{ mV} \), \( E_h \).
Figure 7 Extracellular ROS production is decreased following SFN treatment

To assess the effect of Nrf2 on changing extracellular ROS production, WT and Nrf2 KO MEFs were treated with SFN for 18 h. Extracellular ROS production was measured via Amplex Red extracellular hydrogen peroxide detection methods (see the Materials and methods section), after which protein concentrations were collected for standardization purposes. Results are Amplex Red RFUs per mg of protein per min. SFN treatments decreased concentrations of extracellular hydrogen peroxide in WT MEFs but not in Nrf2 KO cells, SFN treatment resulted in an increase in hydrogen peroxide levels. *P < 0.05 for SFN-treated cells compared with each respective untreated control. †P < 0.05, between each untreated control. Values are means ± S.E.M. and are from three independently performed experiments.

Figure 8 Extracellular redox state is re-regulated to a more reduced state in cells pretreated with the Nrf2 stimulant SFN

NIH 3T3 cells in normal growth medium were pretreated with SFN for 18 h after which they were placed in either 0 (A) or −150 (B) mV extracellular redox medium. Changes to the extracellular Cys/CySS Eh were monitored over a 24 h period. Untreated NIH 3T3 cells re-regulated their redox state to a more oxidizing extracellular condition compared with cells pretreated with SFN for 18 h, regardless of which initial extracellular redox condition was used. Values are means ± S.E.M. and are from three independently performed experiments. *P < 0.05.

Extracellular redox conditions mediate Nrf2 translocation to the nucleus

Isolates from NIH 3T3 cells incubated under different redox conditions showed that under the more oxidizing conditions (0 and −46 mV), Nrf2 is translocated to the nucleus (Figure 10). Under these oxidizing conditions, cytosolic fractions show a decrease in Nrf2 accumulation, whereas nuclear fractions show a significant increase in Nrf2. The more reducing conditions of −80 to −150 mV showed no significant changes in Nrf2 localization. These results suggest that oxidizing conditions are capable of inciting Nrf2 activation and translocation to instigate an antioxidant response.

Extracellular redox conditions increase the expression of Nrf2-related genes, but not in cells overexpression Trx2

As a final measure of Nrf2 activity, WT and Trx2 Tg MEFs were incubated in the extracellular redox media at various redox potentials, and RNA was collected and processed for analysis via real-time quantitative fluorescence PCR. Genes selected for assay in this experiment have been shown previously to be regulated by Nrf2 activation, including GCLc, Xc−, Trx1 and NQO1. For WT MEFs, gene-expression patterns were relatively similar between all genes analysed, where the highest and most significantly different level of expression was observed in cells incubated at 0 mV compared with the −80 mV condition (Figure 11). For GCLc, Xc−, Trx1 and NQO1, there was an increase in expression of approx. 2.7-, 3.2-, 4.1- and 6.5-fold respectively. Similarly, statistically significant differences also were observed in cells incubated under the −46 mV condition, but these changes were not as great as those at 0 mV. Conversely, Trx2 Tg MEFs showed no statistically significant increase in the expression of any of these genes as a result of the oxidizing extracellular redox environment.

DISCUSSION

Although numerous studies have elucidated redox changes within subcellular organelles, there are few that have addressed the
Extracellular redox control of Nrf2

Figure 9  Nrf2 activity is activated in WT but not in Trx2 Tg MEFs maintained in oxidizing extracellular Cys \( E_h \)

EMGAs using an oligonucleotide encoding a NQO1-ARE were used to determine the activation of Nrf2 in WT and Trx2 Tg MEFs. Cells were incubated under the various extracellular conditions for 30 min, after which nuclear isolates were collected and the assay performed. The results show that in WT MEFs, nuclear translocation of Nrf2 was apparent in either the 0 or \(-46\) mV extracellular redox conditions. In Trx2 Tg MEFs, Nrf2 translocation was inhibited under all extracellular conditions. The results suggest that mitochondrial Trx2 can inhibit the extracellular redox-induced activation of Nrf2.

extracellular compartment, especially as a factor capable of affecting signal transduction. However, there are numerous diseases that have been shown to promote changes in the extracellular redox environment [8–10,12–14,35], which has been suggested as a potentially useful biomarker to identify disease processes. Since oxidation of the extracellular environment is associated with many diseases, a primary focus of the present study was to determine whether extracellular redox states could affect intracellular signalling as a mechanism to possibly contribute to disease pathologies.

We demonstrate that extracellular redox status is associated with a decrease in cellular proliferation at more oxidizing conditions. Previous work has demonstrated that intracellular GSH redox status is relatively reduced under cell-culture conditions that would promote proliferation [36,37], but in experiments where cells were cultured under oxidizing conditions, GSH redox status remained unchanged even with an increase in ROS production [15]. Interestingly, cell proliferation can be enhanced with very small doses of hydrogen peroxide (0.1–1 \( \mu \)M), but is inhibited at subsequently higher concentrations [38–40], producing a biphasic effect, suggesting that at some concentrations peroxides stimulate proliferation, whereas at higher concentrations it is inhibited. However, these studies used single bolus doses of hydrogen peroxide to evaluate its effect. Because extracellular redox state is more constant, taking many hours to re-equilibrate, the production of ROS in response to changes in extracellular redox state is more constant, yielding a cell model of extracellular stress that is relevant to physiological conditions. Persistent and prolonged periods of extracellular redox disruption may result in the excessive production of intracellular ROS that inhibits redox-sensitive processes involved in proliferation. The results of the present study support this hypothesis.

Since intracellular ROS production appears to be critical for changes in cell function, it becomes important to understand where ROS originate. A novel finding shown in the present study is that oxidizing extracellular environments cause an increase in mitochondrial superoxide production, suggesting that intracellular ROS, at least in part, originate from these organelles. The mitochondria are known to produce ROS as a consequence of oxidative phosphorylation and in response to specific physiological stimuli [i.e. TNFα (tumour necrosis factor α)] [6]. Although extracellular redox states have not been shown to induce apoptosis directly, there is evidence that they may predispose cells to undergo apoptosis more readily when exposed to an oxidant [15].

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To manage mitochondrial ROS, in particular hydrogen peroxide, the mitochondria has evolved distinct antioxidant systems, including the Trx2 system. Trx2 is distinct from Trx1, in that Trx2 participates in ROS detoxification through mitochondrial-specific peroxiredoxins (Prx-3) and is reduced by its own reductase, TR2, thereby constituting a functional Trx system specific to the mitochondria. Trx2-KO mice die in utero on approx. gestational day 10, the period of development that coincides with mitochondrial maturation and the shift from anaerobic to aerobic metabolism, illustrating the importance of the Trx2/Prx3/TR2 system in the metabolism of mitochondrial ROS [41]. The findings of the present study show that oxidizing extracellular conditions promote Trx2 oxidation, which correlates with increases in intracellular ROS and emphasize the compartmental oxidation of the mitochondria in response to extracellular redox states. Furthermore, in cells that overexpress Trx2, an increase in ROS was not observed, denoting the key role that Trx2 plays in mediating extracellular environment-induced intracellular ROS as a signalling intermediate in the Nrf2 system.

Nrf2 is a redox-sensitive transcription factor that is activated in response to oxidative stress and up-regulates antioxidant and phase II detoxification enzymes. Nrf2 can be activated by oxidizing extracellular conditions that promote ROS production, but activation can be inhibited by Trx2 overexpression and the associated decrease in mitochondrial ROS. A decrease in available ROS through Trx2 overexpression and the related decrease in the activation of Nrf2 are further supported by a decrease in the expression of genes regulated by Nrf2. Taken together, it appears that extracellular redox states mediate Nrf2 signal transduction through ROS generated in the mitochondria and may be regulated by relative concentrations of Trx2. It is noteworthy that other signal transduction pathways, such as that delineated with TNFα signalling, are also reliant on mitochondrial ROS production and have shown Trx2 to function as a modulator of signal transduction [6].

Since ROS production is increased as a consequence of oxidizing extracellular conditions and the inhibition of ROS generation decrease Nrf2 activation, naturally, these results suggest that ROS may be directly responsible for modification of Keap1 residues on route to the activation of Nrf2. However, it is currently unknown whether mitochondrial ROS act directly on the Keap1/Nrf2 system or whether mitochondrial ROS affect other regulatory machinery that may affect signal transduction indirectly, through (de)activation of other redox-sensitive components. Because extracellular conditions are evident in the pathogenesis of many different diseases, furthering our understanding on the mechanistic control of extracellular-induced Nrf2 activation requires further study to possibly develop potential therapeutic interventions.

Figure 11  Nrf2-regulated gene expression is increased in WT, but not Trx2 Tg MEFs maintained in oxidizing extracellular Cys Eh conditions

To determine whether extracellular redox conditions influence the expression of Nrf2-regulated genes, both WT and Trx2 Tg MEFs were incubated under the various extracellular redox conditions for 4 h. Real-time quantitative PCR was used to determine fold changes from normal extracellular conditions (−80 mV). Under both of the 0 and −46 mV extracellular conditions, genes were significantly up-regulated. *P < 0.05, compared with −80, −109, −131 and −150 mV Eh in WT. Values are means ± S.E.M. and are from three independently performed experiments.
Although there have been many proposed mechanisms by which Nrf2 is regulated [19], the results of the present study show that extracellular redox-status-dependent activation of Nrf2 does not increase the overall concentrations of Nrf2. Rather, the Nrf2 concentrations do not appear affected by any extracellular redox condition, suggesting that direct interaction with Keap1 is responsible for the activation and subsequent translocation of Nrf2 to the nucleus.

Activation of Nrf2 causes the re-regulation of the extracellular redox environment and may provide a mechanism by which extracellular oxidative stress may be alleviated. SFN treatments allowed cells to re-regulate their extracellular redox potentials to a more reducing state, approx. 10 mV more reducing by 8 h. These changes may be due to the many downstream functional consequences of Nrf2 activation. Nrf2 up-regulates antioxidant enzymes that could reduce ROS availability and thus allow for the re-regulation of the extracellular redox environment to a more reducing condition. SFN has been shown to cause an increase in intracellular GSH concentrations as mediated through Nrf2 [42]. Increased GSH concentrations may provide a mechanism to remove excess ROS and thereby decrease extracellular concentrations. Moreover, increased GSH concentrations may act to rapidly remove intracellular SFN, as SFN can be transported as a GSH conjugate, and thereby cause decreased Nrf2 signalling [43]. Nrf2 has also been shown to increase the expression of the CySS/glutamate exchange transporter, X(–) [28]. X(–) is important for maintaining intracellular GSH homeostasis [44], but there is some evidence that it also affects extracellular redox environments. In X(–)-deficient mice, plasma CySS concentrations were substantially higher (82 compared with 33 μM), but Cys concentrations were unchanged (18.4 compared with 18.6 μM), illustrating a more oxidized extracellular compartment, determined as being +11 mV more oxidizing [11,28].

Furthermore, SFN treatments also caused a decrease in the production of extracellular ROS. Since production of ROS directly into the extracellular compartment is likely to contribute to a shift in the extracellular environment, the decrease of ROS production may also be an important redox regulatory mechanism. Since Nrf2 is capable of up-regulating X(–) expression and reduces extracellular ROS levels, both of which in turn cause the re-regulation of extracellular redox potentials, Nrf2 stimulation via extracellular stress may be a means by which extracellular redox environments are regulated back to normal baseline values.

These results demonstrate a mechanism by which an oxidizing extracellular redox state, which is associated with numerous disease states, can increase antioxidant systems and cellular defences. Changes in extracellular redox state can thus act to initiate a systemic antioxidant response and protect normal, non-diseased, cells through the increased expression of phase II and antioxidant enzyme systems. Thus diseases that have a systemic effect may provide short-term protection from disease-related oxidative injury. Furthermore, extracellular redox changes as a consequence of pharmacological therapy (i.e. chemotherapy) may serve as a point of intervention to protect cells from unexpected effects of the drug.

**AUTHOR CONTRIBUTION**

Jason Hansen planned and assisted experiments, analysed data and wrote the manuscript. Barry Limhoo was responsible for performing most experiments and assisted in their analysis.

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