Published in final edited form as:

Free Radic Biol Med. 2010 January 15; 48(2): 275–283. doi:10.1016/j.freeradbiomed.2009.10.050.

A key role for mitochondria in endothelial signaling by plasma cysteine/cystine redox potential

Young-Mi Go^{a,*}, Heonyong Park^b, Michael Koval^a, Michael Orr^a, Matthew Reed^{c,d}, Yongliang Liang^a, Debra Smith^a, Jan Pohl^{c,d}, and Dean P. Jones^{a,*}

^a Division of Pulmonary Medicine, Emory University, Atlanta, GA 30322, USA

^b Department of Molecular Biology, Dankook University, Yongin, Korea

^c Microchemical and Proteomics Facility, Department of Medicine, Emory University, Atlanta, GA 30322, USA

^d Biotechnology Core Facility, DSR, Center for Disease Control and Prevention, Atlanta, GA 30333, USA

Abstract

The redox potential of the plasma cysteine/cystine couple ($E_h\text{CySS}$) is oxidized in association with risk factors for cardiovascular disease (CVD), including age, smoking, type 2 diabetes, obesity, and alcohol abuse. Previous in vitro findings support a cause–effect relationship for extracellular $E_h\text{CySS}$ in cell signaling pathways associated with CVD, including those controlling monocyte adhesion to endothelial cells. In this study, we provide evidence that mitochondria are a major source of reactive oxygen species (ROS) in the signaling response to a more oxidized extracellular $E_h\text{CySS}$. This increase in ROS was blocked by overexpression of mitochondrial thioredoxin-2 (Trx2) in endothelial cells from Trx2-transgenic mice, suggesting that mitochondrial thiol antioxidant status plays a key role in this redox signaling mechanism. Mass spectrometry-based redox proteomics showed that several classes of plasma membrane and cytoskeletal proteins involved in inflammation responded to this redox switch, including vascular cell adhesion molecule, integrins, actin, and several Ras family GTPases. Together, the data show that the proinflammatory effects of oxidized plasma $E_h\text{CySS}$ are due to a mitochondrial signaling pathway that is mediated through redox control of downstream effector proteins.

Keywords

Actin; Cytoskeleton; Endothelial cells; Extracellular redox state; Mitochondrial thioredoxin 2; Proinflammatory signaling; Redox proteomics; Redox ICAT; Free radicals

Introduction

Excessive or sustained increases in ROS levels are implicated in the pathogenesis of cardiovascular diseases (CVD) such as atherosclerosis, hypertension, ischemia–reperfusion injury, and diabetic vascular complications [1], whereas moderate ROS levels contribute to regulation of vascular cell function [2,3]. Predominant ROS sources include NADPH

oxidases, xanthine oxidase, uncoupled endothelial nitric oxide synthase (eNOS), and the mitochondrial electron transfer chain [1,4]. In contrast to the extensive studies of NADPH oxidases, xanthine oxidase, and uncoupled eNOS enzyme systems, less is known about the functional significance of mitochondrial ROS in vascular cells. There is increasing evidence that mitochondria-derived ROS can contribute to endothelial cell dysfunction and atherosclerosis [5–7]. Also, Liu et al. demonstrated that shear-stress-induced H_2O_2 production and vasodilation were mediated by superoxide ($O_2^{\cdot-}$) from the mitochondria [8], suggesting a role for mitochondrial ROS in normal vascular physiology.

We developed methods to study mitochondrial thiol/disulfide redox potentials as part of a more comprehensive analysis of the subcellular compartmentalization of redox signaling and oxidative stress [9]. The results show a surprising heterogeneity in redox potentials among compartments and specific thiol/disulfide couples within compartments [9]. Thioredoxin-1 and -2 (Trx1 and Trx2) are more reduced than GSH/GSSG in the respective cytoplasmic and mitochondrial compartments [9]. In addition, the redox couples in the mitochondrial compartment are more reduced than those in nuclei, cytoplasm, and the extracellular environment [9] and more susceptible to oxidation [10]. Despite the lack of redox equilibration of these couples, redox communication between compartments is suggested by experiments that show that a more oxidized extracellular E_h CySS enhances signaling of mitochondria-mediated apoptosis [11].

Human cells in culture regulate extracellular E_h CySS to -80 mV [12], a value similar to the plasma E_h CySS of young healthy adults [13] but considerably more oxidized than cellular pools, which are about -160 mV for Cys/CySS, -230 mV for GSH/GSSG, and -270 mV for Trx1[$(-SH)_2/(-SS-)$] [9]. Studies of plasma E_h as a biomarker of oxidative stress show that Cys/CySS and/or GSH/GSSG is oxidized in association with risk factors for CVD, including age [13,14], type 2 diabetes [15], carotid intima media thickness [16], brachial artery reactivity [17], smoking [18], and alcohol abuse [19]. In vitro studies with systematic variation in E_h CySS in vascular endothelial cells showed that a more oxidized E_h (0 mV) is sufficient to trigger cellular ROS production, proinflammatory signaling, and monocyte adhesion [20]. However, the source of the ROS production and the related thiol/disulfide signaling mechanisms are unknown.

The evidence for mitochondria-derived ROS in endothelial dysfunction, as well as evidence that Trx2 is critical to protect against mitochondrial ROS [10,21] and protect endothelial function in vivo [22], led us to hypothesize that mitochondrial ROS generation could provide a mechanistic link between oxidized plasma E_h and early proinflammatory events of atherogenesis. To test this hypothesis, isolated aortic endothelial cells from Trx2 transgenic mice (Tg MAEC) and cells from littermate control mice (WT MAEC) were exposed to E_h values for Cys/CySS over the range found in human plasma [-150 mV (most reduced value), -80 mV (average value), 0 mV (most oxidized value)]. The results show that E_h CySS-stimulated monocyte adhesion to endothelial cells was prevented by Trx2 overexpression via a mechanism involving decreased mitochondrial ROS. The mechanism was dependent upon cell-surface thiols and was signaled to mitochondria without detectable oxidation of cytoplasmic Trx1 or GSH. However, redox proteomic analyses showed oxidation of a number of proteins associated with membranes and the cytoskeleton. Consequently, a more oxidized extracellular E_h CySS is mechanistically linked to proinflammatory signaling through a mitochondrial pathway mediated by redox-sensitive membranal and cytoskeletal proteins.

Materials and methods

MAEC culture and treatments

MAEC were isolated from Trx2 Tg and WT mice [23] as described [24,25] and confirmed by fluorescence microscopy with DiI-Ac-LDL (Biomedical Technologies, Stoughton, MA, USA) labeling. V5-epitope-tagged human Trx2 (V5-hTrx2) expression was verified by PCR and Western blotting [23]. MAEC were maintained in 20% fetal bovine serum (FBS) and endothelial mitogen (Biomedical Technologies) in DMEM. THP1 monocytes were cultured in 10% FBS in RPMI (37°C, 5% CO₂).

Acetylated LDL (AcLDL), 6-carboxy-2',7'-dichlorofluorescein diacetate (DCF-DA), MitoSOX, 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid, disodium salt (AMS), and monobromotrimethylammoniumbromide (qBBR) were from Invitrogen (Carlsbad, CA, USA). Primary antibodies were used to determine Trx1 (BD Biosciences, San Jose, CA, USA), Trx2 (raised from rabbits by Covance Research Products, Denver, PA, USA, using purified recombinant human Trx2), Myc-epitope-tagged NLS-incorporated Trx1 (NLS-Trx1; Myc antibody from Cell Signaling Technology, Danvers, MA, USA), and V5-hTrx2 (Invitrogen) for Western and redox Western analyses. Secondary antibodies, Alexa Fluor 680 and Alexa Fluor 800, and other fluorescent reagents were from Invitrogen. Mitochondrial electron transport inhibitors, rotenone, and antimycin A were purchased from Sigma-Aldrich (St. Louis, MO, USA).

MAEC incubation with extracellular E_hCySS

E_hCySS was obtained by appropriate Cys and CySS addition to cyst(e)ine-free DMEM (Mediatech, Herndon, VA, USA) with a constant total Cys equivalent (200 μM) [12,20]. MAEC were treated with E_h for 3 h except for redox ICAT (isotope-coded affinity tag; see below), which was for 1 h, and monocyte adhesion assay, which was for 36 h. Different time exposures were selected because E_hCySS caused sequential cell surface protein oxidation, ROS generation, and mitochondrial Trx2 oxidation and increased cell adhesion molecules and selectins, which increase monocyte binding [20]. Extracellular and intracellular Cys and CySS were analyzed by HPLC, and E_hCySS was calculated with the Nernst equation as reported previously [20], $E_h\text{CySS} = E_o\text{CySS} + RT/2F \ln ([\text{CySS}]/[\text{Cys}]^2)$, with −250 mV for E_oCySS at pH 7.4 [13]. The redox potentials as given are for the half-cell, two-electron reduction reactions, i.e., cystine, 2H⁺/2 cysteine.

Immunocytochemistry and fluorescence microscopy

MAEC grown to 70% confluency on a coverslip were preincubated with MitoSOX, MitoTracker Red CMXRos, or MitoTracker Red CMH2-XRos for 45 min before extracellular E_hCySS treatment. After treatment for 1 h, cells were washed, fixed, stained with Alexa Fluor 488 phalloidin to visualize actin, and stained with Hoechst 33342 to visualize nuclei. Mitochondrial localization of V5-hTrx2 was examined by immunofluorescence labeling with V5 antibody followed by Cy2-conjugated goat anti-mouse antibody (Jackson ImmunoResearch). Immunofluorescence was visualized using an Olympus X-70 microscope system, with filter packs suitable for Hoechst (ex BP360–370 nm/dichroic DM400 nm/em BA420–460 nm), Alexa Fluor 488 and Cy2 (ex BP460–490/dichroic DM505/em BA515–550), and MitoTracker red (ex BP530–550/dichroic DM570/em BA590–800+). Cells were imaged with a Hamamatsu Orca-1 CCD camera and acquired and quantified using Image Pro image analysis software (Media Cybernetics, Silver Spring, MD, USA).

Assays

To examine the effects of E_h CySS on adherence of monocytes to MAEC, calcein AM-labeled THP1 cells were added to MAEC after treatment with E_h CySS for 36 h. The fluorescence-attached monocytes were visualized by fluorescence microscopy ($\times 20$), and fluorescence was independently quantified using an M2 plate reader (Molecular Devices, Sunnyvale, CA, USA) [20]. To measure ROS levels in total cells and the mitochondrial compartment, MAEC treated with E_h CySS for 3 h were incubated with DCFH-DA (50 μ M) for 30 min and then with MitoSOX (5 μ M) for 10 min and measured using an M2 plate reader. To measure the redox state of cytoplasmic and nuclear Trx1, MAEC were transfected with or without Myc-epitope-tagged NLS-Trx1. Forty-eight hours after transfection, the cells exposed to E_h CySS for 3 h were examined for the redox state of Trx1 by redox Western blotting [26,27]. Reduced and oxidized forms of Trx1 were detected by probing with Trx1 antibody or Myc antibody. DTT (5 mM) and H_2O_2 (5 mM) were used for reduced and oxidized controls, respectively. To measure the effects of E_h CySS on Trx2, MAEC treated with E_h CySS for 3 h were lysed with a 10% trichloroacetic acid (TCA) solution and proteins were derivatized following the procedures described in the previous study [26]. The reduced and oxidized forms of Trx2 were separated by 15% SDS-PAGE under nonreducing conditions. After blotting, the membranes were probed with antibodies specific to Trx2, and bands corresponding to Trx2 were visualized using an Odyssey scanner and the Odyssey 2.1 software (Li-Cor) [26]. Trx1 and Trx2 redox potentials were calculated from the reduced and oxidized forms of these proteins using the Nernst equation [26]. Steady-state GSH/GSSG redox potentials (E_h GSSG) were calculated from cellular GSH and GSSG concentrations using the Nernst equation (cell volume 5 μ l/mg cells, pH 7.4) [13], E_h GSSG = $E_o + RT/2F \ln([GSSG]/[GSH]^2)$, using -264 mV for E_o at pH 7.4. The redox potentials are for the half-cell two-electron reactions, e.g., GSSG, $2H^+/GSH$.

Proteomic analysis of plasma-membrane-enriched fraction

Three 10-cm plates with confluent MAEC treated with E_h CySS (150 or 0 mV) for 1 h were used for isolation of plasma-membrane-enriched fractions [28]. Briefly, cells were washed, homogenized in 250 mM sucrose containing 10 mM Hepes (pH 7.5) and 1 mM EDTA, and centrifuged at 700 g for 10 min. The resulting supernatant was centrifuged at 10,000 g for 35 min to obtain a pellet that contained primarily plasma membrane and associated proteins. Pellets were resuspended in HBSS containing 2% Zwittergent 3-10 (Calbiochem), and redox ICAT analysis [29,30] was used to identify redox-sensitive proteins. One hundred micrograms of protein from each E_h treatment was labeled with the first biotin-labeled thiol reagent (Heavy ICAT, H; Applied Biosystems) for 2 h. Proteins were then precipitated by 10% TCA for 30 min on ice, pelleted by centrifugation, washed with acetone, and resuspended in denaturing buffer provided by the manufacturer. Unlabeled oxidized thiols (disulfide form) in the proteins were then reduced by tris-(2-carboxyethyl phosphine) and finally labeled with the second biotin-labeled thiol reagent (Light ICAT, L) for 2 h. Each sample from -150 and 0 mV was digested with trypsin for 18 h. The peptides were cleaned, fractionated by cationic exchange followed by avidin purification, and analyzed by mass spectrometry. Using this approach, membrane- and actin-cytoskeleton-associated proteins from each treatment were identified with the H-to-L ratio as a measure of the reduction state of Cys residues in identified proteins.

Mass spectrometry analysis of redox ICAT

An Ultimate 3000 nanoHPLC system (Dionex) with a nanobore column (0.075 $\times$ 150-mm Pepmap C18 100 $\text{\AA}$, 3 μ m; Dionex) was used with the liquid chromatography eluent being sprayed directly into a QSTAR XL system using a nanospray source from Proxeon Biosystems. The data from each salt cut were combined and processed by ProteinPilot software (Applied Biosystems). All quantification was performed by the ProteinPilot version

2.0.1 software using the Swiss-Prot database. Quantification for proteins of interest was manually validated by examination of the raw data.

Results

Effect of hTrx2 expression on endogenous mouse thioredoxins (mTrx2 or mTrx1) and GSH in MAEC

Isolated aortic endothelial cells were incubated with fluorescence-labeled and acetylated LDL for 4 h at 37°C, and images were taken by fluorescence microscopy to verify the identity of endothelial cells (Fig. 1A). Cultured Trx2 transgenic MAEC and MAEC from wild-type littermates were tested for V5-epitope-tagged hTrx2 expression by RT-PCR (Fig. 1B, top left) and Western blotting (Fig. 1B, bottom left). RT-PCR results showed the expected PCR product in Tg MAEC compared with that in Trx2-positive mouse tail extracts loaded as a positive control. Mitochondrial expression of V5-hTrx2 in Tg MAEC but not in WT was shown by immunofluorescence labeling with an antibody against V5 (Fig. 1B, right, see typical punctate mitochondrial pattern in Tg). This result confirmed our previous finding showing mitochondrial localization of hTrx2 in Trx2 Tg mice [23]. To examine whether hTrx2 expression affected endogenous cellular antioxidant systems, the total amounts of mitochondrial Trx2, cytoplasmic and nuclear Trx1, and cellular GSH were determined. Results confirmed V5-epitope-tagged hTrx2 expression in Tg MAEC (V5-hTrx2, Fig. 1B) and showed that expression of this protein had no effect on the abundance of endogenous mouse Trx2 (mTrx2, Fig. 1B), Trx1 (mTrx1, Fig. 1B), or total cellular GSH combining both reduced GSH and oxidized GSSG (14.5 ± 0.5 in WT, 14.7 ± 0.7 in Tg; Fig. 1D).

A more oxidized E_h CySS stimulates mitochondrial Trx2 oxidation

Increasing evidence suggests that oxidized extracellular E_h CySS stimulates inflammatory signaling and sensitizes cells to oxidative stress [11,20]. To test whether this oxidative signaling affects mitochondrial redox status, the Trx2 redox potential was examined by redox Western analysis (Fig. 1C). MAEC (WT and Tg) treated with various E_h CySS (−150, −80, 0 mV) showed that Trx2 in WT (−150 mV, -311 ± 9 mV; −80 mV, -294 ± 5 mV; 0 mV, -277 ± 2 mV) but not in Tg cells (−150 mV, -306 ± 3 mV; −80 mV, -311 ± 4 mV; 0 mV, -315 ± 6 mV) was significantly oxidized by oxidized extracellular E_h (0 mV; Fig. 1C, top). This result shows that the previously observed increase in endothelial cell ROS production in response to a more oxidized E_h CySS is associated with oxidation of mitochondrial Trx2 redox state.

To determine whether this effect was specific to mitochondria, we examined the effect of a more oxidized extracellular E_h CySS on the redox states of other compartments including cytoplasm and nuclei. The redox states of Trx1 in cytoplasm (−150 mV treatment, -261 ± 8 mV; −80 mV treatment, -260 ± 5 mV; 0 mV treatment, -258 ± 4 mV) were measured using redox Western blotting (Fig. 1C, bottom). To examine the nuclear Trx1 redox state, cells transfected with a Myc-epitope-tagged and nuclear-targeted Trx1 (NLS-Trx1) were analyzed after exposure to various extracellular E_h CySS (−150 mV treatment, -259 ± 1 mV; −80 mV treatment, -254 ± 4 mV; 0 mV treatment, -258 ± 3 mV) (Fig. 1C). In contrast to mitochondrial Trx2 oxidation, there was no detectable change in cytoplasmic or nuclear Trx1 redox state (Fig. 1C, bottom) in a more oxidized extracellular E_h CySS. The amounts of GSH and GSSG (Fig. 1D, bottom) and the redox potential of the cellular GSH/GSSG couple (Fig. 1D, top) were also unaffected by a more oxidized extracellular E_h CySS in WT or Tg cells. Thus, the results show that mitochondrial Trx2 is oxidized in response to a more oxidized extracellular E_h CySS without concomitant oxidation of cytoplasmic or nuclear Trx1 and GSH.

No effect of extracellular Cys/CySS redox state on intracellular Cys, CySS, or E_h CySS

Initial extracellular E_h CySS values in cell culture medium calculated from measured Cys and CySS concentrations were comparable to the desired values (Fig. 2, initial values: -150 mV, -154 ± 3 mV; -80 mV, -89 ± 5 mV; 0 mV, -7 ± 2). The extracellular E_h CySS changed as a function of time, with the more reduced E_h CySS becoming oxidized and the more oxidized E_h CySS becoming more reduced over an 8-h time course (Fig. 2). We also examined changes in the cellular levels of Cys and CySS by extracellular E_h CySS. Although the results were limited by a poor signal to noise for measurement of Cys and CySS in cell extracts (about 4:1), the data showed no significant changes in Cys and CySS concentrations over a 4-h time course [respective values for -150 , -80 , and 0 mV for Cys (μ M): 1.2 ± 0.6 , 0.3 ± 0.1 , 0.3 ± 0.1 ; respective values for CySS (μ M): 0.3 ± 0.1 , 0.2 ± 0.1 , 0.3 ± 0.1]. The result suggests that extracellular E_h CySS-dependent redox signaling is not mediated by Cys/CySS transport or cellular levels of Cys/CySS.

A more oxidized extracellular E_h CySS elevates mitochondrial ROS level depending on plasma membrane thiols and Trx2

Our previous study showed that a more oxidized extracellular E_h CySS stimulated an increase in ROS measured by the DCF assay [20] and that this redox-dependent increase in ROS was prevented by pretreatment of cells with nonpermeative alkylating reagents [20]. Because Trx2 oxidation occurred without cytoplasmic Trx1, GSH, or Cys oxidation, we tested the possibility that mitochondria are the source of increased ROS by comparing ROS production in WT and Tg MAEC. WT and Tg MAEC were treated with various extracellular E_h CySS values for 3 h, and the DCF assay was used to measure ROS (Fig. 3A). In WT, an E_h CySS at 0 mV elevated ROS ($138\pm 6\%$) compared with that at -150 ($100\pm 6\%$) or -80 mV ($104\pm 5\%$; Fig. 3A, WT). However, in Tg MAEC, no increase in ROS level was observed at 0 mV ($110\pm 6\%$) compared to -80 ($102\pm 7\%$) or -150 mV ($100\pm 4\%$; Fig. 3A, Tg). Thus, increased Trx2, which has been previously shown to be exclusively in the mitochondria [23], inhibits the increase in cellular ROS.

To further test whether mitochondria are the source of ROS generation in response to a more oxidized extracellular E_h CySS, MAEC were exposed to -150 , -80 , or 0 mV for 3 h and then incubated with the mitochondrial ROS indicator MitoSOX (Fig. 3B). Results showed a significant increase in fluorescence of MitoSOX at 0 mV in WT MAEC compared to -80 and -150 mV in WT MAEC (-150 mV, $131\pm 10\%$; -80 mV, $133\pm 2\%$; 0 mV, $174\pm 7\%$; Fig. 3B, bar graph). Fluorescence was also increased in WT compared to Tg MAEC at 0 mV ($174\pm 7\%$ vs $132\pm 13\%$; Fig. 3B). Increased red fluorescence of MitoSOX in WT MAEC treated with 0 mV compared with WT treated with -150 mV and Tg MAEC treated with 0 mV was visualized by fluorescence microscopy (Fig. 3B, right).

To determine whether the mitochondrial ROS increase in response to extracellular E_h CySS was regulated by cell-surface thiols, MAEC were pretreated with non-cell-permeative thiol-reacting reagents, including positively charged qBBR and negatively charged AMS, for 1 h before being treated with E_h CySS for 3 h. Pretreatment with either qBBR or AMS inhibited the increase in MitoSOX fluorescence (Fig. 3C, see 4, 5, and 6). Thus, the data show that the stimulated ROS generation measured by MitoSOX is mediated through thiols at the plasma membrane or otherwise accessible to extracellular reagents.

Additional experiments to confirm increased mitochondrial oxidation by oxidized extracellular E_h were performed using MitoTracker CMH2XROS. MAEC were prelabeled with MitoTracker CMH2XROS, exposed to -150 or 0 mV, and processed for immunocytochemistry (Fig. 3D). Increased fluorescence of MitoTracker was observed in WT MAEC treated with 0 mV compared to -150 mV (Fig. 3D, red-stained images on left).

This increase in red fluorescence was blocked by AMS pretreatment (AMS+0 mV) and by Trx2 overexpression (Tg+0 mV), consistent with the results from MitoSOX experiments (Fig. 3C). Staining of F-actin with phalloidin and nuclei with Hoechst dye is given for comparison. Taken together, the results show that oxidized extracellular redox signaling stimulates mitochondrial ROS generation and mitochondrial thiol oxidation in a process mediated by plasma-membrane protein thiols and inhibited by mitochondrial Trx2.

Increase in ROS level by oxidized E_h CySS is altered by rotenone but not antimycin A

To examine the source of ROS induced by oxidized E_h CySS, cells pretreated with the mitochondrial electron transport inhibitors rotenone and antimycin A were exposed to extracellular E_h CySS (−150, −80, 0 mV) and analyzed for ROS levels by DCF assay (Fig. 4). Pretreatment with antimycin A, a complex III inhibitor, substantially elevated ROS levels in a pattern that seemed to be independent of extracellular E_h CySS [Fig. 4, Δ increased ROS (%): 118 at −150 mV, 109 at −80 mV, 108 at 0 mV]. In contrast, the complex I inhibitor rotenone increased ROS levels in a pattern that was dependent upon extracellular E_h CySS (Fig. 4). Specifically, the increased amount in the presence of rotenone was much less at 0 mV (Δ increased ROS: 17.1%) than that at −150 mV (Δ increased ROS: 51.5%). Although further study is needed to understand the mechanistic basis for this finding, the results suggest that ROS production by complex I is stimulated by oxidized extracellular E_h CySS. Experiments with Trx2 Tg cells showed that overexpression of Trx2 blocked both rotenone- and antimycin A-increased ROS levels (data not shown).

Proteomic analysis of redox-sensitive plasma-membrane-associated proteins using redox ICAT and mass spectrometry

The inhibition of MitoSOX and MitoTracker oxidation by pre-incubation of endothelial cells with membrane-impermeative thiol reagents (Figs. 3C and D) indicates that signaling could occur through plasma-membrane proteins that are sensitive to extracellular E_h CySS [20]. To identify redox-sensitive proteins associated with the plasma membrane that could potentially function as redox sensors, we applied a proteomics method using ICAT technology [29,30]. This method is based on the rapid and irreversible modification of accessible cysteine residues in proteins with the thiol-reactive reagent iodoacetamide [29]. First, redox ICAT analysis was performed using WT MAEC to examine extracellular E_h CySS-dependent changes in the redox states of cell-surface proteins of the plasma-membrane-enriched fraction exposed to either −150 or 0 mV of E_h CySS for 1 h. In the redox ICAT analysis, the ratio of heavy to light isotopic labeling reflects the ratio of the reduced to the oxidized form of a given peptide, and tandem mass spectrometry identifies the respective peptides containing cysteines modified by the H and L ICAT reagents. Using this approach, we identified 144 proteins with an H:L value identified in both −150 and 0 mV-treated samples (Supplementary Data 1). Because of the large number of proteins determined to be redox sensitive, we examined the distribution of the change in protein oxidation state using a histogram analysis (Fig. 5). The H:L values of 1756 (−150 mV) and 1888 (0 mV) peptides representing the 144 proteins were used. The mean values of the oxidized forms were $42 \pm 16\%$ at 0 mV and $33 \pm 20\%$ at −150 mV (Fig. 5A). A subset of these proteins was recognized to be actin–cytoskeleton-associated proteins and was analyzed separately. This subset consisted of H:L values for 416 (−150 mV) and 453 (0 mV) peptides corresponding to 34 proteins (Supplementary Data 2). The subset showed a comparable difference in distribution of redox states, with the mean value for protein oxidation at 0 mV being $42 \pm 15\%$ compared to $30 \pm 14\%$ at −150 mV (Fig. 5B). These results show that the redox states of multiple cell surface and actin–cytoskeletal proteins respond to changes in extracellular E_h CySS. Whether one or more of these proteins function in signaling mitochondrial ROS production remains to be determined.

Ingenuity Pathway Analysis software (www.ingenuity.com) was used to further evaluate the redox ICAT data for all 144 proteins to determine whether redox-sensitive protein networks could be identified. In this analysis, the difference in percentage oxidation at 0 and -150 mV was determined as “ $\Delta\%$ oxidation.” The data show that the oxidized state varied among cysteine residues within the same protein, i.e., there is a differential redox sensitivity of the cysteines within the same protein as well as differences between proteins. Such differences have previously been shown to depend upon the characteristics of the functional groups of neighboring amino acids in the protein [31]. Proteins with $\Delta\%$ oxidation greater than 5% are shown in Supplementary Data 3 and include proteins in networks that regulate cell structure and organization, cell signaling, and cell adhesion proteins. Proteins with $\Delta\%$ oxidation of 10% or greater include annexin A1, annexin A2, calpain-2, dynein, Ras GTPase activating-like protein, vimentin, integrin- α 3, protein S100-A11, vascular cell adhesion protein 1, GTP-binding protein G(i) subunit α 2, and myoferlin. Several of these proteins are potential candidates for functioning as sensors for extracellular E_h CySS because they are plasma membrane associated and directly exposed to the extracellular compartment.

Discussion

Cys and CySS constitute the most abundant low-molecular-weight thiol/disulfide couple in human plasma, and accumulating evidence shows that E_h CySS is oxidized in association with risk factors for CVD. In addition, the results from in vitro cell models testing whether there is a cause-effect relationship of the plasma Cys/CySS redox state in CVD suggest that oxidation of E_h CySS in vivo could activate inflammatory signaling mechanisms and thereby contribute to CVD. Model studies in animals have further shown that nutritional deficiencies and exposure to bleomycin and endotoxin [32,33] cause oxidation of plasma E_h CySS. Thus, Cys oxidation could provide a common mechanism whereby multiple factors could contribute to a proinflammatory state and result in CVD.

The finding that extracellular E_h CySS controls inflammatory signaling in MAEC is consistent with previous results that cell-surface thiols have critical functions in transferring extracellular redox signals downstream to intracellular redox signaling mechanisms [20]. The present mass spectrometry analysis identifies redox-potential-sensitive proteins from the plasma-membrane-enriched fraction. The results show that a relatively large number of proteins are redox sensitive, including actin-cytoskeleton-associated proteins known to function in regulating cell structure and organization. Further studies are required to examine the detailed mechanisms and establish whether the redox changes in actin cytoskeletal proteins and their associated proteins are directly linked to mitochondrial ROS production or endothelial dysfunction. Such identification of critical cysteine residues would be of great interest for the development of therapeutic targets for control of proinflammatory responses.

Actin dynamics have been found to depend upon endothelial redox changes in association with ROS production [34]. In this study, we found that changes in extracellular E_h CySS altered the redox state of several actin-associated proteins, which is the condition that also regulates ROS generation by mitochondria. Consistent with this, mitochondria were in close association with actin filaments of cells exposed to either oxidized or reduced E_h CySS (data not shown). Whether interactions between mitochondria and the cytoskeleton are required to have extracellular E_h CySS influence the redox state of actin-associated proteins is not known. Nonetheless, several studies have shown that mitochondria bind to multiple cytoskeletal elements, including actin, intermediate filaments, and microtubules, which can influence their function [35,36]. Consequently, the present data support the concept that cytoskeletal elements could interact with mitochondria to stimulate ROS generation. Whether oxidation of cytoskeletal proteins is a critical upstream component of the

mechanism of signaling for mitochondrial ROS production remains to be determined. Furthermore, there is a possibility that increased ROS generated by mitochondria may contribute to downstream protein oxidation that signals NF- κ B activation and increases expression of cell adhesion molecules.

Our study provides evidence for a critical role for Trx2 in endothelial cell function, which is consistent with the previous findings by Zhang et al., which demonstrate that Trx2 improves endothelial cell function and reduces atherosclerotic lesions in ApoE-deficient mice [22]. The results of this study, taken together, support the idea that oxidative extracellular E_h CySS-dependent signaling is a specifically localized pattern of mitochondria-derived oxidative stress. This signaling potentially contributes to endothelial reorganization due to changes in redox states of actin-associated proteins examined by redox ICAT analysis and subsequent changes in mitochondrial function. A possible mechanism could involve cytoskeletal proteins that functionally link to the mitochondrial membrane. A proposed scheme incorporating this concept along with the data provided in this study is shown in Fig. 6. Oxidized extracellular E_h CySS induces oxidation of plasma-membrane proteins, which in turn signals structural changes in cytoskeletal proteins. The cytoskeletal changes lead to mitochondrial functional changes in association with H_2O_2 elevation and Trx2 oxidation in mitochondria. Experiments with rotenone suggest that the ROS generation may occur at complex I. H_2O_2 from mitochondria stimulates inflammatory signaling such as NF- κ B activation and subsequent gene expression, including cell adhesion molecules and selectins shown previously [20]. As a downstream effect of this signaling, monocyte recruitment to endothelial cells is stimulated. Consistent with this model, Trx2 overexpression prevented mitochondrial ROS elevation and stimulation of monocyte adhesion to endothelial cells.

In summary, oxidation of extracellular E_h CySS stimulated oxidation of the plasma-membrane and associated actin and actin-related cytoskeletal proteins. This oxidative signaling resulted in oxidation of mitochondrial Trx2 without concomitant oxidation of Trx1 or GSH redox systems. Oxidative signaling led to stimulation of monocyte adhesion to endothelial cells. Trx2 overexpression prevented monocyte adhesion by blocking increases in mitochondrial ROS. The results identify a novel signaling pathway whereby an oxidized plasma thiol/ disulfide redox state contributes to early atherosclerosis development by stimulating mitochondrial ROS production. Consequently, prevention of plasma Cys oxidation, blocking membranal oxidant signaling, and prevention of mitochondrial oxidation provide potential targets to protect from oxidative stress-induced cardiovascular disease development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH Grants ES011195, ES009047, and HL083120.

Abbreviations

AMS	4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid
CySS	cystine
CVD	cardiovascular disease
DCF	dichlorofluorescein

DTT	dithiothreitol
E_hCySS	Cys/CySS redox potential
GSH	glutathione
GSSG	glutathione disulfide
ICAT	isotope-coded affinity tag
MAEC	mouse aortic endothelial cell
NLS	nuclear localization signal
ROS	reactive oxygen species
Trx	thioredoxin
Tg	transgenic
WT	wild type

References

1. Li JM, Shah AM. Endothelial cell superoxide generation: regulation and relevance for cardiovascular pathophysiology. *Am. J. Physiol., Regul. Integr. Comp. Physiol* 2004;287:R1014–1030. [PubMed: 15475499]
2. Felty Q, Xiong WC, Sun D, Sarkar S, Singh KP, Parkash J, Roy D. Estrogen-induced mitochondrial reactive oxygen species as signal-transducing messengers. *Biochemistry* 2005;44:6900–6909. [PubMed: 15865435]
3. Kimura S, Zhang GX, Nishiyama A, Shokoji T, Yao L, Fan YY, Rahman M, Suzuki T, Maeta H, Abe Y. Role of NAD(P)H oxidase- and mitochondria-derived reactive oxygen species in cardioprotection of ischemic reperfusion injury by angiotensin II. *Hypertension* 2005;45:860–866. [PubMed: 15824196]
4. Zhang DX, Gutterman DD. Mitochondrial reactive oxygen species-mediated signaling in endothelial cells. *Am. J. Physiol., Heart Circ. Physiol* 2007;292:H2023–2031. [PubMed: 17237240]
5. Doughan AK, Harrison DG, Dikalov SI. Molecular mechanisms of angiotensin II-mediated mitochondrial dysfunction: linking mitochondrial oxidative damage and vascular endothelial dysfunction. *Circ. Res* 2008;102:488–496. [PubMed: 18096818]
6. Puddu P, Puddu GM, Galletti L, Cravero E, Muscari A. Mitochondrial dysfunction as an initiating event in atherogenesis: a plausible hypothesis. *Cardiology* 2005;103:137–141. [PubMed: 15665536]
7. Wenzel P, Schuhmacher S, Kienhofer J, Muller J, Hortmann M, Oelze M, Schulz E, Treiber N, Kawamoto T, Scharffetter-Kochanek K, Munzel T, Burkle A, Bachschmid MM, Daiber A. Manganese superoxide dismutase and aldehyde dehydrogenase deficiency increase mitochondrial oxidative stress and aggravate age-dependent vascular dysfunction. *Cardiovasc. Res* 2008;80:280–289. [PubMed: 18596060]
8. Liu Y, Zhao H, Li H, Kalyanaraman B, Nicolosi AC, Gutterman DD. Mitochondrial sources of H_2O_2 generation play a key role in flow-mediated dilation in human coronary resistance arteries. *Circ. Res* 2003;93:573–580. [PubMed: 12919951]
9. Go YM, Jones DP. Redox compartmentalization in eukaryotic cells. *Biochim. Biophys. Acta* 2008;1780:1273–1290. [PubMed: 18267127]
10. Chen Y, Cai J, Jones DP. Mitochondrial thioredoxin in regulation of oxidant-induced cell death. *FEBS Lett* 2006;580:6596–6602. [PubMed: 17113580]
11. Jiang S, Moriarty-Craige SE, Orr M, Cai J, Sternberg P Jr, Jones DP. Oxidant-induced apoptosis in human retinal pigment epithelial cells: dependence on extracellular redox state. *Investig. Ophthalmol. Vis. Sci* 2005;46:1054–1061. [PubMed: 15728565]

12. Jonas CR, Ziegler TR, Gu LH, Jones DP. Extracellular thiol/disulfide redox state affects proliferation rate in a human colon carcinoma (Caco2) cell line. *Free Radic. Biol. Med* 2002;33:1499–1506. [PubMed: 12446207]
13. Jones DP, Mody VC Jr, Carlson JL, Lynn MJ, Sternberg P Jr. Redox analysis of human plasma allows separation of pro-oxidant events of aging from decline in antioxidant defenses. *Free Radic. Biol. Med* 2002;33:1290–1300. [PubMed: 12398937]
14. Hildebrandt W, Kinscherf R, Hauer K, Holm E, Droge W. Plasma cystine concentration and redox state in aging and physical exercise. *Mech. Ageing Dev* 2002;123:1269–1281. [PubMed: 12020948]
15. Samiec PS, Drews-Botsch C, Flagg EW, Kurtz JC, Sternberg P Jr, Reed RL, Jones DP. Glutathione in human plasma: decline in association with aging, age-related macular degeneration, and diabetes. *Free Radic. Biol. Med* 1998;24:699–704. [PubMed: 9586798]
16. Ashfaq S, Abramson JL, Jones DP, Rhodes SD, Weintraub WS, Hooper WC, Vaccarino V, Harrison DG, Quyyumi AA. The relationship between plasma levels of oxidized and reduced thiols and early atherosclerosis in healthy adults. *J. Am. Coll. Cardiol* 2006;47:1005–1011. [PubMed: 16516085]
17. Ashfaq S, Abramson JL, Jones DP, Rhodes SD, Weintraub WS, Hooper WC, Vaccarino V, Alexander RW, Harrison DG, Quyyumi AA. Endothelial function and aminothiol biomarkers of oxidative stress in healthy adults. *Hypertension* 2008;52:80–85. [PubMed: 18504327]
18. Moriarty SE, Shah JH, Lynn M, Jiang S, Openo K, Jones DP, Sternberg P. Oxidation of glutathione and cysteine in human plasma associated with smoking. *Free Radic. Biol. Med* 2003;35:1582–1588. [PubMed: 14680681]
19. Brown LA, Ping XD, Harris FL, Gauthier TW. Glutathione availability modulates alveolar macrophage function in the chronic ethanol-fed rat. *Am. J. Physiol* 2007;292:L824–832.
20. Go YM, Jones DP. Intracellular proatherogenic events and cell adhesion modulated by extracellular thiol/disulfide redox state. *Circulation* 2005;111:2973–2980. [PubMed: 15927968]
21. Chen Y, Cai J, Murphy TJ, Jones DP. Overexpressed human mitochondrial thioredoxin confers resistance to oxidant-induced apoptosis in human osteosarcoma cells. *J. Biol. Chem* 2002;277:33242–33248. [PubMed: 12032145]
22. Zhang H, Luo Y, Zhang W, He Y, Dai S, Zhang R, Huang Y, Bernatchez P, Giordano FJ, Shadel G, Sessa WC, Min W. Endothelial-specific expression of mitochondrial thioredoxin improves endothelial cell function and reduces atherosclerotic lesions. *Am. J. Pathol* 2007;170:1108–1120. [PubMed: 17322393]
23. He M, Cai J, Go YM, Johnson JM, Martin WD, Hansen JM, Jones DP. Identification of thioredoxin-2 as a regulator of the mitochondrial permeability transition. *Toxicol. Sci* 2008;105:44–50. [PubMed: 18550601]
24. Chen S, Segal M, Agarwal A. “Lumen digestion” technique for isolation of aortic endothelial cells from heme oxygenase-1 knockout mice. *BioTechniques* 2004;37:84–86. 88–89. [PubMed: 15283205]
25. Kobayashi M, Inoue K, Warabi E, Minami T, Kodama T. A simple method of isolating mouse aortic endothelial cells. *J. Atheroscler. Thromb* 2005;12:138–142. [PubMed: 16020913]
26. Halvey PJ, Watson WH, Hansen JM, Go YM, Samali A, Jones DP. Compartmental oxidation of thiol–disulphide redox couples during epidermal growth factor signalling. *Biochem. J* 2005;386:215–219. [PubMed: 15647005]
27. Watson WH, Pohl J, Montfort WR, Stuchlik O, Reed MS, Powis G, Jones DP. Redox potential of human thioredoxin 1 and identification of a second dithiol/disulfide motif. *J. Biol. Chem* 2003;278:33408–33415. [PubMed: 12816947]
28. van der Flier A, Sonnenberg A. Function and interactions of integrins. *Cell Tissue Res* 2001;305:285–298. [PubMed: 11572082]
29. Go, Y-M.; Phol, J.; Jones, DP. Quantification of redox conditions in the nucleus.. In: Hancock, R., editor. *The Nucleus*. Humana Press; Totowa, NJ: 2008. p. 303-317.
30. Sethuraman M, McComb ME, Huang H, Huang S, Heibeck T, Costello CE, Cohen RA. Isotope-coded affinity tag (ICAT) approach to redox proteomics: identification and quantitation of

- oxidant-sensitive cysteine thiols in complex protein mixtures. *J. Proteome Res* 2004;3:1228–1233. [PubMed: 15595732]
31. Snyder GH, Cennerazzo MJ, Karalis AJ, Field D. Electrostatic influence of local cysteine environments on disulfide exchange kinetics. *Biochemistry* 1981;20:6509–6519. [PubMed: 6796114]
 32. Hansen JM, Zhang H, Jones DP. Differential oxidation of thioredoxin-1, thioredoxin-2, and glutathione by metal ions. *Free Radic. Biol. Med* 2006;40:138–145. [PubMed: 16337887]
 33. Iyer SS, Jones DP, Brigham KL, Rojas M. Oxidation of plasma cysteine/cystine redox state in endotoxin-induced lung injury. *Am. J. Respir. Cell Mol. Biol* 2009;40:90–98. [PubMed: 18664641]
 34. Moldovan L, Moldovan NI, Sohn RH, Parikh SA, Goldschmidt-Clermont PJ. Redox changes of cultured endothelial cells and actin dynamics. *Circ. Res* 2000;86:549–557. [PubMed: 10720417]
 35. Anesti V, Scorrano L. The relationship between mitochondrial shape and function and the cytoskeleton. *Biochim. Biophys. Acta* 2006;1757:692–699. [PubMed: 16729962]
 36. Reipert S, Steinbock F, Fischer I, Bittner RE, Zeold A, Wiche G. Association of mitochondria with plectin and desmin intermediate filaments in striated muscle. *Exp. Cell Res* 1999;252:479–491. [PubMed: 10527638]

Fig. 1.

Oxidized extracellular E_h CySS (0 mV) stimulates mitochondrial Trx2 oxidation in MAEC. (A) MAEC from Trx2 transgenic and littermate control mice were verified by AcLDL labeling. (B) Trx2 overexpression in Tg MAEC was verified by PCR (top left) and Western blotting (bottom left) with no effect on endogenous mouse Trx2 (mTrx2) or Trx1 (mTrx1) expression. Western blots were probed with antibodies specific to V5 epitope, mTrx2, and mTrx1. Mitochondria-localized expression of V5-hTrx2 in Tg is shown (right; red, V5-hTrx2; blue, Hoechst). (C) Trx2 (top) and Trx1 (bottom) redox states at their respective extracellular E_h values. Cytoplasmic (Trx1) and nuclear (NLS-Trx1) Trx1 redox states were examined in cells transfected with or without NLS-Trx1. DTT- and H_2O_2 -treated samples were used for fully reduced and oxidized controls, respectively. (D) Cellular GSH (bottom), GSSG (bottom), and GSH/GSSG redox states (top) at various E_h . Data presented are means $\pm$ SEM of triplicates from two experiments.

Fig. 2.

Extracellular $E_h\text{CySS}$ changed with time. Confluent WT MAEC were incubated with various initial $E_h\text{CySS}$ (intended redox values -150 , -80 , 0 mV) and assayed for Cys and CySS in culture medium as a function of time. $E_h\text{CySS}$ calculated from the Nernst equation are shown as means $\pm$ SEM; $n=3$.

Fig. 3.

Proinflammatory response of MAEC to oxidized extracellular E_h is signaled through a pathway dependent upon membranal thiols and mitochondrial ROS. WT and Tg MAEC exposed to E_h CySS (-150, -80, 0 mV) for 3 h were examined for cellular and mitochondrial ROS by quantifying fluorescence of (A) DCF and (B) MitoSOX, respectively. Cell images (B, right) to visualize mitochondrial ROS increase are representative of WT MAEC treated with -150 mV (1) and 0 mV (2) and Tg MAEC treated with 0 mV (3) from three experiments. (C) WT MAEC pretreatment with the membrane-nonpermeable thiol-alkylating reagents qBBR and AMS (500 μ M each) prevented E_h -dependent mitochondrial ROS increase measured by MitoSOX fluorescence (left) and visualized by fluorescence

microscopy (right, compare 5 and 6 to 4). Data are means $\pm$ SEM ($n=8$). * $P<0.05$ vs group of WT treated with 0 mV. (D) Increase in mitochondrial ROS by 0 mV was verified by increase in MitoTracker fluorescence (red, 0 mV), whereas AMS pretreatment (red, AMS+0 mV) and Trx2 overexpression (red, Tg+0 mV) inhibited ROS increase. No changes in actin filaments (F-actin) or nuclei were observed with Alexa Fluor 488 phalloidin and Hoechst 33342 staining, respectively.

Fig. 4.

Increase in ROS level by oxidized $E_h\text{CySS}$ is regulated by mitochondrial electron transport complex I. WT MAEC were pretreated with rotenone (2 μM) or antimycin A (5 μM) for 2 h before oxidized $E_h\text{CySS}$ treatment. Cells were then examined for ROS levels by measuring DCF fluorescence. (A) ROS levels induced by various $E_h\text{CySS}$ (-150, -80, 0 mV) were quantified without (None) or with rotenone or antimycin A. (B) Rotenone (left) and antimycin A (right)-stimulated levels of ROS at various $E_h\text{CySS}$ were quantified.

Fig. 5.

Extracellular E_h -dependent redox changes in plasma-membrane- and actin-associated proteins. The membrane-enriched fraction isolated from MAEC (WT) treated with -150 or 0 mV was analyzed by redox ICAT-based mass spectrometry. (A) Distribution of the oxidized states of the peptides (1756 peptides at -150 mV; 1888 peptides at 0 mV) relevant to 144 proteins (-150 and 0 mV) from the membrane-enriched fraction. The mean values of oxidation were 33 (SD of 17%) and 42% (SD of 20%) for the -150 and 0 mV treatments, respectively. (B) Redox changes in 34 actin and actin-related proteins (416 peptides at -150 mV; 453 peptides at 0 mV) were examined. Mean values of the percentage of oxidized state for -150 and 0 mV were 30 (SD of 14%) and 42% (SD of 15%), respectively.

Fig. 6. Proposed scheme for mitochondrial redox signaling in response to oxidized extracellular E_h . E_h -induced oxidation of plasma membrane (PM) and cytoskeleton proteins stimulates ROS generation in mitochondria that is blocked by Trx2. H_2O_2 from the mitochondria triggers inflammatory signaling including NF- κ B activation and subsequent gene expression (cell adhesion molecules, integrins, cytoskeletal proteins). H_2O_2 can affect cell structure by affecting actin dynamics. Changes in the endothelial cell structure and increases in cell adhesion molecules result in an increase in monocyte recruitment as an early event of atherosclerosis.