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Cysteine/cystine redox signaling in cardiovascular disease

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Abstract

Extracellular thiol/disulfide redox environments are highly regulated in healthy individuals. The major thiol/disulfide redox couple in human plasma is cysteine (Cys) and its disulfide form, cystine (CySS). Oxidation of this redox couple measured as a more positive steady-state redox potential (E_h) is associated with risk factors for cardiovascular disease (CVD), including aging, smoking, obesity, and alcohol abuse. Rodent and vascular cell studies show that extracellular redox state of Cys/CySS (E_hCySS) can play a vital role in controlling CVD through proinflammatory signaling. This inflammatory signaling is regulated by cell surface protein redox state and involves mitochondrial oxidation, nuclear factor-κB activation, and elevated expression of genes for monocyte recruitment to endothelial cells. Gene array and proteomics studies reveal the global nature of redox effects, and different cell types, e.g., endothelial cells, monocytes, fibroblasts, and epithelial cells, show cell-specific redox responses with different phenotypic traits, e.g., proliferation and apoptosis, which can contribute to CVD. The critical nature of the proinflammatory redox signaling and cell biology associated with E_hCySS supports the use of plasma levels of Cys, CySS, and E_hCySS as key indicators of vascular health. Plasma redox state-based pharmacologic interventions to control or improve E_hCySS may be effective in preventing CVD onset or progression.

Keywords

Thiol/disulfide; Plasma redox state; Inflammatory mechanism; Atherosclerosis; Endothelial cells; Monocytes

I. Introduction

Oxidative stress has been implicated in the progression of many diseases including cardiovascular disease (CVD), cancer, and neurodegenerative disease. Numerous studies supporting an important role of free radicals in disease provided a basis to use antioxidants such as vitamin C and E in prevention and treatment trials for CVD; however, these trials showed that these antioxidant vitamins have limited benefits [1-3]. Moreover, a systematic review on the effects of antioxidant supplements indicated that treatment with β-carotene, vitamin A, and vitamin E may increase mortality rather than improve health in randomized primary and secondary prevention trials [4]. Therefore, development of therapeutics for preventing CVD using different approaches is critical.

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Proteins present in extracellular fluids and on the surface of cells are susceptible to oxidation through reactive thiols in cysteine (Cys) residues. Reactive thiols often form transient catalytic intermediates in the reaction cycle of many enzymes or serve as a site of covalent modification to regulate biological activity. Alterations in protein activity by modifying the redox state of functionally essential thiols affects cellular signaling mechanisms, which couples protein redox state directly to functional activity. Oxidation and modification of extracellular thiols have a significant effect on lymphocyte proliferation and function, illustrating the importance of maintaining extracellular thiols in cell signaling [5-7]. These changes in lymphocyte function were implicated in CVD [8,9]. In addition to CVD, the progression of the numerous diseases involves oxidation and modification of thiols. For instance, oxidation of thiols sensitized radiation-induced cell death, indicating that thiol plays a critical role in protecting cells from a pathologic event [10]. Available data indicates that control of protein redox state via thiol-disulfide switching is critical for normal cellular activities and for maintaining physiological functions. Consequently, thiol oxidation offers an alternative mechanism by which oxidative stress could contribute to disease with little or no dependence upon free radicals.

Earlier studies suggesting that high plasma homocysteine is associated with other risk factors for CVD [11,12]. More recent evidence has suggested that these risk factors are specifically associated with the redox state of thiol/disulfide systems [11,13-16]. This fact was supported by multiple studies suggesting that increased plasma homocysteine levels of patients with peripheral vascular disease and decreased plasma albumin levels were associated with oxidation of plasma redox state [14,15]. High methionine level in association with hyperhomocystenemia leads to atherosclerosis in the coronary artery, and is exacerbated when combined with high dietary cholesterol [17,18]. Met intake positively associated with CAD and death while protein intake negatively associated and no relation to homocysteine levels [17]. Earlier studies also showed that increased total Cys was associated with pathologic conditions such as CVD [19-22]. However, this interpretation was not correct due to lack of separate quantification of the reduced form, Cys, the oxidized form, CySS, or the mixed protein Cys disulfide. Later, the increased level of total Cys was redefined as increased oxidized form of Cys (CySS) and the protein-bound disulfide form. Since Cys/CySS is the most abundant low molecular weight thiol/disulfide couple in human plasma [14,23], the value of the plasma redox state is largely determined by the redox state of Cys/CySS.

In addition to the critical function of Cys in proteins, redox state of free Cys and CySS in human plasma has attracted attention as a means to measure oxidative stress in the clinical setting. The present review summarizes relevant literature showing that the balance of Cys and CySS modulates cellular events relevant to CVD, including early proinflammatory signaling-controlled cell adhesion [24-26], cell proliferation [27] and resistance to apoptosis [28]. The clinical research showing that Cys becomes more oxidized in association with age [23,29], smoking [30], and age-related diseases [31] are summarized. These include evidence that increased oral intake of zinc and sulfur amino acid supplements modulates the Cys and CySS concentrations in plasma [32], thereby providing possible approaches to decrease risk of CVD. The advances in the understanding of Cys/CySS redox signaling and control suggest that free radical scavenging trials may have failed because these antioxidants do not correct oxidative stress-associated disruption of thiol/disulfide systems in vascular diseases.

II. Effects of oxidized extracellular E_hCySS on proinflammatory signaling

The innermost cell layer of blood vessels is the endothelium. These cells play a vital role in maintaining vascular health by responding to physical and biochemical changes in the blood.

The normal endothelium promotes vasodilatation, is anti-inflammatory and inhibits thrombosis. When the endothelium becomes dysfunctional, many of these properties are altered. Endothelial injury and dysfunction, highly associated with increased oxidative stress and inflammation, stimulate atherosclerotic events by initiating monocyte adhesion to endothelium, thereby leading to development of atherosclerosis [33,34]. A number of clinical studies are discussed below which provide evidence that CVD is associated with oxidation of plasma redox state. In this section, we focus on recent advances which show that oxidized extracellular E_hCySS stimulates inflammatory redox signaling associated with mechanisms in the pathogenesis of the vascular disease (Fig. 1).

Downstream signaling due to changes in extracellular E_hCySS has been studied using a redox clamp in which Cys and CySS concentrations are used to obtain a series of controlled E_hCySS [35]. The extracellular/plasma E_h is controlled mainly by Cys/CySS and GSH/GSSG, while redox states of other subcelluar compartments (cytoplasm, mitochondria, nucleus, endoplasmic reticulum) are maintained by different thiol/disulfide couples (See Fig. 2). To test whether oxidation of extracellular E_hCySS affects inflammatory signaling and vascular function, redox clamp approach was used to approximate the range of human plasma E_hCySS with aortic endothelial cells and monocytes. [25,26]. Subcellular compartmentation of thiol systems has been studied little in association with CVD. However, research with endothelial cells shows the same general characteristics as other cell types and supports an important role for mitochondrial oxidation. This work has established a mechanistic sequence whereby extracellular E_hCySS has a central role in regulation of early atherosclerotic events. As summarized in Fig.1 endothelial cells and monocytes stimulate inflammatory signaling in response to oxidation of extracellular E_hCySS. This signaling involves several key events relevant to atherosclerosis development such as redox changes in cell surface and actin cytoskeleton proteins, mitochondrial oxidation, NF-κB activation, increased expression of cell adhesion molecules and a cytokine, IL-1β, and monocyte adhesion. These key findings are highlighted as follows;

1) Oxidation of extracellular redox environment elevated H_2O_2, a non-radical signaling molecule that activates downstream effectors [24-26]

Increased fluorescence of dichlorofluorescin (DCF) as a measure of oxidant generation was observed in aortic endothelial cells and monocytes after exposure to oxidized E_hCySS. Introducing polyethylene glycol-conjugated catalase into endothelial cells prior to oxidized E_hCySS treatment inhibited DCF fluorescence and indicated that oxidized extracellular E_hCySS stimulates H_2O_2 generation in endothelial cells.

2) Oxidized extracellular E_hCySS stimulated H_2O_2 generation in mitochondria [26]

Both MitoSOX and MitoTracker fluorescence were enhanced in cells after 3h exposure to oxidized E_hCySS suggesting that mitochondria are the source of oxidant generation in response to a more oxidized extracellular E_hCySS. In addition, mitochondrial thioredoxin, Trx2, was substantially oxidized by oxidized extracellular E_hCySS whereas the redox state of cytoplasmic and nuclear thioredoxin, Trx1, was not altered. Enhanced MitoSOX and MitoTracker fluorescence by oxidized E_hCySS was inhibited by overexpression of mitochondrial Trx2 [26]. H_2O_2 produced by the mitochondria stimulates monocyte adhesion to endothelial cells. However, this signaling was blocked by Trx2 overexpression, suggesting that NF-κB – dependent activation of proinflammatory signaling by oxidized extracellular E_hCySS is abrogated by Trx2 overexpression (Y.-M. Go and D. P. Jones, unpublished data). Taken together with studies of transcriptional activation and cell adhesion, the data support a pathway in which mitochondria are a key mediator to transfer extracellular E_hCySS signaling to downstream mechanisms to activate the transcription

factor NF-κB and increase inflammatory gene expression including cell adhesion molecules, selectins, and cytokines [26].

3) Intracellular responses to extracellular E_hCySS are regulated by plasma membrane protein thiols

qBBr and AMS are bimane and maleimide derivatives, respectively, that are readily conjugated to thiols of cell surface proteins to block thiol/disulfide interactions. The high polarity of qBBr (positively charged) and AMS (negatively charged) decreases their membrane permeability and makes these reagents useful for determining whether redox-sensitive thiols are exposed at extracellular sites. Endothelial cells pretreated with these thiol-reacting reagents had decreased H_2O_2 production in response to oxidized E_hCySS as measured by DCF, MitoSOX, and MitoTracker [25,26]. Additionally, quantification of cell surface thiols after extracellular E_hCySS treatment by monitoring fluorescence of AMS-bound thiols showed that thiols were detected more at reduced E_hCySS than at oxidized state [25]. Therefore, these results show that key redox events of the inflammatory processes are controlled by the redox state of Cys/CySS at the extracellular surface of endothelial cells. Redox processes at cell surfaces have been extensively studied, especially related to superoxide anion radical, H_2O_2 and nitric oxide generation [36-38]. Thiol proteins such as Trx1 [39], protein disulfide isomerase [40] and related proteins [41] are known to be present at cell surface. Available data support the interpretation that the E_hCySS effects on the plasma membrane are specific. For instance, E_hCySS effects on endothelial cell signaling were blocked by both qBBr and AMS [25] while Caco2 signaling was only sensitive to AMS [42]. Additionally, proliferative signaling in fibroblasts and smooth muscle cells differed considerably from that observed in endothelial cells, monocytes and human retinal pigment epithelial cells (See below). Finally, alveolar type 2 cells signaling in response to E_hCySS differed from that provided by E_hGSSG at identical E_h values (J. M. Hansen, D. P. Jones, L. A. Brown, unpublished data). However, identification of specific targets of oxidation is challenging because of the presence of many proteins with reactive surface thiols, the large size and complexity of these proteins (e.g. integrins), and the specialized microdomains of the plasma membrane.

4) The redox states of multiple cell surface and actin–cytoskeleton proteins respond to changes in extracellular E_hCySS [26]

Redox-sensitive proteins associated with plasma membrane that could potentially function as redox sensors have been studied with a redox proteomics method using Isotope-Coded Affinity Tag (ICAT) technology [43]. Proteins exhibiting extensive oxidation include vascular cell adhesion molecule, integrins, actin, and several Ras family GTPases that control cell structure and cytoskeletal organization. Several of these proteins are candidates as sensors of extracellular E_hCySS. Ingenuity Pathway Analysis indicates that proteins oxidized in response to changes in extracellular E_hCySS are in networks that modulate cell signaling and morphology (Fig. 3).

The mechanisms controlling the redox dependences of these proteins are largely unknown. Variations in Cys and CySS concentrations to maintain the same redox potential with 100 μM total Cys equivalents instead of 200 μM did not affect the enhancement of monocyte adhesion to endothelial cells or H_2O_2 generation at E_hCySS, 0 mV [25]. This suggests that key events are linked to redox potential rather than to concentration of Cys or CySS. However, imported CySS from the extracellular compartment could also have an effect on global thiol oxidation in cells and trigger oxidative signaling. Additionally, it is presently unclear whether the measured redox dependences are catalyzed by specific proteins or are thiol-disulfide exchange reaction of a subset of reactive protein thiols.

There has been an increasing interest in autophagy as a determinant of cellular protein turnover, and its role in aging and various diseases [44]. Excessive self-digestion and degradation of essential cellular components by autophagy promote cell death; however, autophagy might also promote cell survival by generating free amino acids and fatty acids required to maintain function during nutrient-limiting conditions [45]. In addition to the great number of extracellular (e.g. hormone, starvation) and intracellular stimuli (e. g. accumulation of misfolded proteins, infection), autophagy is modulated in a redox-dependent manner [44]. For instance, the cysteine protease Atg4 is regulated by a redox-dependent mechanism during starvation-induced autophagy [46]. Changes in redox state can cause rapid activation and inactivation of Atg4, and induce the autophagic process. The potential contribution of autophagy to variations in plasma E_hCySS is interesting since autophagy generates free amino acids including Cys and regulates protein synthesis. This might be especially important when considering diurnal variations in plasma E_hCySS, as autophagy is also known to have a circadian rhythm.

5) Both aortic endothelial cells and monocytes respond to extracellular E_hCySS

Both endothelial cells and circulating cells play a key role in maintaining vascular health. In addition to endothelial cells, monocytes (THP1 and U937) also respond to extracellular E_hCySS [24,25]. Monocytes exposed to the oxidized extracellular E_hCySS increased adhesion to endothelial cells, similar to the results obtained by endothelial cells exposed to the oxidized extracellular E_hCySS. These results support a mechanism of inflammatory signaling in early atherosclerosis due to oxidation of extracellular E_hCySS (Fig 1). A more oxidized E_hCySS values results in oxidized plasma membrane protein thiols, elevates H_2O_2 production by mitochondria, activates NF-κB, increases expression of adhesion molecules (intercellular adhesion molecule-1, platelet endothelial cell adhesion molecule-1, P-selectin), and stimulates THP1 monocytes adhesion to endothelial cells [25,26].

Studies in human monocyte cell line (U937 cells) also showed that monocytes exposed to more oxidized E_hCySS stimulated pro-Interleukin (IL)-1β secretion to the extracellular compartment [47]. In the same study, in vivo data from humans and LPS-challenged mice showed strong positive associations of the level of IL-1β with plasma E_hCySS oxidation. These results are consistent with the findings from endothelial cell studies above that oxidation of E_hCySS causes inflammation signaling associated with vascular disease.

Phenotypic studies of THP-1 cells further showed that a cell stress response occurred with more oxidized E_hCySS and that cell proliferation was stimulated with more reduced E_hCySS [24]. Microarray analysis and mass spectrometry-based proteomics showed that components of stress/detoxification and cell death pathways were increased by oxidized E_hCySS, while components of cell growth and proliferation pathways were increased by a reduced E_hCySS. These studies suggest that extracellular redox events in the subendothelial space could also contribute to monocytic proliferation, differentiation to macrophages, and/or activation of apoptosis in CVD mechanisms.

Alternative redox-sensitive processes could also contribute to CVD mechanisms, but these have not been studied in the vascular system. For instance, fibrotic signaling is activated by oxidized E_hCySS, and the effects of oxidized plasma E_hCySS on lung fibrosis has been investigated [48] as a model for life-threatening pulmonary toxicity of bleomycin therapy [49,50]. In mouse studies, bleomycin-induced lung fibrosis was associated with distinct changes in plasma E_hCySS and E_hGSSG. E_hGSSG was selectively oxidized during the proinflammatory phase, while E_hCySS oxidation was observed at the fibrotic phase. Although not studied in atherosclerosis, a parallel effect on profibrotic signaling could contribute to plaque pathophysiology. This will be discussed further in a later section.

III. Oxidation of plasma redox in association with risk factors for CVD

The concept that E_hCySS modulation can affect cell signaling provides a clear alternative to free radical mechanisms for oxidative stress in CVD. Oxidized E_hCySS is sensed by cell surface thiols in endothelial cells and monocytes. In endothelial cells, oxidants activate NF-κB and increase expression of cell adhesion molecules. In monocytes, oxidation activates proinflammatory cytokine IL-1b and TNFα production. Together, these processes enhance the critical early event of vascular monocyte adhesion. Thus, a critical question concerns the in vivo conditions in which E_hCySS is oxidized. Several studies have shown that oxidation of E_hCySS, and/or related increases in CySS concentration and oxidation of E_hGSSG are associated with risk factors for CVD. These are summarized in Table 1 and discussed in the following section.

1) Age-related oxidation

Age is an important risk factor for CVD [51] and human studies have demonstrated oxidation in plasma E_h, with an age-dependent increase of the plasma CySS [13,14,29,52]. A study of 122 healthy individuals aged 19-85 years showed a linear oxidation of E_hCySS with age at a rate of 0.16 mV/year over the entire age span. In contrast, E_hGSSG was not oxidized prior to 45 years and subsequently was oxidized at a nearly linear rate of 0.7 mV/year [23] suggesting that E_hCySS and E_hGSSG need to be considered differently. The data indicate that there is a continuous increase in oxidative events throughout adult life but that the capacity of the GSH antioxidant system is maintained until middle age and then declines relatively rapidly. The data further suggest that E_hCySS and E_hGSSG should be considered separately to distinguish causative oxidative events and decreased GSH antioxidant functions.

Age-dependent plasma oxidation has also been observed in patients with other pathologic conditions. In addition to coronary heart disease or hyperlipidemia [53], patients with cancer [14] and healthy subjects after intensive physical exercise [29] showed comparable oxidations in plasma E_h at a relatively younger age. Age-related oxidation of plasma E_hCySS was associated with higher plasma concentrations of amino acids, which were also associated with a lower rate of protein synthesis. The results therefore suggest that age-related plasma E_h oxidation is perhaps due to a decrease in the rate of protein synthesis and corresponding amino acid clearance together with failure of Cys or CySS transport from tissue to blood. However, as discussed below, more systematic research is needed to understand how plasma E_h is maintained, why it becomes oxidized in association with age, and how its oxidation contributes to age-related disease.

2) Plasma oxidation by smoking

Cigarette smoking contributes to the development of numerous chronic age-related disease processes such as CVD. Patients and healthy subjects who smoked showed decreased GSH levels in platelet, bronchial tissues, serum and plasma compared with non-smokers [30,54,55]. A study of hemodialysis (HD) patients who smoked showed that the circulating plasma levels of GSH and non-GSH plasma thiols, including Cys, were substantially lower than those in non-smoking HD patients [56]. Chronic smokers had impaired platelet-derived nitric oxide (PDNO) production and augmented platelet aggregation. These events were associated with alteration in platelet redox state [54]. PDNO release and plasma and platelet GSH levels were lower in smokers than non-smokers, which could be correlated with the results that smokers showed greater platelet aggregation than that of nonsmokers [54]. Importantly, the latter study shows evidence that impaired PDNO activity and elevated platelet aggregation in smokers are caused by failure to maintain redox balance.

In unrelated studies, platelet activation has been found to be directly mediated by E_hGSSG through integrin αIIbβ3 [57]. Plasma E_hCySS and E_hGSSG were significantly oxidized in smokers (n = 43) compared to nonsmokers (n = 78) [30]. These studies support the interpretation that smoking-stimulated oxidation of E_hCySS or other perturbations of Cys metabolism plays a key role in vascular disease. However, the detailed mechanisms by which smoking-stimulated vascular disease progression alters plasma E_hCySS or vice versa, smoking-induced oxidation of E_hCySS stimulates CVD progression have not been investigated.

3) High body mass index (BMI)

Obesity is accompanied by a high incidence of chronic diseases including type II diabetes and vascular disease. Oxidative stress could be an underlying mechanism for development of obesity and could contribute to metabolic disorders associated with obesity. Increasing evidence shows that the plasma redox states, E_hGSSG and E_hCySS, and concentration of GSH, GSSG, Cys, and CySS are affected by BMI [58-60]. Although Cys and CySS levels were not measured separately, studies by El-Khairy et al. and Elshorbagy et al. showed that plasma total Cys level (Cys + CySS) was strongly correlated with BMI [19,61]. These results suggest that BMI is positively correlated with plasma E_hCySS oxidation since CySS is the major disulfide form in human plasma. Plasma levels of adiponectin and interleukin-6 in obese patients (BMI > 30) were significantly higher than those in healthy normal weight controls (BMI < 25) showing a potential role of high BMI in inflammatory signaling [60]. E_hGSSG was correlated with the level of adiponectin, suggesting that high BMI significantly increases oxidative stress. Conversely, an in vivo rat study demonstrated that the adipose tissues of obese animals showed a specifically higher content of GSH and a reduced E_hGSSG compared with lean animals. However, decreased GSH level and oxidation of E_hGSSG were observed in the erythrocytes of obese rats. [59]. Corresponding data are not available for Cys/CySS and adiponectin.

Results from a Mediterranean diet study using a sample of monozygotic and dizygotic middle-aged male twins also support a close association between plasma GSH/GSSG redox state and CVD [62]. The Mediterranean diet is a healthy eating pattern with cardioprotective effects. The traditional Mediterranean dietary pattern is inversely associated with BMI and obesity [63,64], i.e., greater adherence to the Mediterranean diet is associated with a lower risk of CVD. Adherence to the Mediterranean diet is associated with reduced plasma E_hGSSG [63]. Study of plasma E_hCySS did not show a similar association. Consequently, these data are also consistent with independent mechanisms of CVD risk associated with plasma E_hGSSG and E_hCySS.

4) Plasma enzymes and cholesterol levels

Plasma Cys/CySS redox state might be also affected by enzymes present in plasma such as paraoxonase (PON1). PON1, a calcium-dependent arylesterase associated with high density lipoproteins (HDL) is responsible for hydrolysis of organophosphates. PON1 functions as an antioxidant by protecting HDL and low density lipoprotein (LDL) from oxidation. Decreased PON1 activity has been attributed to increased oxidation of plasma protein thiols [65,66], suggesting that PON1 could also affect thiol/disulfide redox state in plasma. In addition to PON1, cholesterol levels appear to be linked to plasma thiol concentrations and thiol/disulfide redox state [53]. The data show a positive correlation of reduced E_hGSSG with HDL amount and a negative correlation with LDL level [53]. Jacob et al. have shown the positive correlation between the total Cys (Cys + CySS) amount and cholesterol level in patients with CVD [11]. These results suggest that plasma cholesterol, HDL and LDL levels, and plasma enzymes such as PON1, might also have an effect on concentrations of Cys, CySS and redox state of Cys/CySS.

5) Carotid intima media thickness (IMT)

Intima media thickness is a measurement of thickness of artery walls by external or internal ultrasound to detect the presence or to track progression of CVD. Ashfaq et al. [58] tested whether plasma redox factors GSH, GSSG, E_hGSSG, Cys, CySS, and E_hCySS are correlated with carotid intima-media thickness (IMT). Plasma levels of these redox factors were analyzed from 114 healthy non-smokers, without known clinical atherosclerosis, along with measurement of carotid IMT. This study showed that plasma redox markers including GSH, E_hGSSG, and CySS were correlated with IMT as were more traditional factors, i.e., BMI, age, and lipids. This finding suggests that CySS and/or E_hGSSG might be useful *in vivo* measures of plasma oxidative stress to predict early stage atherosclerosis.

6) Endothelial function

The use of plasma CySS as an independent predictor of vascular disease is further supported by a study of endothelial function [67]. In a study of 124 healthy nonsmokers, endothelial function was examined by ultrasound measurement of brachial artery flow-mediated vasodilation. This study showed significant and independent correlations between flow-mediated vasodilation and high-density lipoprotein level, BMI, gender, and the Framingham risk score. Importantly, an increased level of oxidized redox factors, CySS and glutathione-cysteine (mixed disulfide) was associated with endothelial dysfunction [67]. This finding supports the in vitro cell studies described above linking inflammatory function to oxidized extracellular E_hCySS in aortic endothelial cells [25,26].

Most recently, another clinical study on plasma CySS level in CVD has been reported at the American Heart Association meeting [68]. This study of more than 1,200 people undergoing cardiac imaging at Emory University due to suspected heart disease show that people with plasma CySS > 118 μM levels were twice as likely to have a heart attack or die over the next few years. Importantly, this concentration range is similar to the 200 μM total Cys used in *in vitro* studies [25] although the latter did not explicitly examine effects of CySS. This finding supports the use of plasma CySS level as an independent variable to predict outcome in CVD.

While far more clinical studies have focused on GSH in disease, accumulating data on the Cys/CySS couple (Table 1) shows that increased CySS and oxidation of E_hCySS are associated with many risk factors for CVD. The finding that oxidized E_hCySS activates proinflammatory signaling indicates that regulation of E_hCySS could be central to oxidative mechanisms of CVD. Thus, factors regulating Cys and CySS metabolism and redox state could be mechanistically important in CVD and are discussed below.

7) Sleep disorders and cerebrospinal fluid (CSF)

Studies of extracellular redox in the central nervous system have been mostly limited to the cerebrospinal fluid (CSF) in the context of neurobiological disorders. Redox state of GSH/GSSG plays an important role in neuronal diseases including amyotrophic lateral sclerosis, Parkinson's disease, Lewy body disease (LBD) and Alzheimer's disease, and GSH is essential for the cellular detoxification of ROS. In the CSF, GSH levels were lower in LBD patients compared to controls while GSSG levels and E_hGSSG in CSF were not altered by LBD [69]. GSH in CSF enhanced neuronal loss due to energy depletion, resulting in the extracellular redox state being more reduced. More reduced extracellular redox causes an increased activation of N-methyl-D-aspartate receptor, which is associated with neurotoxicity [70]. These findings suggest that increased GSH level in CSF is more likely to be deleterious rather than protective. Cys in CSF is also involved in the reducing environment of neurons leading to neuronal death [71], but the data available for the Cys and CySS in CSF are limited. Total Cys including Cys and CySS was reported to be less

than 2.5 μM in human CSF [72]. The ratio of Cys to CySS in the CSF is very high compared with that in plasma suggesting that redox balance of Cys/CySS could serve as a major redox buffer in the CSF [73]. In analogy to the circadian rhythm of plasma E_h values [74], changes in redox balance of Cys/CySS in CSF might occur and suggest a need for research to determine whether E_hCySS is associated with sleep disorders.

IV. Cys/CySS redox interactions with sulfur amino acid metabolism

Previous studies have extensively addressed the roles of homocysteine and GSH in CVD [19,75]; these two molecules are connected with Cys metabolism in multiple ways and the quantitative relationships have not been fully explored (Fig. 3). Cys and CySS metabolism involves both synthesis and degradation of the carbon skeleton and also reversible oxidation-reduction of the thiol. Studies of Cys/CySS turnover using stable isotopic tracer methods in humans have addressed only the carbon skeleton turnover, mostly focused on the inter-organ cycling of Cys and GSH in which dietary Met and Cys are converted to GSH in the liver as means to provide a short-term storage of Cys [76-78]. E_hCySS regulation with sulfur amino acid metabolism summarized in Fig. 4, regulation of Cys, CySS, and E_hCySS and their interactions are discussed below.

Sulfur amino acids, Met and Cys, are derived mostly from dietary protein. The average American diet contains a combined sulfur amino acid intake of about 4 g/d [79], and Cys is present mostly as disulfide forms. 1) During digestion, the thiol/disulfide redox potential in the lumen is maintained by GSH secretion by enterocytes and in bile (not shown), and by a Cys-CySS shuttle mechanism [80] (Fig. 3). 2) Cys and CySS are transported into enterocytes by Na^+-dependent and -independent transporters [81]; Cys is transported into plasma, but it is unclear whether CySS is directly released into plasma (indicated by broken arrow) or reduced and released as Cys. 3) Cys is rapidly cleared from circulation by reversible transport systems, which differ among cell types and serve to maintain a balanced supply of Cys to all organs [81]. 4) CySS is cleared more slowly by uptake into cells, and the biological reversibility of this process is uncertain. 5) Ongoing oxidation of Cys to CySS maintains plasma E_hCySS more oxidized than tissue E_hCySS; oxidation is a major component of Cys clearance from circulation when plasma Cys is increased in postprandial periods [82]. 6) A substantial amount of Cys is maintained in plasma as a disulfide with Cys34 in albumin; this pool weakly correlates with E_hCySS [83]. 7) An interorgan GSH-Cys cycle has been extensively discussed as a means for liver to support Cys homeostasis throughout the body [84]. In this mechanism, the very high concentration of hepatic GSH supports concentration-dependent release into plasma (7a); in the plasma, the GSH is either directly hydrolyzed to yield Cys (not shown) or undergoes thiol-disulfide exchange (7b) with the high concentration of CySS to yield Cys and the disulfide CySSG [85]. The CySSG is then hydrolyzed to yield CySS [83]. 8) An analogous thiol-disulfide exchange reaction (8b) occurs with homocysteine (HCys) and CySS to form the disulfide HCys-Cys. HCys is released (8a) from liver and other tissues [86], and also reacts with Cys34 of albumin to produce an HCys disulfide (not shown). 9) In a typical American diet, Met is consumed in considerable excess of the Recommended Dietary Allowance; Met is used to support a large number of methylation reactions (not shown) [84]. HCys is a product of the methylation cycle and is increased under a number of nutritional deficiencies, and total HCys has been associated with increase CVD risk [87]. 10) HCys is converted to Cys through the transulfuration pathway [84]. 11) CySS conversion to Cys in tissues appears to be a key process in controlling systemic E_hCySS together with mechanisms of 3), 4), and 5), but there are no known specific redox systems in mammalian cells to catalyze this reduction [82]. 12) Conversion of Cys to GSH is a quantitatively important component of Cys turnover. 13) Under conditions of excess Cys, a cysteine dioxygenase system is rapidly activated [84]. 14) Decarboxylation and oxidation produce taurine. Sulfate is the

predominant final product of sulfur amino acid metabolism and is produced from taurine, cysteine sulfinic acid and other precursors (not shown) [84]. 15) Hydrogen sulfide (H_2S), a recently recognized protective agent against CVD, is produced by cystathionase in the transulfuration pathway, as well as by other pathways. Mechanisms of protein sulfhydration are not completely understood, but could involve thiocysteine as an intermediate. 16) Although conversion of Cys to GSH has received the most attention in the research literature, the rate of incorporation of Cys into protein probably exceeds rates of incorporation into GSH in most cells under most conditions [88]. 17) Oxidation of thiols to disulfides is a common step in protein folding and is especially important in the secretory pathway [89]. Accumulating evidence indicates that ongoing oxidation of a subset of proteins, as described in this review, function in cell signaling. 18) Reduction of proteins by thioredoxin- and GSH-dependent systems function in cell signaling and also in protection against abnormal loss of protein activity due to oxidative stress [90,91]. Proteins are also reduced upon endocytosis in antigen processing [92]. 19) Proteolysis yields both Cys and CySS; the relative fractions are not known. 20) Protein cysteinylation is a covalent modification which can alter protein function [93]. However, little is known about the precise mechanisms and controls of this modification.

As suggested by the large number of reactions in Fig. 4, multiple factors can control the dynamics of the Cys/CySS system and its interaction with GSH/GSSG system and other thiol systems. Two key aspects must be included in redox systems biology descriptions, carbon skeleton flux and electron transfer flux [94]. Although carbon skeleton flux data are available, these frequently focus on GSH-Cys interconversions and do not include rates of Cys incorporation and release from protein. Moreover, redox reactions often occur at faster rates than interconversion of Cys and GSH by synthesis and degradation. The relationships of the carbon skeletons and redox interactions are depicted in Fig. 5. The rates of protein cysteinylation/decysteinylation have been studied very little and are not included. The whole body synthesis/degradation turnover of GSH in humans occurs at 24-31 μmol/kg/h [95]. The carbon skeleton turnover of the total Cys (Cys + CySS) in plasma is about 2 times faster (38-80 μmol/kg/h) [82], but the rate of CySS turnover was estimated to be only 18 μmol/kg/h. These data support earlier rodent studies indicating that GSH efflux from tissues is an important contributor to the plasma Cys pool. However, radiotracer studies of Ookhtens using ^{35}S-GSH showed rapid appearance of ^{35}S-CySS without corresponding labeling of Cys (^{35}S-Cys) [96], suggesting a pathway involving GSH reduction of CySS to generate CySSG which was subsequently metabolized to CySS [83]. In human postprandial studies, Cys clearance was 8 times faster than CySS clearance [82], indicating rapid oxidation of Cys to CySS following eating. Together, the data indicate that oxidation of the thiol pools is an ongoing process involving the interaction of the GSH and Cys/CySS pools. With the observed redox disequilibrium between plasma E_hCySS (−80 mV), plasma E_hGSSG (−140 mV), tissue E_hCySS (−160 mV) and tissue E_hGSSG (−220 mV), these results indicate 1) currently poorly defined oxidative reactions of Cys and reductive reactions of CySS are important in the overall redox interactions and 2) that thiol/disulfide equilibration of Cys and GSH systems does not occur. The disequilibrium of the systems highlights a need to address protein-cysteine redox reactions, as a counterpart to the extensively studied protein glutathionylation reactions.

In most extracellular compartments including plasma, CySS is a major amino-thiol. In the presence of a CySS transporter, x_C^- on the cell surface, CySS is imported from the oxidizing extracellular environment and then reduced to Cys within cells. The Cys, obtained from CySS reduction and produced from Met via transmethylation and transsulfuration mechanisms, is then utilized for protein synthesis and other cellular functions. Tight regulation of Cys level has been shown in rat liver even when sulfur amino acid intakes are deficient or excessive [97]. Meanwhile, total Cys levels in the plasma and serum vary with

life style, diet, and risk of vascular disease [19]. The total Cys levels including Cys and CySS in serum/plasma from healthy subjects is about 250 μM (PrS-SCys, CySS, CyS-SG, Cys, CysGly-Cys, HCys-Cys), which is 20-fold higher than the total plasma homocysteine [86] and is associated with a wide range of factors including age, body mass index (BMI), sex, cholesterol and diastolic blood pressure [20]. A recent study showed that total plasma homocysteine and total plasma cysteine are associated with body composition [75]. Total cysteine including Cys and CySS showed positive associations with BMI, and fat mass, independent of diet, exercise, and plasma lipids. Total homocysteine was not associated with lean mass, and it was significantly inversely associated with BMI and fat mass after adjustment for total Cys [75].

V. Current understanding of regulation of plasma E_hCySS

The quantitatively important mechanisms involved in regulation of plasma and extracellular E_hCySS are not known, but several human, rodent, and cellular studies have provided possible mechanisms of regulation. Studies with cellular systems indicate that the balance of "reduction" and "oxidation" is largely determined by transport. Both GSH and Cys release from cells probably contribute to supplying thiol while thiol loss occurs by Cys uptake and by oxidation to form disulfide. Cystine uptake also occurs and may be a critical determinant of E_h under some conditions. Rodent and human studies discussed below provide some insight into this regulation, but quantitative tracer studies which simultaneously measure carbon skeleton flux and redox changes will be required to delineate which of these processes controls E_h in vivo.

CySS, the predominant form in the plasma is usually present at >40 μM while Cys is typically present at values of about 8-10 μM in the fasting state [74]. The average E_hCySS value of this couple in young healthy subjects is −80 ± 9 mV [83], and E_hCySS is altered to be more reduced following ingestion of Cys [82] and more oxidized in a variety of pathophysiologic conditions [98-100]. Hildebrandt et al. predicted that the rate of amino acid clearance could be a major determinant of the plasma thiol/disulfide redox state [29]. Any pathologic conditions with decreased insulin responsiveness and protein synthesis would thereby be expected to diminish the clearance rate of amino acids from blood and elevate the CySS level within the blood [29]. Consequently the processes of cellular utilization of Cys and CySS appear to be critical for E_hCySS regulation. In this section, we discuss current understanding of plasma and extracellular E_hCySS regulation using data from human studies, *in vivo* rodent studies, and *in vitro* cell studies.

1) Human studies

Kinscherf et al. have described the critical role of plasma CySS to maintain the amino acid reservoir and body cell mass [101]. A study of young healthy subjects showed a negative correlation of CySS in plasma with CySS and amino acid amounts present in peripheral tissue [102]. This suggests that plasma CySS level may be regulated by CySS and other amino acids present in peripheral tissue. Amino acid exchange studies controlling for Cys/CySS levels were undertaken to examine a mechanistic link between plasma thiol (Cys) and protein catabolism [103]. The control mechanism was markedly impaired in elderly, obese, and hyperlipidemic subjects, which were observed with a decrease in Cys level [103]. An age-related decrease in plasma Cys and increase in CySS were found, suggesting that plasma Cys and CySS are regulated by age-related increase in oxidative stress as discussed earlier [14,23]. Middle-aged obese and hyperlipidemic patients show a significant decrease in Cys level in plasma, but no increase in the plasma CySS concentration [103]. Although the latter study did not measure plasma redox state of Cys/CySS, E_hCySS of these patients calculated using the Nernst equation and Cys and CySS concentrations demonstrated in this study (Fig.2 in [103]) shows that plasma E_hCySS of both middle-aged obese and

hyperlipidemic patients were substantially more oxidized (obese, 20 mV; hyperlipidemia,10 mV) compared with those in young healthy group and middle-age healthy group. Given the Cys/CySS-dependent redox signaling mechanisms described above, these results directly implicate Cys/CySS as a mechanistic intermediate in the pathophysiology.

Animal studies have shown that plasma thiol/disulfide pools, both E_hCySS and E_hGSSG undergo diurnal variations associated with dietary intake of sulfur amino acids [104,105], and this was also shown in human studies [74]. Results show that plasma Cys and GSH concentrations varied with the time of day, and E_hGSSG and E_hCySS couples varied in association with the concentrations of the thiol forms, GSH and Cys. Maximal reduction and oxidation of the E_hCySS occurred at 21:30 and 06:30, whereas the respective values for the E_hGSSG occurred at 03:30 and 13:30. The mean diurnal variation for E_hCySS in persons aged ≥ 60 year was 1.8-fold that in persons aged < 40 year. The findings from this study suggest that the magnitude of variation in E_hCySS in older persons could alter sensitivity to oxidative stress over a course of hours.

Recent studies showed that vascular oxidative stress and inflammation increased with age in F344 rats and that these effects could be prevented by supplementation with (R)-alpha-lipoic acid (LA) [106]. LA caused a significant decrease in mRNA of NOX4 and VCAM-1 and reversed age-dependent changes in aortic SOD activity and plasma MCP-1 levels. At least 2 mechanisms related to CySS and/or E_hCySS could contribute to these effects, namely direct extracellular reduction of CySS or activation of the transcription factor Nrf2. LA has an E_o of −300 mV and can directly reduce CySS [107,108]. Alternatively, transcription of the CySS transport protein x_C^- and the GSH synthetic enzyme, glutamate cysteine ligase, is controlled by Nrf2, which is activated by LA [109].

Other studies show that zinc, dietary sulfur amino acid intake level and acetaminophen use can affect plasma E_h in humans. The effects of zinc (Zn) supplementation on plasma thiol metabolites and their redox status was studied in a cohort of patients with age-related macular degeneration [32]. Results showed that patients receiving Zn supplementation for 5 years had significantly less plasma CySS compared with those not receiving zinc [32]. Therefore, this study suggests that Zn supplementation could be useful to decrease plasma CySS and should be studied for possible benefits in CVD.

Metallothioneins (MT), low molecular weight, Cys-rich Zn-binding proteins belong to a superfamily of cellular metal-binding proteins. Most widely expressed in mammals are MT-1 and MT-2 and induced by metals, drugs and inflammatory mediators [110]. Alterations in MT expression were observed when the dietary Zn supply is restricted or supplemented [111-113]. The association of Zn with sulfur ligand and the biological significance of this cluster have not been clearly identified. MT becomes a redox protein, allowing the Cys sulfur ligand to Zn to be oxidized and reduced with concomitant release and binding of Zn [114]. The GSH redox system including GSH/GSSG and NADP+/NADPH is known to modulate Zn transfer in the cell [115]. A more oxidized redox environment or enzymes with disulfides react with MT and release Zn [114]. Although the study by Moriarty-Craige et al. [32] did not include data for the effect of Zn supplementation on MT, based on previous studies above, MT levels might be increased in response to Zn supplementation with a positive correlation with Cys and negative correlation with CySS.

Other interaction with metal ions and metalloproteins could be important in controlling plasma E_hCySS. Among these, the plasma Cu^{2+} protein, ceruloplasmin (ferroxidase) and a plasma membrane-associated thiol oxidase activity has been described for rat and porcine kidneys and rat small intestine [116]. Myeloperoxidase also has thiol oxidase activity [117].

A recent human study of 8 healthy individuals also has provided evidence that plasma redox is controlled by sulfur amino acids (SAA) load [82]. This study showed that provision of a meal with SAA resulted in increased plasma Cys and CySS levels and more reduced plasma E_hCySS compared with the postprandial values following the same meal without SAA. The difference in postprandial plasma E_hCySS value due to meal content of SAA was 10 mV, a value sufficient to alter physiologic signaling [28,118]. Thus, findings from this human study support previous *in vivo* rodent studies of an important role of SAA in regulation of plasma Cys, CySS, and E_hCySS levels. Additional clinical studies show that plasma E_hCySS level was affected by therapeutic doses of acetaminophen (APAP) by altering SAA metabolism in healthy human adults [119]. APAP administration oxidized the plasma E_hCySS but not the plasma E_hGSSG, suggesting that APAP regulates plasma E_hCySS by affecting SAA metabolism independent of GSH metabolism.

2) Rodent studies

In vivo studies using rat and mouse models have provided additional important information about regulation of plasma E_hCySS under pathologic conditions. Many studies have used N-acetylcysteine as a means to support GSH synthesis [120,121]. However, these studies have rarely provided information on Cys, CySS or E_hCySS. In a study of the effect of dietary supplementation with sulfur amino acids (SAA) on short bowel syndrome (SBS), SAA supplementation was found to cause reduction in plasma redox state [122]. SAA supplementation was associated with a greater reduction in plasma E_hCySS and ileal E_hGSSG and increased adaptive ileal growth [122]. This reduced environment could contribute to the protection of gut system from oxidative stress associated with SBS [122]. The data suggest that similar mechanisms could exist to promote vascular function and repair.

Oxidation of E_hCySS associated with inflammation has been discussed earlier in this review. As discussed, oxidized E_hCySS functions as a determinant of proinflammatory cytokine, interleukin (IL)-1β, in a mouse model challenged with LPS [47]. In the same study, the effect of dietary SAA on oxidative stress in LPS-challenged mice was examined. The results showed that preservation of plasma E_hCySS from oxidation by dietary sulfur amino acid (SAA) supplementation was associated with a 1.6-fold decrease in plasma IL-1β compared to control mice. These findings of dietary SAA-prevented oxidation of redox state have translational importance by raising the possibility that SAA-based nutritional therapies may be a strategy to improve cellular and plasma redox states and protect from oxidation under pathologic conditions.

3) Cell studies

Reed and Beatty [85] showed that GSH released from tissues can react with CySS to contribute to maintenance of Cys/CySS balance. To test whether cellular GSH alters regulation of extracellular E_hCySS, HT-29, cells were treated with l-buthionine-[S,R]-sulfoximine (BSO) to inhibit GSH synthesis [88]. Decrease of GSH by > 90% showed no effect on the rate of reduction of extracellular E_hCySS to achieve a stable E_hCySS in the culture medium. Limiting Cys and CySS in the culture medium also substantially decreased cellular GSH but resulted in no significant effect on extracellular E_hCySS, suggesting that regulation of extracellular E_hCySS in colonic epithelial cells is a GSH-independent mechanism [88]. Addition of CySS to these cells showed that extracellular E_hCySS approached −80 mV at 4 h while cellular and extracellular E_hGSSG recovered more slowly. This study suggested that transport systems for Cys and CySS are more important than cellular GSH in regulation of extracellular E_hCySS. Many transport systems exist for Cys and CySS [123]. Inhibitor studies with Caco2 cells showed that y^+L and x_C^- systems contribute to extracellular E_hCySS [124].

The transporter x_c^- is found in the plasma membrane of many mammalian cell types and is highly specific for exchanging amino acids, CySS and glutamate [125]. A Na^+ -independent x_c^- system is composed of two distinct proteins, 4F2hc and xCT. The xCT controls activity of x_c^- by forming heteromultimeric complex with 4F2hc. This system transports extracellular CySS into cells coupled to export of glutamate (Glu) to the outside of cells. Intracellular CySS is then reduced to Cys for proteins and GSH synthesis. Activity of x_c^- is known to be regulated by electrophiles, CySS depletion, O_2, and lypopolysaccharide [125].

The presence of multiple Cys and CySS transporters indicates that in vivo regulation is likely to be complex due to the varied abundance of transporters in different organ systems. Studies of E_h effects on cell proliferation in Caco2 cells also suggest that E_h regulation is dependent upon growth factors. Redox mechanisms function in regulation of cell growth, and variation in E_hCySS of plasma occurs in various physiologic conditions, including diabetes, chemotherapy, and aging. To determine whether a systematic variation in extracellular E_hCySS over a range (0 mV to −150 mV) that occurs in human plasma altered proliferation of cells, Caco2 cells exposed to E_hCySS (0 mV to −150 mV) were examined for cell proliferation [27]. Incorporation of bromo-2-deoxyuridine (BrdU) was lowest at the most oxidized extracellular E_hCySS (0 mV) and increased as a function of E_h, attaining a 100% higher value at the most reduced E_hCySS condition (−150 mV). Addition of insulin-like growth factor-1 (IGF-1) or epidermal growth factor (EGF) increased the rate of BrdU incorporation at more oxidizing redox conditions but had no effect at reduced E_hCySS. In the absence of growth factors, extracellular E_hCySS values were largely maintained for 24 h. However, addition of growth factor, IGF-1 or EGF stimulated alteration in extracellular E_hCySS to values similar to that of plasma E_hCySS in young and healthy individuals. The results suggest that growth factor signaling may be an important physiological regulator of E_hCySS. Similar results were observed in THP1 monocytes [24] and endothelial cells (Go and Jones unpublished observation).

VI. Global effects on cell signaling mechanisms and functions controlled by extracellular E_hCySS

In addition to the proinflammatory signaling in monocytes and endothelial cells described above, a number of other redox signaling pathways have been described. These are summarized in Fig. 6 and briefly discussed because of possible relevance to the integrated effects of diet, life style and metabolism in CVD. Global effects of E_h on signaling in other cell types and in organ systems could have important indirect effects on vascular function and thereby impact CVD.

The earlier findings on more reduced extracellular E_hCySS-stimulated proliferation of colon carcinoma cells led to studies to identify the underlying mechanisms induced by extracellular E_hCySS. Since cell proliferation involves activation of epidermal growth factor receptor (EGFR) and mitogen-activated protein kinase (MAPK), the studies were performed to examine whether extracellular E_hCySS controlled MAPK activation via EGFR [42]. Results showed that in the absence of added growth factors, the most reduced E_hCySS (−150 mV) stimulated EGFR phosphorylation followed by a marked increase in phosphorylation of p44/p42 MAPK (Fig. 6). Blocking p44/p42 phosphorylation with inhibitors of EGFR and a cell membrane impermeable, thiol alkylating reagent indicated that E_hCySS signaling is mediated by EGFR and thiols accessible to the extracellular space. The EGFR ligand transforming growth factor (TGF)-α level was increased in culture medium at more reduced E_hCySS, and redox-dependent proliferation was partially blocked by an anti-TGFα antibody. Additional data suggested that reduced extracellular E_hCySS signaling involved metalloproteinase-dependent release of cell surface associated TGF-α. Although

studied in an epithelial cell line, this cell proliferation mechanism could occur in many cell types and be relevant to CVD.

In addition to stimulation of the proinflammatory signaling by oxidized extracellular E_hCySS in vascular cell models [24-26,47,48,126], oxidized extracellular E_hCySS signaling potentiated oxidant-induced human retinal pigment epithelial (hRPE) cell death [28] (Fig. 6). The hRPE cells under oxidized extracellular E_hCySS environment elevated apoptotic events such as loss of mitochondrial membrane potential, release of cytochrome c, and activation of caspase 3. These results suggest that a more oxidized extracellular redox environment increased susceptibility to oxidant-induced apoptosis signaling through the intrinsic mitochondrial pathway and could contribute to an age-related decline in cell populations in the retina. It is not currently known whether this is a general mechanism which contributes to sensitivity in other cell types; however, we have found a similar result in aortic endothelial cells (Go and Jones, unpublished observation) suggesting that the mechanism could be relevant to homeostasis of endothelial cell populations.

Additional redox studies have been performed to define E_hCySS-dependent signaling mechanisms that link oxidative stress to fibrogenesis using primary murine lung fibroblasts [118]. This study showed that oxidized extracellular E_hCySS stimulated lung fibroblast proliferation and elevated fibronectin, a matrix glycoprotein associated with fibrotic lung diseases by mediation of protein kinase C activation involving increased levels for NF-κB and SMAD3 (mothers against decapentaplegic homolog 3) (Fig. 6). Furthermore, fibronectin expression by oxidized E_hCySS was associated with expression of TGF-β1. These studies suggest that extracellular E_hCySS controls lung fibroblast proliferation and matrix expression through TGF-β1 regulation. Additionally, this proliferative cellular response to oxidized extracellular E_hCySS has been also observed in vascular smooth muscle cells. This signaling involves increased NADPH oxidase-1 (Nox1) expression regulated by EGFR [127]. Consequently, studies are needed to determine whether profibrotic signaling in response to E_hCySS contributes to CVD.

The marked contrast between oxidized E_hCySS-dependent a signaling pathway for fibroblast proliferation and stimulating proinflammatory signaling and apoptosis in other cell types (see above) is consistent with known differences in responses of cells to extracellular stimuli. Differential effect of oxidative stress (e.g. oxidized E_hCySS, hypoxia, angiotensin II) on endothelial/epithelial cells and fibroblast leading to apoptosis [28,128-130] and proliferation [118,131,132], respectively, has been speculated in Fig. 7.

The differential responses of cell types to extracellular redox potential would appear to be part of the intercellular signaling necessary to complex injury-repair cycles in tissues. Such coordination of signaling may be critical in CVD related to fibrous cap formation. Fibrous cap formation arises from migration and proliferation of vascular smooth muscle cells and from matrix deposition. And this process requires endothelial activation and infiltration of monocyte macrophages [133]. Impaired cap formation or an unstable plaque phenotype caused by smooth muscle senescence and mummification can rupture and result in thrombosis. Thus, stability of plaque is now recognized as a key player in patients with CVD. A recent study by Zulli et al. using a rabbit model shows that high dietary methionine (Met) could be protective in CVD for stable fibrous cap formation [134] while Met stimulates atherosclerosis development in coronary artery [17,18]. The role of dietary Met in the development of atherosclerosis and stabilization of cap formation is unclear. As we discussed earlier in sulfur amino acid metabolism, Met is used to generate homocysteine associated with CVD. In this regard, the result of oxidized extracellular E_hCySS-stimulated proliferation of fibroblasts is interesting. Since in vitro data provided stimulation of proinflammatory signaling by oxidized E_hCySS, there could be a close link between the

plasma redox state and the fibrous cap formation. More detailed studies with control of thiol/disulfide systems will be needed to address this possibility.

Lastly, oxidized extracellular E_hCySS was associated with activation of Nrf2 (nuclear factor-erythroid 2-related factor 2) in mouse embryonic fibroblasts [135] (Fig. 6). Results from this study suggest that extracellular E_hCySS plays an important role in up-regulation of antioxidant and detoxification systems. The results of this study showed that signaling was dependent on mitochondrial redox state, consistent with the finding in endothelial cells [26]. Together, the multiple signaling pathways affected by E_hCySS indicate that thiol/disulfide redox mechanisms associated with CVD are complex and will require additional study to discover effective means for therapeutic intervention.

VII. Conclusions

Accumulating data on Cys/CySS signaling provide a mechanism for oxidative stress in CVD. Both endothelial and monocytic cells in culture respond to oxidized Cys/CySS with proinflammatory signaling and increased cell adhesion. The signaling responses occur over an oxidized range of E_hCySS which occurs in humans in association with age, cigarette smoking, obesity and alcohol abuse, known risk factors for CVD. While free radical mechanisms have long been considered central to CVD, these processes may largely be bystanders to more critical thiol redox signaling mechanisms. Renewed focus on therapies to regulate thiol-dependent redox signaling may provide a useful alternative to free radical scavengers as means to prevent and manage CVD risk.

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List of Abbreviations

AMS	4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid
BMI	body mass index
CVD	cardiovascular disease
Cys	cysteine
CySS	cystine
E_hCySS	redox state of cysteine/cystine
E_hGSSG	redox state of GSH/GSSG
DCF	dichlorofluorescin
Trx	thioredoxin
GSH	glutathione
GSSG	glutathione disulfide
ICAT	isotope coded affinity tag
IL-1β	interleukin-1β
HCys	homocysteine
SAA	sulfur amino acid

RPE cells	retinal pigment epithelial cells
EGFR	epidermal growth factor receptor
TGF	transforming growth factor
Nrf2	nuclear factor-erythroid 2-related factor 2

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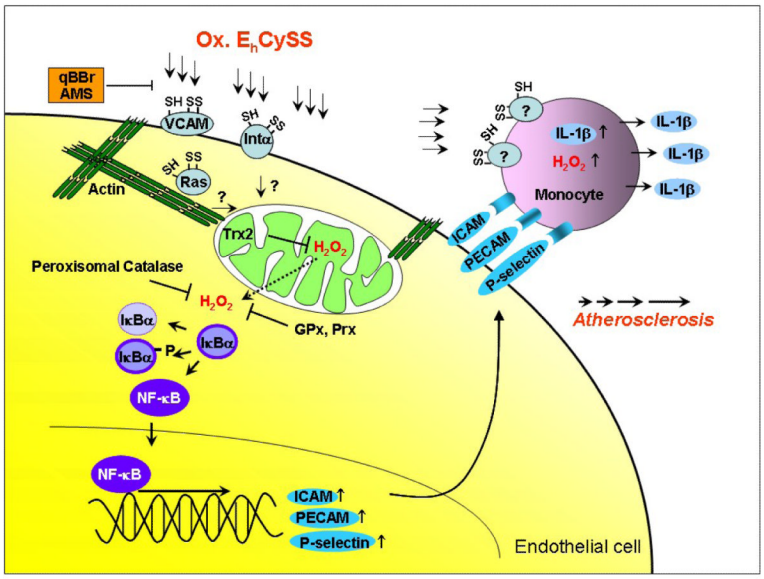

Figure 1.

Proposed scheme for inflammatory signaling in response to oxidized extracellular E_hCySS. Extracellular E_hCySS-induced oxidation of plasma membrane and cytoskeleton proteins stimulates ROS generation in mitochondria that is blocked by Trx2. This signaling mechanism was also blocked by inhibiting plasma membrane thiol/disulfide regulation by AMS and qBBr. H_2O_2 from the mitochondria triggers inflammatory signaling including NF-κB activation and increased expression of cell adhesion molecules. H_2O_2 can affect cell structure by affecting actin dynamics. Monocytes stimulate proinflammatory signaling by generating inflammatory cytokine, IL-1β and H_2O_2 in respond to extracellular E_hCySS. Changes in the endothelial cell structure and increases in cell adhesion molecules and cytokines result in an increase in monocyte recruitment as an early event of atherosclerosis [24-26,47]. GPx, glutathione peroxidases; ICAM, intercellular cell adhesion molecule; IL-1β, interleukin 1β; PECAM; platelet endothelial cell adhesion molecule; Prx, peroxiredoxins

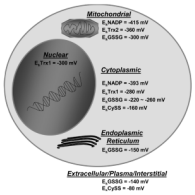

Figure 2.

Steady-state redox potentials of the major redox couples in subcellular compartments. Results from multiple cell types provide evidence for stably non-equilibrium relationships between redox couples in different compartments. Representative values for $NADP^+$/NADPH, GSH/GSSG, Trx(SH)2/Trx(SS), and Cys/CySS in cytoplasm, mitochondria, nucleus, cytoplasm, endoplasmic reticulum, and extracellular space are based upon data reviewed in detail [92]. The presence of these couples in each distinct compartment allows for the independent, specific control of redox processes that occur in these regions.

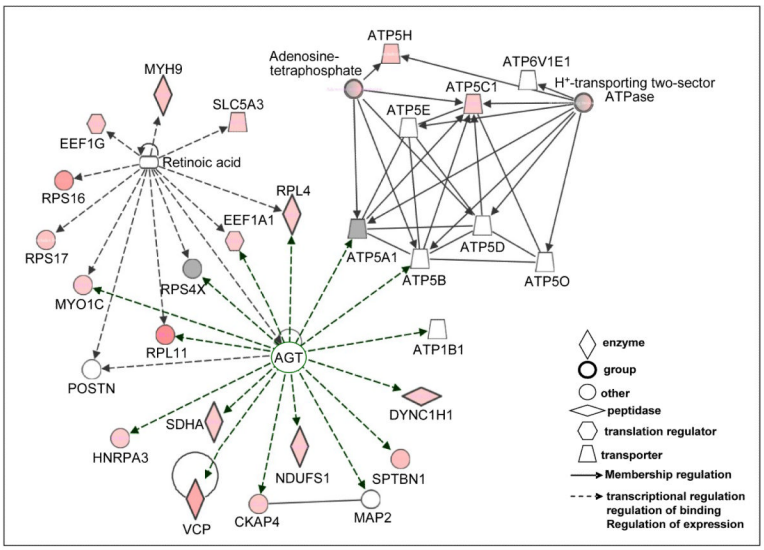

Figure 3.

Proteins oxidized by extracellular E_hCySS control cell signaling and morphology. Redox ICAT-based mass spectrometry identified endothelial proteins that alter redox state in response to extracelluar E_hCySS (supplementary data from the previous study [26]). Ingenuity Pathway Analysis shows that proteins oxidized by more positive extracellular E_hCySS (red) are in networks for cell signaling and cell morphology in association with angiotensin- and retinoic acid-dependent pathways. Protein names of symbols are as follows: AGT, angiotensinogen; ATP1B1, ATPase, Na^+/K^+ transporting, beta-1 polypeptide; ATP5C1; ATP synthase, H^+ transporting, mitochondrial F_1 complex, gamma polypeptide 1; ATP5H, ATP synthase, H^+ transporting, mitochondrial F_0 complex, subunit d; CKAP4, cytoskeleton-associated protein 4; DYNC1H1, dynein, cytoplasmic 1, heavy chain 1; EEF1A1, eukaryotic translation elongation factor 1 alpha 1; EEF1G, eukaryotic translation elongation factor 1 gamma; HNRPA3, heterogeneous nuclear ribonucleoprotein A3; MAP2, microtubule-associated protein 2; MYH9, myosin, heavy chain 9, non-muscle; MYO1C, myosin IC; NDUFS1, NADH dehydrogenase (ubiquinone) Fe-S protein 1; POSTN, periostin, osteoblast specific factor; RPL4, ribosomal protein L4; RPS16, ribosomal protein S16; SDHA, succinate dehydrogenase complex, subunit A; SLC5A3, solute carrier family 5, member 3; VCP, valosin-containing protein.

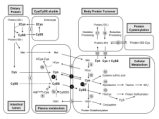

Figure 4.
Cys/CySS redox interactions with sulfur amino acid metabolism. Metabolic regulation of plasma Cys, CySS, and E_hCySS is controlled by diet, body protein turnover, sulfur amino acid metabolism in peripheral tissues and cells, and Cys/CySS shuttle mechanism. 3), 4), 5), and 11) indicate a key process in controlling systemic E_hCySS in plasma. See text for additional details.

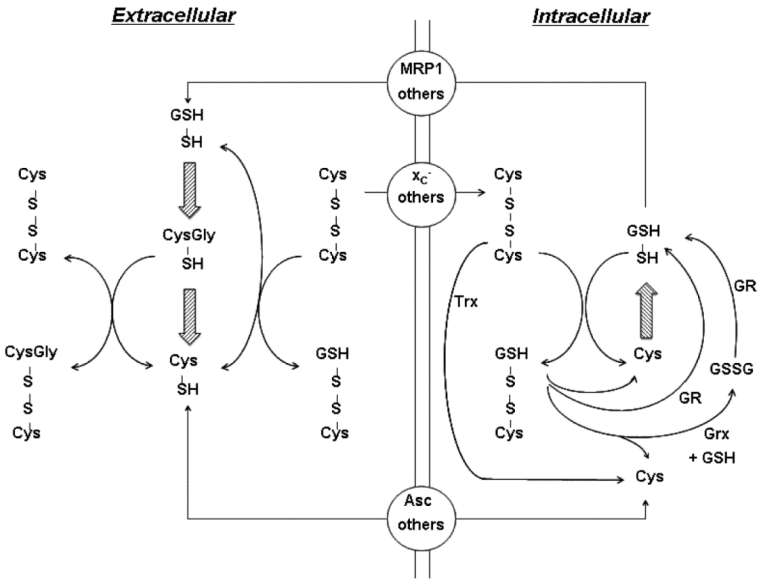

Figure 5.

Interrelationships between Cys and GSH systems. Cysteine (Cys) and glutathione (GSH) are interconnected by both carbon skeleton changes and oxidation-reduction of thiols. The carbon skeleton changes involve extracellular hydrolysis of GSH to CysGly and then to Cys (large and hatch style arrows, left) and intracellular synthesis of GSH in 2 steps from glutamate, cysteine and glycine (large and hatch style arrow, right). The oxidation-reduction reactions in the plasma involve GSH and CysGly reactions with CySS. The products are hydrolyzed by the same enzymes that hydrolyze GSH and CysGly. The cellular reactions are less clear. No enzyme is known that reduces CySS in mammalian tissues. Reaction with GSH can occur, but the non-catalyzed rate appears to be too slow to account for the rate of CySS reduction [88]. The product of this reaction is CySSG, which is a relatively poor substrate for GSSG reductase (GR). CySSG can also transfer the GSH moiety to glutaredoxin (Grx) with release of Cys. Reaction of the GSH-Grx with GSH results in release of GSSG, which is a substrate for GSSG reductase. Thioredoxin (Trx) also has a relatively low capacity to reduce CySS. The x_C^-, Asc, and MRP1 transporter systems for CySS, Cys, and GSH respectively are shown, and there are multiple transporters for each.

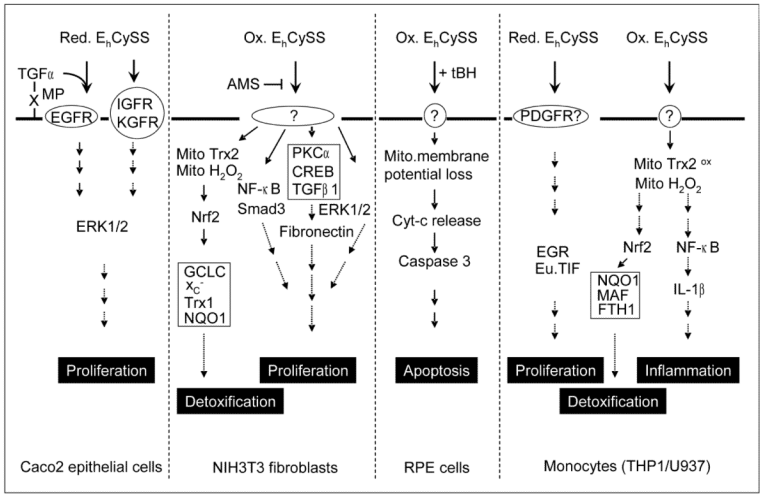

Figure 6.

Diverse signaling responses by extracellular E_hCySS. Reduced E_hCySS stimulates growth and proliferation mechanisms in Caco2 epithelial cells [27,42,98] and vascular cells, monocytes [24]. Oxidized E_hCySS stimulates detoxification and proliferation mechanisms in NIH3T3 fibroblasts [118,135]. Oxidized E_hCySS stimulates tBH-induced cell death mechanism in human retina pigment epithelial cells [28] and stimulate inflammatory mechanisms in vascular cells including human monocytes and endothelial cells [24-26,47].

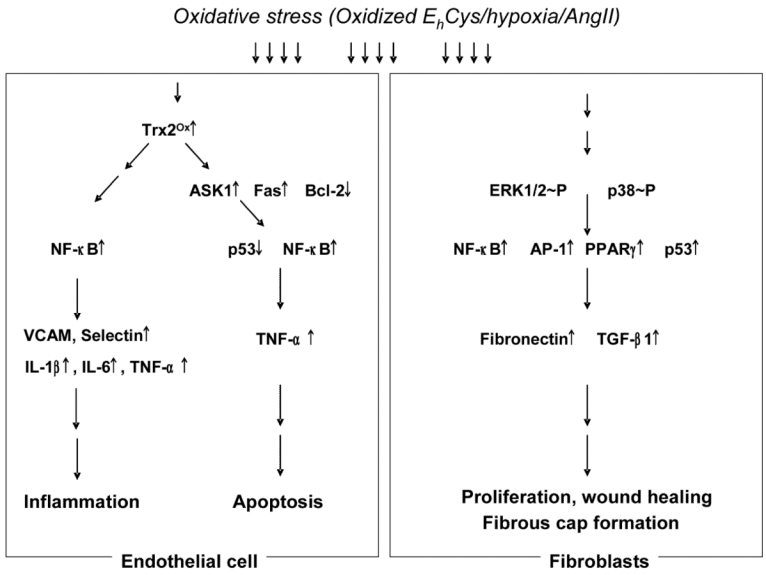

Figure 7.

Integration of extracellular E_hCySS with other oxidative stimuli in cellular responses of CVD. Responses of endothelial cells and fibroblasts to oxidative stimuli of hypoxia and angiotensin II have been well described [128,129,131]. Accumulating evidence suggests that oxidized E_hCySS can contribute to the cellular responses of CVD. Possible effects in endothelial cells (left) include activation of proinflammatory signaling via NF-κB and result in increased VCAM-1, P-selectin and proinflmmatory cytokines. Additional cell stress responses are mediated through mitochondria and possibly p53, which can result in apoptosis. Effects on fibroblasts (right) include proliferative signaling and activation of profibrotic signaling by TGF-β1. This increased activity can contribute to wound healing and also to fibrous cap formation.

Table 1

Summary of plasma redox system in association with risk factors and other causes of CVD (NS, no significant association; ND, no data were reported; cGSSG/cEhGSSG, cellular GSSG/E_hGSSG; ↓, decrease in concentration; ↑, increase in concentration; oxidation ↑, increased oxidation in redox potential)

Risk factor	Plasma thiol/disulfide redox system						Ref
	Cys	CySS	E_hCySS	GSH	GSSG	E_hGSSG	
Old age	↓	↑	oxidation↑	↓	↑	oxidation↑	[13,23,29]
Diabetes	ND	ND	ND	↓	↑	oxidation↑	[136,137]
High cholesterol	↓		oxidation↑	NS	↑ in cGSSG	Oxidation ↑ in cE_hGSSG	[53]
Smoking	↓	↑	oxidation↑	↓	NS	oxidation↑	[30,54]
Diet (Mediterranean)	ND	ND	ND	NS	↓	oxidation↓	[62]
Obesity	ND	ND	ND	↓	ND	oxidation↑	[59,60]
Alcohol	↓	↑	oxidation↑	↓	↑	oxidation↑	[55]
Flow-mediated dilation	NS	↑	NS	NS	NS	NS	[67]
Persistent atrial fibrillation	ND	ND	oxidation↑	ND	ND	oxidation↑	[138]
Carotid IMT	NS	↑	NS	↓	NS	↑	[58]