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Cysteine Redox Potential Determines Pro-Inflammatory IL-1β Levels

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Abstract

Background: Cysteine (Cys) and its disulfide, cystine (CySS) represent the major extracellular thiol/disulfide redox control system. The redox potential (Eh) of Cys/CySS is centered at approximately −80 mV in the plasma of healthy adults, and oxidation of Eh Cys/CySS is implicated in inflammation associated with various diseases.

Methodology/Principal Findings: The purpose of the present study was to determine whether oxidized Eh Cys/CySS is a determinant of interleukin (IL)-1β levels. Results showed a 1.7-fold increase in secreted pro-IL-1β levels in U937 monocytes exposed to oxidized Eh Cys/CySS (−46 mV), compared to controls exposed to a physiological Eh of −80 mV (P<0.01). In LPS-challenged mice, preservation of plasma Eh Cys/CySS 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 fed an isonitrogenous SAA-adequate diet (P<0.01). Analysis of Eh Cys/CySS and IL-1β in human plasma revealed a significant positive association between oxidized Eh Cys/CySS and IL-1β after controlling for age, gender, and BMI (P<0.001).

Conclusions/Significance: These data show that oxidized extracellular Eh Cys/CySS is a determinant of IL-1β levels, and suggest that strategies to preserve Eh Cys/CySS may represent a means to control IL-1β in inflammatory disease states.

Introduction

Interleukin (IL)-1β is a pro-inflammatory cytokine that functions as a critical regulator of host defense in response to infection and injury. However when present in excess, IL-1β is extremely toxic [1]. Elevated systemic levels of IL-1β cause hypotension during septic shock and induce capillary leak in acute lung injury [2]. IL-1β is also involved in chronic inflammation associated with arthritis, lung fibrosis, and atherosclerosis [3,4,5]. Therefore, strategies to modulate IL-1β production in inflammatory diseases are of therapeutic interest.

IL-1β activation and induction are associated with inflammation, a process with enhanced generation of reactive oxygen and nitrogen species [6]. These reactive species serve multiple biological functions, which include removal of cell debris and cell signaling necessary for host defense. Recent advances in redox signaling mechanisms have revealed that functional pathways utilize diffusible oxidants such as peroxide and redox-sensitive thiols in specific proteins as sensors [7]. The redox states of these sensors are controlled by rates of oxidation of specific amino acid residues and their reduction by thiol/disulfide control systems.

The thiol/disulfide control systems are compartmentalized; glutathione/glutathione disulfide (GSH/GSSG) and thioredoxin provide control mechanisms within cells, while cysteine/cystine (Cys/CySS) and GSH/GSSG control the redox state of proteins in the extracellular space and on the cell surface [8]. The Cys/CySS couple predominates in the extracellular fluid and the steady-state redox potential (Eh) of Cys/CySS is oxidized in acute and chronic inflammatory disease states [9]. In vitro, oxidized Eh Cys/CySS induces upregulation of nuclear factor-kappa B (NF-κB) [10,11], increases adhesion of leukocytes to the endothelium [10], and sensitizes epithelial cells to apoptosis [12]. Based on these observations, we hypothesized that extracellular Eh Cys/CySS is a determinant of pro-inflammatory cytokine production.

We tested this hypothesis by modifying extracellular Eh Cys/CySS and determining IL-1β levels in vitro and in vivo. In vitro results showed that oxidized extracellular Eh Cys/CySS is sufficient to increase pro-IL-1β levels in monocytes, and in vivo results showed that dietary treatment to protect against plasma Eh Cys/CySS oxidation is associated with decreased IL-1β levels in LPS-challenged mice. Analysis of Eh Cys/CySS and IL-1β in human plasma revealed a significant positive association between oxidized

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Eh Cys/CySS and IL-1β, independent of age, gender, and BMI. Together, the data show that oxidized extracellular Eh Cys/CySS is one of the determinants of IL-1β levels, and suggest that strategies to preserve Eh Cys/CySS may represent a means to control IL-1β in inflammatory disease states.

Materials and Methods

Ethics Statement
All protocols involving human subjects were reviewed and approved by the Emory Institutional Review Board. All protocols involving mice were reviewed and approved by the Institutional Animal Care and Use Committee at Emory University.

Materials
Except as indicated, all chemicals were purchased from Sigma Chemical Corporation (Sigma, St. Louis, MO). Distilled, deionized water was used for analytical purposes. HPLC quality solvents were used for HPLC.

Cell culture
Human monocyteic cells (U937, ATCC, Rockville, MD) were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Norcross, GA) and 10 U/ml penicillin and streptomycin sulfate. Cells were transferred to 0.5% FBS media 8-12 h prior to experimental manipulations.

To generate the desired range of extracellular redox potentials, the extracellular thiol/disulfide pool was altered by varying concentrations of Cys and CySS, added to cyst(e)ine-free RPMI, as previously described [11]. In these experiments, the total extracellular pool size of Cys+CySS was set at 200 µM, while concentrations of Cys and CySS were varied to obtain initial Eh values from −80 mV (physiological) to −46 mV (oxidized). The measured Cys/CySS redox range in human plasma is −120 to −20 mV [13]. Eh Cys/CySS is centered at −80 mV in healthy individuals and becomes oxidized with age, lifestyle factors such as smoking and alcohol abuse [14,15], and pathologies such as atrial fibrillation [16], and age-related macular degeneration [9]. Therefore, the experimental range of −80 to −46 mV covers the physiological range and extends to oxidizing values that are reachable under pathological conditions.

Production of reactive oxygen species (ROS) was detected using 6-Carboxy-2',7'-dichlorofluorescin diacetate (DCFH-DA, Molecular Probes, Eugene, OR, USA) [10]. U937 cells, plated into 96-well plate, were washed with KRH buffer and incubated with 100 µM DCFH-DA for 30 minutes (37°C, 5% CO2). Cells were washed and exposed to physiological (−80 mV) and oxidized (−46 mV) Cys/CySS redox media at 37°C. Oxidation of DCFH-DA to fluorescent DCF was measured on a microplate reader (excitation, 485 nm; emission, 530 nm) [17].

Experimental animals and dietary intervention
Experiments were conducted using 10–14 week old, female C57BL/6j mice [Jackson Laboratories, Bar Harbor, ME]. Mice were housed in cages and maintained on a 12-h light-12-h dark cycle at the Division of Animal Resources at Emory University. All experiments were initiated during the light cycle. All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee.

Prior to the dietary intervention, all animals were fed pelleted rodent food (Test Diet 5015, Lab Diet Inc., Richmond, IN). Semi-purified diets were custom-prepared (Harlan-Teklad, Madison, WI, USA) in order to test the specific effects of sulfur amino acid (SAA) supplementation [18]. The SAA-adequate and SAA-supplemented diets were isocaloric and isonitrogenous and contained adequate and identical quantities of energy, nitrogen, carbohydrate, fat, fiber, micronutrients and essential amino acids. The SAA and nitrogen content was controlled at the desired experimental levels by varying the amount of cystine and methionine and the non-essential amino acids L-alanine, L-aspartic acid, glycine and L-serine. The SAA-supplemented diet contained 3-fold cystine, and 1.8-fold methionine compared to the SAA-adequate diet. Animals had free access to water at all experimental time points.

LPS Administration
Escherichia coli O55:B5 LPS, dissolved in sterile PBS (100 µg/ml) was administered intraperitoneally to unanaesthetized animals at a dose of 1 mg LPS/kg body weight. Animals were sacrificed at 2 h post-LPS. The dose of LPS was used based on previous observations that it substantially increases plasma and lung IL-1β levels [19], and oxidizes plasma Eh Cys/CySS at 2 h [20].

Sample collection and analysis of Cys, CySS, GSH and GSSG
Mice were anesthetized by isoflurane inhalation (Baxter Pharmaceuticals, Deerfield, IL). Opening of the blood brain barrier is reported at high concentrations of isoflurane [21]. The implications of this deleterious effect of isoflurane to measurements made in the current study are presently unknown. Subsequent to anesthesia, blood was collected by submandibular bleeding using a 4 mm mouse bleeding lancet (Medipoint, Inc., Mineola, NY). 0.18 ml of collected blood was immediately transferred to 0.02 ml of preservation solution containing γ-glutamyl-glutamate (γ-Glu-Glu) as an internal standard [22].

Samples were centrifuged at 16000 g for 60 seconds to remove precipitated protein, and 0.1 ml of the supernatant was immediately transferred to an equal volume of ice-cold 10% (w/v) perchloric acid. Samples were immediately stored at −80°C.

For HPLC analysis (Gilson Medical Electronics, Middleton, WI), derivatized samples were centrifuged, and 50 µl of the aqueous layer was applied to the Supercosil LC-NH2 column (25 cm x 4.6 mm; Supelco, Bellefonte, PA). Derivatives were separated with a sodium acetate gradient in methanol/water and detected by fluorescence [23]. Concentrations of thiols and disulfides were determined by integration relative to the internal standard. Redox potential (Eh) of the GSH/GSSG and Cys/CySS pools, given in millivolts (mV), were calculated from the following forms of the Nernst equation for pH 7.4: GSH/GSSG, Eh = −0.264+30 log ([GSSG]/[GSH])2, Cys/CySS, Eh = −2.50+30 log ([CySS]/[Cys])2 [24].

Quantitative Real-Time PCR Analysis
Lung samples were excised, snap frozen in liquid N2 and stored at −80°C. Total RNA was extracted from tissue using an RNaseasy Midi Kit (QIAGEN Inc., Valencia, CA) according to manufacturer’s instructions. DNase treatment was performed to remove contaminating genomic DNA. RNA concentration was spectrophotometrically determined at 260 nm, and 0.5 µg of total RNA was used to synthesize 20 µl of cDNA (Invitrogen, Carlsbad, CA). Quantitative real-time PCR was performed on cDNA using gene-specific primers on an iCycler IQ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). Primers were designed using Beacon Designer Software 4.00 (PREMIER Biosoft International, Palo Alto, CA/ Table 1). Samples containing serial dilutions of known concentrations of cDNA, encoding the gene of...
interest, were amplified in parallel. Data were analyzed using the iCycler Software, and starting quantities of message levels of each gene were determined from constructed standard curves. Melt curves were examined to ensure amplification of a single PCR product. Expression level of IL-1β was normalized to β-actin in monocytes and 18S ribosomal RNA in mouse lung tissue.

Cytokine and Western blot analysis

Levels of IL-1β in the cell-supernatant were detected by ELISA (R&D Systems, Minneapolis, MN), and are expressed relative to total protein content in the supernatant. Western Blot analysis of the cell extract from U937 cells was performed to detect the precursor form of IL-1β. After washing with PBS, cells were lysed in homogenization buffer (50 mM NaCl, 50 mM NaF, 50 mM NaPO₄, 10 mM EDTA, 5 mM EGTA, 2 mM Na₃ V₀₄, 0.5 mM PMSF, 0.01% Triton X-100, 10 ug/ml leupeptin, 10 mM HEPES, pH 7.4) and the concentration of proteins was determined by using a Bradford reagent (Bio-Rad, Hercules, CA). Equal amounts of protein were loaded onto 10% acrylamide SDS/PAGE gradient gels (Bio-Rad).

Proteins were transferred onto nitrocellulose membranes using a semi-dry trans-blot apparatus set at 25 V for 1 h (Bio-Rad). Membranes were subsequently incubated overnight with an anti-human IL-1β polyclonal antibody (Cell Signaling Technology, Danvers MA). Rabbit polyclonal anti-β-actin (Abcam Cambridge, MA) was used as a loading control. After washing with tris buffered saline (TBS) (10 mM Tris-HCL, pH 8.0, 150 mM NaCl, 0.05% Tween-20), membranes were incubated for about 1 h at room temperature with goat anti-rabbit IgG conjugated to horseradish peroxidase-coupled secondary antibody. After further washing, immunoreactive signals were determined by chemiluminescence. Protein bands were quantified by densitometric scanning using a GS-800 Calibrated laser densitometer (Bio-Rad).

To better assess the inflammatory status in vivo, levels of IL-1β, and tumor necrosis factor (TNF-α) were measured. IL-1β and TNF-α in human plasma, mouse plasma, and mouse lung homogenates were detected by immunofluorescence using a multiplex panel assay (R&D Systems, Minneapolis, MN). Briefly, the antibody coupled beads were incubated with the sample followed by incubation with a detection antibody. Fluorescence intensity was read on a Bioplex beads were incubated with the sample followed by incubation with a detection antibody. Fluorescence intensity was read on a Bioplex (Bio-Rad, Hercules, CA).

IL-1β luciferase reporter assay

To evaluate IL-1β gene transcription, U937 monocytes were electroporated with a pIL-1 (4.0 kb) luciferase promoter construct, as previously described [25]. Cells were plated in 6-well plate and were incubated for 8 h in specific redox media. Cells were lysed in reporter lysis buffer (Promega, Madison, WI) and assayed for luciferase activity. Measurements were done using a Labsystems Luminoskan Ascent Plate Luminometer. Results were recorded as luciferase units and were adjusted for total protein content.

Human study protocol

Participants were instructed not to eat after 10 pm the night prior to the inpatient visit in order to standardize baseline levels of metabolites. Participants were admitted to the GCRC at 7:00 on Day 1 and a heparin-lock catheter was placed in a forearm vein for blood sampling at 8.00. After a 30-minute supine resting period, 3 ml blood samples were drawn every hour for 24 consecutive hours. Participants were given breakfast at 9:30, lunch at 13:30, dinner at 17:30, an evening snack was provided at 21:30 immediately following the timed blood draw for that hour. The composition of the meals and snacks was standardized for all conditions.

Human subjects

This study was reviewed and approved by the Emory Institutional Review Board. A total of 16 healthy volunteers were recruited by posting fliers in public locations in the Atlanta/Emory University community. Following written informed consent, participants were admitted to the outpatient unit of the Emory University Hospital General Clinical Research Center (GCRC), where potential subjects were screened using a medical history, physical examination, urinalysis, standard chemistry profile and a complete blood count. Eligibility was established based on the following criterion: absence of acute or chronic illness (other than a medical history of well-controlled hypertension), BMI<30, non-smokers, and compliance in discontinuing nutritional supplements, if consumed, 2 weeks prior to study entry. Eligible participants were then scheduled for a 24 h inpatient visit within 2 weeks of screening in the GCRC. Characteristics of study subjects are presented in Table 2.

Table 1. Details of PCR primer sequences used in the analyses of extracted RNA for quantitative real-time PCR.

| Target       | Genbank accession number | Forward primer | Reverse primer | Product size, bp |
|--------------|--------------------------|----------------|----------------|-----------------|
| IL-1β human  | NM_000576                | TGGACTGTATTTACTCAGGGCAATG | GTAGTGGTGGTCAGAGATTCG | 140             |
| β-actin human| NM_001101                | GCGTCAATGAGGGAGAAG | GAAGGAGGGCTGAGAGGAG | 172             |
| IL-1β mouse  | NM_008361                | ATCTCGACAGACGGTAC | CAGGACGGGTACATCAGTAC | 192             |
| 18S mouse    | NR_003278                | CGCTGCGCTTCTCACTTTCG | GCCTGCTGCTTCTCCTTTG | 130             |

Table 2. Characteristics of study subjects.

| Demographics              |          |
|---------------------------|----------|
| Age, mean±SD              | 60.3±17.9|
| BMI, mean±SD, kg/m²       | 24.2±3.1 |
| Females, %                | 56       |
| Fasting markers, mean±SD  |          |
| Cy₅, μM                   | 9.2±2.8  |
| Cy₅S, μM                  | 66.6±13.9|
| EₕCy₅/Cy₅S, mV            | (-)71.7±9.2|
| GSH, μM                   | 1.2±0.4  |
| GSSG, μM                  | 0.07±0.02|
| EₕGSH/GSSG, mV            | (-)122.8±9|
| TNF-α, pg/mL              | 4.5±3.7  |
| IL-1β, pg/mL              | 3.6±3.6  |

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subjects, as previously reported [26]. Water was provided ad libitum throughout the study period.

**Statistical Methods**

Data are presented as means±SEM. Statistical analysis was done using SAS v 9.1 (SAS Institute Inc., Cary, NC, US). Data from *in vitro* experiments were analyzed using an unpaired t-test. Data from *in vivo* marine experiments were analyzed using a one-way ANOVA with treatment specified as the main effect. To analyze data from human plasma, a linear mixed model was used. The association of Cys, CySS, and Eh$_b$ Cys/CySS with IL-1β, and TNF-α was determined after controlling for time of day, BMI, age, and gender. Because none of the potential confounders had statistically significant regression coefficients, parameters for BMI, age, and gender were excluded to arrive at the most parsimonious model. The residuals for thiol/disulfide redox were normally distributed; therefore thiol/disulfide redox was specified as the response variable in all constructed models to improve the stability of the computed regression coefficients. The human study by its very design cannot establish a cause-effect relationship; causality is therefore not invoked by specification of thiol/disulfide redox as the independent variable. The best model from all subsets was determined using the Akaike Information Criterion (AIC). Significance was set at a P value <0.05 for all tests.

**Results**

**Oxidized extracellular Eh$_b$ Cys/CySS increases pro-IL-1β and IL-1β mRNA levels in human monocytes**

Measurements of Cys and CySS in human plasma show that Eh$_b$ Cys/CySS is centered at approximately −80 mV in healthy young individuals [21] and is oxidized in association with inflammatory disease states [9]. *In vitro*, oxidized extracellular Eh$_b$ Cys/CySS activates nuclear factor (NF)-κB, a key pro-inflammatory transcription factor [10,11]. Together, these data indicate that oxidized Eh$_b$ Cys/CySS is likely to modulate pro-inflammatory cytokine production. Because peripheral blood monocytes are constantly exposed to the extracellular redox environment, we determined whether levels of IL-1β, a major monocyte-derived cytokine, are increased by oxidized extracellular Eh$_b$ Cys/CySS.

U937 monocytes were exposed to Eh$_b$ of −80 mV (physiological) or −46 mV (oxidized) for 8 h and levels of IL-1β were determined in the cell extract by western blot using an antibody that detects both the precursor and mature forms of IL-1β. Figure 1A shows an increase in cellular levels of the IL-1β precursor at −46 mV compared to −80 mV (lane 1 versus lane 3; P<0.05). IL-1β levels were also determined in cells treated with LPS (100 µg/ml) under different redox conditions, to assess whether IL-1β is increased in oxidized extracellular Eh$_b$ under inflammatory states. The data show increased cellular levels of the IL-1β precursor at −46 mV compared to −80 mV with LPS treatment (lane 2 versus lane 4; P<0.05).

Because an increase in the precursor form of IL-1β was observed in the cell extract under oxidized conditions, we next attempted to quantify secreted IL-1β levels in the cell supernatant by ELISA. In monocytes and macrophages, the release of mature IL-1β is dependent on stimulation of caspase-1 by extracellular ATP [27]. Because ATP was not added to the media, the level of IL-1β measured by ELISA is likely the precursor form. Figure 1B shows a 1.7-fold increase in precursor form of IL-1β in cells treated with Eh$_b$ of −46 mV compared to −80 mV (P<0.01).

To determine whether increased pro-IL-1β in response to oxidized Eh$_b$ occurred due to increase in mRNA abundance, IL-1β mRNA was quantified by real-time PCR. As shown in Figure 1C, IL-1β mRNA was more abundant in response to Eh$_b$ of −46 mV, 4 h after treatment (P<0.05). Experiments with U937 cells expressing the human IL-1β promoter fused to a luciferase reporter gene revealed significant increase in luciferase activity at −46 mV, suggesting transcriptional induction of IL-1β by oxidized Eh$_b$ (Figure 1D; P<0.05).

**Oxidized extracellular Eh$_b$ Cys/CySS induces intracellular ROS production in monocytes but has no effect on cellular Eh$_b$ GSH/GSSG**

Previous studies have shown that induction of IL-1β by extracellular stimuli, such as ATP, is associated with generation of ROS and up-regulation of genes involved in GSH synthesis [27]. Therefore, we determined whether increase in IL-1β in response to oxidized extracellular Eh$_b$ Cys/CySS is associated with intracellular redox changes. Measurement of cellular GSH (Figure 2A) and Eh$_b$ GSH/GSSG (Figure 2B) revealed no significant differences between the −80 and −46 mV treatment groups at 2 h and 8 h.

Next, we examined whether oxidized extracellular Eh$_b$ Cys/CySS stimulated cellular ROS production. Cells were pre-incubated with a ROS-sensitive dye, DCFH-DA, prior to treatment with −80 mV and −46 mV Cys/CySS redox media. Oxidation of DCFH-DA to fluorescent DCF was measured on a microplate reader. Cells treated with glucose oxidase served as positive controls. Within 5 minutes, cells exposed to oxidized Eh$_b$ of −46 mV showed a 5-fold increase in DCF fluorescence compared to cells exposed to a physiological redox potential of −80 mV (P<0.001). (Figure 2C). When measured 2–6 h after treatment, the magnitude of DCF fluorescence increased in both −80 and −46 mV conditions, with at least a 2-fold higher fluorescence at −46 mV (P<0.001, data not shown). This indicates that oxidized Eh$_b$-induced ROS production was sustained over-time, and occurred in the absence of oxidation to cellular Eh$_b$ GSH/GSSG.

We subsequently determined whether increase in ROS, in response to oxidized extracellular Eh$_b$ Cys/CySS, was sensitive to the oxidation of redox-sensitive membrane-bound thiols. To this end, monocytes were pre-treated with 0.25 mM 4-acetamidofluoro-amiclovarine (AMS), a non-permeant alkylating agent, prior to exposing the cells to given Eh$_b$ Cys/CySS. Pre-treatment with AMS decreased (P<0.001), but did not completely inhibit, cellular ROS levels in response to oxidized Eh$_b$ indicating that increase in cellular ROS occurs in part due to oxidation of membrane-bound thiols [10].

Taken together the *in vitro* data show that oxidized extracellular Eh$_b$ Cys/CySS induces a rapid and sustained increase in ROS in monocytes compared to physiological Eh$_b$, which could be involved in oxidized Eh$_b$ mediated increase in IL-1β. Based on these *in vitro* findings, we wanted to determine whether increase in IL-1β during inflammation *in vivo* can be attenuated by preservation of Eh$_b$ Cys/CySS from oxidation.

**Dietary SAA supplementation protects against endotoxin-induced perturbations in plasma Cys, CySS and Eh$_b$ Cys/CySS**

Based on previous research showing that supplementation with sulfur amino acids (SAA) increases plasma Cys and shifts the Eh$_b$ to a more reduced potential [18], we applied this approach to a mouse model of inflammation in which LPS/endotoxin is administered to increase IL-1β [19]. In this design, mice were fed either a SAA-adequate diet or an isonitrogenous SAA-supplemented diet for 7 days prior to endotoxin challenge. Mice were sacrificed 2 h post-endotoxin, a time point that coincides with a peak increase in IL-1β expression.
with maximal IL-1β production [19]. Plasma was collected prior to sacrifice for analysis of Cys, CySS, GSH and GSSG by HPLC. Thiol/disulfide redox parameters in untreated controls, fed pelleted rodent chow, are shown for comparison. Redox potential values for GSH and Cys are approximately 10–15 mV more reduced in mice plasma compared to human plasma.

SAA-supplementation protected against endotoxin-induced decrease in plasma Cys (Figure 3A; Cys (μM) - SAA supplemented, 19.6±1.9; SAA-adequate, 13.5±1.2; P<0.05). The decrease in plasma Cys in response to endotoxin is likely due to clearance of Cys, and oxidation of Cys to CySS. Ex vivo studies in hepatic membrane vesicles show that LPS stimulates hepatic influx of Cys to support gluconeogenesis and synthesis of acute phase proteins [28]. LPS also increases peroxide levels [29], and this could decrease plasma Cys via oxidation to CySS, an interpretation supported by the increase in plasma CySS levels in mice fed SAA-adequate diet (Figure 3B). However, because the magnitude of increase in CySS exceeds the decrease in Cys, mechanisms related to altered transport or recycling of Cys and/or CySS may also contribute to alterations in Cys/CySS homeostasis during endotoxemia.

The difference in plasma Cys between SAA-supplemented and SAA-adequate group is consistent with the predicted increase in Cys due to excess SAA intake, based on previous work done in our laboratory [18]. However, plasma CySS levels were not affected by excess SAA intake [18]. Thus, the lack of increase in plasma CySS in the SAA-supplemented group, 2 h post endotoxin, suggests additional effects on ROS homeostasis due to SAA supplementation (CySS (μM) - SAA-adequate, 141±9.7; SAA supplemented, 85.2±1.2; P<0.01). As a consequence of the higher Cys and decreased CySS, Δ0 Cys/CySS was on average 15 mV more reduced in response to endotoxin in the SAA-supplemented group compared to SAA-adequate group (Figure 3C; SAA-adequate, -76.4±2.6; SAA supplemented, -91.5±3.7; P<0.01).
Production was measured in monocytes treated with glucose oxidase (2)

increase in ROS production (P < 0.001). Data are mean ± SE of 4 replicates of a representative experiment repeated 3 times.

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Dietary SAA supplementation protects against endotoxin-induced IL-1β and TNF-α

Next, we determined whether the more reducing plasma Eν Cys/CySS, in SAA-supplemented animals is associated with a decrease in plasma IL-1β, in response to endotoxin. Plasma IL-1β levels in untreated controls were un-detectable (not shown). As shown in Figure 4A, plasma IL-1β decreased by 1.6-fold in SAA-supplemented animals (IL-1β (pg/ml) - SAA adequate, 190 ± 22; SAA supplemented, 116 ± 12; P < 0.01). Induction of IL-1β in the lung is an early response to endotoxin [20], so we determined whether a protective effect on IL-1β was also observed in the lung, in response to a reduced extracellular Eν Cys/CySS. Lung IL-1β levels were decreased by 2-fold in the lung homogenate from the SAA-supplemented group (Figure 4B; P < 0.001). Measurements of IL-1β message levels by quantitative real-time PCR revealed a 3-fold decrease in lung IL-1β mRNA levels (Figure 4C; P < 0.05). Thus, preserving extracellular Eν Cys/CySS during endotoxemia decreased tissue and circulating levels of IL-1β.

Tumor necrosis factor (TNF-α) is another prototypical pro-inflammatory cytokine that is induced in concert with IL-1β in response to infection, injury, and immunological challenge [30]. To better assess the inflammatory status in vivo, we determined whether attenuation of TNF-α also occurred in SAA-supplement- ed mice. Results showed a greater than 2-fold decrease in plasma TNF-α levels with SAA-supplementation (Figure 4D; P < 0.05). TNF-α in the lung was also significantly decreased (Figure 4E; P < 0.05). Thus, the in vivo observations show that preservation of plasma Eν Cys/CySS during endotoxia is associated with a decrease in IL-1β levels. These data extend the in vitro observations, and the combined findings support a mechanistic role for Cys redox potential in determining IL-1β levels.

IL-1β and TNF-α in plasma of healthy adults are increased in association with oxidized Eν Cys/CySS

To investigate whether Cys redox potential could represent a determinant of pro-inflammatory cytokine levels in humans, we examined IL-1β, TNF-α, Cys, and CySS in plasma samples from 16 healthy adults. The characteristics of the study participants are shown in Table 2.

Because plasma Cys and Eν Cys/CySS, and cytokines exhibit well documented circadian rhythms [26,31], the study was designed to examine whether diurnal variation in Eν Cys/CySS was associated with variation in IL-1β and TNF-α. For this purpose, hourly samples were collected for an entire 24 h period from 16 individuals. Diurnal variation in plasma Cys and Eν Cys/CySS was closely related to meal intake, as reported previously (data not shown) [26]. However, due to considerable inter-individual variation in plasma IL-1β and TNF-α, we did not detect significant time-dependent variations in cytokine levels. Therefore, we used a secondary analysis to determine whether variation in Eν Cys/CySS correlated with variation in IL-1β, independent of time of day.

Because repeated measures were obtained from the same individual, we used a linear mixed procedure to model variation in redox parameters with cytokine levels controlling for time of day, BMI, age, and gender. As none of the potential confounders had
statistically significant regression coefficients, parameters for BMI, age, and gender were excluded to arrive at the most parsimonious model. Cys, CySS and Eh Cys/CySS were specified as response variables in the analyses because the residuals for these biomarkers were normally distributed. Regression coefficients for the mixed model are presented in Table 3.

Examination of redox parameters for Cys redox potential revealed a strong positive association between Eh Cys/CySS and IL-1β (P < 0.001). As seen in Figure 5A, oxidized values of Eh Cys/CySS correlate with higher levels of plasma IL-1β. The regression parameter for IL-1β indicates that a 1 unit increase in plasma IL-1β is associated with a 1.1 mV oxidation of plasma Eh Cys/CySS. Analysis of the association between plasma Cys and IL-1β revealed a significant negative association indicating that low levels of plasma Cys correlate with high IL-1β levels (Figure 5B; P < 0.05). No significant correlation was observed between plasma CySS and IL-1β. While an association does not establish causality, together with our in vitro and in vivo data the present findings strongly suggest that Cys redox potential is an important determinant of IL-1β. Plasma TNF-α was also significantly associated with Eh Cys/CySS (Figure 5C) and CySS levels (Figure 5D) (P < 0.05) suggesting that oxidized Cys redox potential is also a determinant of TNF-α. It must be noted that a caveat that may limit extrapolation of the results to the general population is the age (62 years) and the BMI (24 kg/m²) of the population studied. Because old age and overweight are risk factors for inflammation [32,33], the association between Eh Cys/CySS and IL-1β and TNF-α may be less strong in a younger population with an optimum BMI range.

Discussion

The current study has three main findings: that oxidized extracellular Eh Cys/CySS is sufficient to induce pro-IL-1β in a monocyte cell line; that preservation of plasma Eh Cys/CySS from oxidation during endotoxin-induced inflammation is associated with a decrease in circulating IL-1β levels in mice; and that oxidized Eh Cys/CySS is positively associated with circulating IL-1β levels in healthy humans. Together, these data suggest that plasma Cys redox potential is not only a biomarker of oxidative stress, but may also be a determinant of immune cell function. Because a number of dietary and behavioral risk factors for disease are known to oxidize Cys redox potential e.g., sulfur amino acid deficiency [18], alcohol [34], and smoking [14]; this provides a mechanistic basis to consider monitoring Cys redox potential as a risk factor for pro-inflammatory states, and consider interventional strategies to control oxidation of Cys redox potential in pro-inflammatory diseases.

Previous studies have shown that induction of IL-1β by non-infectious extracellular stimuli, such as ATP and cigarette smoke condensate, occurs by activation of membrane-bound receptors [27,35]. The mechanism by which oxidized extracellular Eh Cys/CySS induces pro-IL-1β is unknown, but likely involves the
generation of intracellular ROS signaled, in part, via oxidation of membrane-bound thiols. Studies in endothelial cells have revealed increased oxidation of cell-surface protein thiols, induction of intracellular ROS, and activation of NF-κB in response to an oxidizing extracellular E\text{h}Cys/CySS [10]. The rapid and sustained induction of cellular ROS by oxidized E\text{h} in the present study is consistent with the same type of mechanism in U937 cells, i.e., the increase in IL-1β by oxidized E\text{h} could be signaled via the membrane and involve ROS-dependent activation of pro-inflammatory transcription factors such as NF-κB. Interestingly, the increase in ROS by oxidized E\text{h} occurred in the absence of changes to intracellular E\text{h} GSH/GSSG. This observation is, however, not unexpected. The major thiol/disulfide control systems GSH, thioredoxin, and Cys exist under non-equilibrium conditions and their redox states are distinctly modified by oxidative stress [14], substrate availability [18,36,37], physiological [26] and pathological stimuli [8,36]. Indeed, the present research shows that the oxidation of plasma E\text{h} Cys/CySS during acute endotoxemia in vivo occurs without the oxidation of plasma E\text{h} GSH/GSSG.

The acute oxidation of E\text{h} Cys/CySS by LPS is attenuated in mice supplemented with sulfur amino acids (SAA). This effect could be solely due to dietary augmentation of Cys reserves, an interpretation supported by the 1.4-fold higher plasma Cys concentrations in SAA-supplemented mice. However, plasma CySS does not increase in response to LPS suggesting the additional effects on ROS homeostasis due to SAA supplementation. The more reduced redox potential of the Cys/CySS couple in SAA-supplemented mice is associated with a significant decrease in circulating and tissue levels of IL-1β and TNF-α. These data suggest that Cys and associated Cys redox potential are critical determinants of cytokine production during activation of the immune system by LPS in mice. Thus, preservation of E\text{h} Cys/CySS by SAA supplementation may be involved in decreased IL-

| Table 3. Mixed model of redox parameters and IL-1β and TNF-α controlling for time of day. |
|---------------------------------------------------------------|
| **Biomarker** | **Regression coefficients** | **P** |
|-----------------|-----------------------------|-------|
| IL-1β           |                             |       |
| Cys, μM         | -0.19                       | 0.09  | <0.05 |
| CySS, μM        | 0.28                        | 0.44  | ns    |
| EhCys/CySS, mV  | 1.08                        | 0.28  | <0.001|
| TNF-α           |                             |       |
| Cys, μM         | -0.06                       | 0.04  | ns    |
| CySS, μM        | 0.41                        | 0.17  | <0.05 |
| EhCys/CySS, mV  | 0.25                        | 0.11  | <0.05 |

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Figure 4. Effect of dietary SAA-supplementation on endotoxin-induced IL-1β and TNF-α. C57BL/6J mice receiving either SAA-adequate diet or SAA-supplemented diet were treated with 1 mg/kg ip endotoxin/LPS. At 2 h, mice were sacrificed and plasma and lung samples were collected for analysis of IL-1β and TNF-α. Plasma IL-1β levels are shown in (A). IL-1β levels in lung homogenate are presented after normalization for total protein (B). In (C) RNA was extracted from whole lung and transcript levels of IL-1β were quantified by quantitative real-time PCR. Plasma TNF-α (D) and lung TNF-α (E) were also determined. Data are mean±SE of 4 replicates of a representative experiment repeated 2 times, * P<0.05.
and TNF-α levels during endotoxemia. It must be noted, however, that the effects of SAA supplementation may also include preservation of intracellular thiol/disulfide redox status.

In human nutrition, Cys is a conditionally essential amino acid because Cys requirements are normally met by the transulfuration of dietary methionine [38]. However, Cys requirements increase during infection, injury, and in conditions associated with limited hepatic transulfuration [39,40]. Because Cys is not routinely added to solutions used in parenteral therapy, patients with sepsis could be particularly susceptible to a deficiency of Cys. Studies in humans have shown that plasma Cys redox potential is modulated fairly rapidly by precursor availability [26]. Furthermore, in the present study we find that plasma Cys in humans in negatively associated with plasma IL-1β levels. Thus, nutritional supplementation with Cys or Cys precursors during early sepsis may be a strategy to alleviate acute inflammation and associated tissue injury.

In addition to the pathology associated with disregulated cytokine production in conditions such as sepsis; elevated cytokine levels in healthy individuals independently predict risk of chronic diseases such as type II diabetes and atherosclerosis [41,42]. Therefore, the association between oxidized Eh Cys/CySS and IL-1β and TNF-α in otherwise healthy individuals suggests that oxidized Eh Cys/CySS may represent a risk factor for chronic inflammatory diseases. Accordingly, maintenance of Cys redox potential may be critical in protecting against subclinical inflammation in healthy individuals, and in ameliorating pathological processes associated with chronic inflammation. Large population studies with detailed measurements of known factors affecting redox potential along with pro-inflammatory cytokine markers and disease risk factors are needed to test this concept. Such data are critical because assays are available to assess plasma Cys redox potential in humans [22,43], and simple and inexpensive interventional strategies are available which could improve Cys redox potential [9,44].

The association between oxidative stress and inflammation is well-recognized and multiple studies have shown that antioxidants such as N-acetyl cysteine, have anti-inflammatory effects. The present observations identify a mechanistic link between oxidative stress and inflammation. The combined in vitro and in vivo observations show that oxidized extracellular Eh Cys/CySS is a previously unrecognized modulator of IL-1β. The findings suggest that strategies to preserve Eh Cys/CySS may represent a means to control IL-1β in inflammatory disease states.

**Author Contributions**

Conceived and designed the experiments: SSI CJ A TZ MR JR DJ. Performed the experiments: SSI KB JDR. Analyzed the data: SSI. Wrote the paper: SSI DJ.

Figure 5. IL-1β and TNF-α in plasma of healthy adults is increased in association with oxidized Eh Cys/CySS. A linear mixed model was used to model variation in plasma Eh Cys/CySS and IL-1β and TNF-α. A strong positive association was observed between Eh Cys/CySS and IL-1β (A; P < 0.001). Plasma Cys was negatively associated with IL-1β (B; P < 0.05). TNF-α was positively correlated with Eh Cys/CySS (C), and CySS (D) (P < 0.05). doi:10.1371/journal.pone.0005017.g005
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