Distribution Agreement

In presenting this dissertation as a partial fulfillment of the requirements for an advanced degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis or dissertation in whole or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of this thesis or dissertation. I retain all ownership rights to the copyright of the dissertation. I also retain the right to use in future works (such as articles or books) all or part of this dissertation.

Signature:

_____ _____

Yanci O. Mannery Date

Sulfur Amino Acid and Acetaminophen Effects on Extracellular Thiol/Disulfide Redox Potentials

By

Yanci O. Mannery

Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences

Molecular and Systems Pharmacology

_____

Dean P. Jones, Ph.D.
Advisor

_____

Thomas R. Ziegler, M.D.
Advisor

_____

Lou Ann Brown, Ph.D.
Committee Member

_____

Edward T. Morgan, Ph.D.
Committee Member

Accepted:

_____

Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____

Date

Sulfur Amino Acid and Acetaminophen Effects on Extracellular Thiol/Disulfide Redox Potentials

By

Yanci O. Mannery
B.S., Dillard University, 2002

Advisor: Dean P. Jones, Ph.D.

Advisor: Thomas R. Ziegler, M.D.

An abstract of
A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in Molecular and Systems Pharmacology
2010

Abstract

Sulfur Amino Acid and Acetaminophen Effects on Extracellular Thiol/Disulfide Redox Potentials

By Yanci O. Mannery

Recent evidence demonstrates the importance of thiol/disulfide redox potential in control of biological processes such as cell proliferation, differentiation, and apoptosis. In human plasma, cysteine (Cys) and cystine (CySS) constitute the most abundant low molecular weight thiol/disulfide couple, and the steady-state redox potential of this couple, E_hCySS, is one of the central physiologic systems controlling redox homeostasis. *In vivo* studies show that plasma E_hCySS becomes oxidized with aging, disease, and lifestyle choices. The purpose of this dissertation was to study the effects of sulfur amino acid (SAA) availability and acetaminophen (APAP) administration on extracellular Cys/CySS redox potential (E_hCySS) and potential mechanisms involved.

The first section of the dissertation used a human intestinal cell culture model to investigate mechanisms for regulation of extracellular E_hCySS. The basal surface more rapidly adjusted E_hCySS toward the physiologic value of -80 mV compared to the apical following exposure to an oxidized condition. Experiments designed to test specific mechanisms for regulation of E_hCySS on the basal surface showed that redox regulation required Na^+ in the culture medium, suggesting a requirement for a transport system. Transport inhibition experiments indicate that E_hCySS on the basal surface is controlled by y^+L and x_c^-.

The second and third sections of the dissertation were designed to test whether a) dietary SAA availability or acute acetaminophen administration regulate plasma E_hCySS and b) SAA insufficiency alters APAP metabolism in healthy adults. With adequate SAA intake, APAP administration oxidized the plasma Cys/CySS redox potential (E_hCySS). However, APAP administration with 0% SAA did not cause further oxidation beyond APAP or 0% SAA alone. While results show that therapeutic drug use can affect plasma E_hCySS, SAA limitation was not sufficient to cause a change in the pharmacokinetic properties for disposition of APAP in humans.

Together, these studies show that Cys and CySS transport mechanisms are important in the regulation of plasma redox potential in human gut epithelial cells and that plasma E_hCySS is dependent both upon SAA intake and exposure to acetaminophen. Future studies are indicated to define relevant signaling pathways for redox regulation of the Cys and GSH pools and the responses to dietary SAA availability during illnesses when acetaminophen is administered.

Sulfur Amino Acid and Acetaminophen Effects on Extracellular Thiol/Disulfide Redox Potentials

By

Yanci O. Mannery
B.S., Dillard University, 2002

Advisor: Dean P. Jones, Ph.D.

Advisor: Thomas R. Ziegler, M.D.

A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in Molecular and Systems Pharmacology
2010

ACKNOWLEDGEMENTS

As I started to write this section of my dissertation, a line from one of my favorite songs began to play in my head. The line simply states: "Gratefulness is flowing from my heart!" This song perfectly describes how thankful I am to have the love, support, and help of so many during this arduous process. First, I would like to thank God for being and providing everything that I needed exactly when I needed it. There is no way that I could have reached this goal without His grace. I would also like to thank the Laney Graduate School, the Division of Biological and Biomedical Sciences, the Molecular and Systems Pharmacology program, and the ACTSI Masters in Clinical Research program for believing enough in my abilities to grant me the opportunity to further my education at Emory University. To Lou Ann Brown, Ph.D. and Edward T. Morgan, Ph.D., I would like to thank you for giving your time to serve on my committee and for pushing me just past my comfort zone in order to help me become a better scientist and critical thinker. Next, I would like to thank the numerous individuals that helped with the cell and human studies that comprise this dissertation. I am truly grateful for the assistance of the members of the Jones and Ziegler labs (past and present), the nursing, bionutrition, and laboratory staff at the Emory University Hospital GCRC, the pharmacists and staff at the Research Pharmacy, and the study volunteers for their commitment!

To Monica, Terri, Tamara, Sheraé, Kroshona, Andrea, Emma, Mary, and Brittney: thank you for being living examples of the true definition of friendship. You have been my confidantes, my counselors, my comedians, my cheerleaders but most importantly, my sisters through all of the ups and downs of life. Words cannot express how thankful I am to have each of you in my life. I love you girls!!! To my friends Rahn Gatewood and Chad Jackson, thank you for always being my sounding board and for letting me know that things were never as bad as they seemed!

I have a very large family and since there is no way that I could make everyone's name fit, I want to thank the Mannery, Johnson, Peale and Blue families for all of your prayers and encouragement! To my grandmother, Mrs. Lela Thurman for reminding me that the race is not given to the swift nor the strong but to the one that endures until the end. Thank you for believing in me Granny! To my parents, Jerry and Sharon Mannery, I would like to thank you for your love and for believing in me when I didn't believe in myself. You taught me at an early age that I could do anything and be anything that I chose if I put my mind to it and worked for it. Because of this lesson, I am standing here today. You are the best parents ever and I love you both so much! Thank you to my brother Jerron for always making me laugh even when I felt like crying. Not only are you an awesome brother but one of my best friends! Thank you to my sister-in-law Kassundra for always being supportive and for providing much needed comic relief when I come home! To my niece Kennedi and nephew Landon, you have brought so much joy into my life and are my inspiration! Thank you for loving your Auntie Yanci unconditionally! To Lenny Bell, your southern belle is finally finished! Thank you so much for wiping away my tears, for helping me to smile during the stressful days, and most of all, for loving and encouraging me!

Last but not least, I would like to thank my advisors, Dean P. Jones, Ph.D. and Thomas R. Ziegler, M.D. Not only are you great mentors but more importantly, you are both awesome individuals. I have learned so much from you about life, both scientific and personal in scope. Thank you for helping me to see that I can't take myself so seriously and to always believe in myself, in my abilities, and my research. I can only hope that one day I will be as good of a mentor to someone as you both have been to me. "Flowing from my heart is gratefulness!"

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ALI	Acute liver injury
AMD	Age-related macular degeneration
AMS	4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid
APAP	Acetaminophen
BCS	Bathocuproine disulfonate
BDPS	Bathophenanthroline disulfonate sodium salt
BMI	Body mass index
BrdU	5-bromo-2-deoxyuridine
BSO	L-buthionine-[S,R]-sulfoximine
CL	Clearance
Cys	Cysteine
CySS	Cystine
DMEM	Dulbecco's modified Eagle's high glucose medium
DPI	Diphenyleneiodonium chloride
E_h	Redox potential
FITC	Fluorescein isothiocyanate
γ-Glu-Glu	γ-glutamylglutamate
GCRC	General Clinical Research Center
GCS	γ-glutamylcysteine synthetase
GSH	Glutathione
HPLC	High performance liquid chromatography
hRPE	Human retinal pigment epithelial cells

k_{elim}	Rate of elimination
Lys	Lysine
Met	Methionine
NAPQI	N-acetyl-p-benzoquinonimine
PAPS	3′-phosphoadenosine-5′-phosphosulfate
RDA	Recommended dietary allowance
REE	Resting energy expenditure
SAA	Sulfur amino acids
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
Sec	Selenocysteine
$t_{1/2}$	Half-life
TER	Transepithelial resistance
THA	Threo-β-hydroxy-aspartate
Trx	Thioredoxin
V_d	Volume of distribution

Chapter 1: Introduction

Electron transfer reactions, termed “oxidation-reduction” or “redox” reactions, are central to all of life because such reactions provide the energy necessary for biological organization, defense and reproduction. The extraction and utilization of energy, as well as the structural, catalytic and signaling functions of life, are highly dependent upon the protein content of organisms. Importantly, protein in the diet of animals provides a necessary supply of amino acids for protein synthesis by the organism, and also a source of energy provided by oxidation of the amino acids.

The regulation of the function of cell proteins is also dependent upon redox reactions. Unlike the other macromolecules, e.g., DNA, RNA, carbohydrates and lipids, proteins contain sulfur atoms which can undergo reversible oxidation-reduction. These reversible redox reactions involve cysteine (Cys), methionine (Met) and selenocysteine (Sec). Selenocysteine is relatively uncommon and mostly involved as a catalytic element in a small number of proteins. Cys and Met function in catalytic and structural roles, but also undergo reversible oxidation-reduction in control of protein function and structure. Consequently, these elements are described as “sulfur switches” (92). The sulfur atoms of both Cys and Met undergo multiple oxidations, but the oxidation of thiols to disulfides has been studied most extensively and appears to be most central in redox control. Changes in redox state of thiol/disulfide couples affect protein conformation, enzyme activity, transporter activity, ligand binding to receptors, protein–protein interactions, protein–DNA interactions, protein trafficking, and protein degradation (59).

The central importance of these sulfur switches has only been recognized in the past decade, and considerable gaps occur in the understanding of the factors which govern their function. Accumulating knowledge indicates that redox state of the Cys

pool in human plasma is an important factor affecting protein redox states (57, 75), and the present dissertation addresses the interaction of nutrition and therapeutic drug use as determinants of cysteine/cystine redox state.

The introduction to this dissertation provides background on the definition of redox potential and the central thiol redox control elements, Cys, glutathione (GSH) and thioredoxins (Trx). These redox elements are controlled differently in the extracellular and cellular compartments, and the factors affecting extracellular Cys/CySS redox homeostasis are briefly reviewed as a basis for one of the central questions addressed within this dissertation. An overview of sulfur amino acid metabolism, including discussion of the Recommended Dietary Allowance (RDA) for sulfur amino acids, is provided as background to study of the effects of dietary sulfur amino acid availability and thiol/disulfide redox homeostasis. Finally, an overview of the non-prescription analgesic, acetaminophen, is provided as a background for study of the effects of acetaminophen on redox homeostasis and sulfur amino acid availability on acetaminophen metabolism and disposition.

1.1. Quantitative Definition of Redox Potential

The redox potential, also known as electromotive force (E_h), is a measure of the tendency of a chemical species to accept or donate electrons. The E_h is formally defined under equilibrium conditions, but in biologic conditions, the redox active centers are often maintained under non-equilibrium steady states (59). The term “redox state” was earlier used in preference to “redox potential” (52). However, in recent years, most

biologists have recognized the non-equilibrium conditions and use the terms interchangeably.

E_h is a measure of the tendency to accept or donate electrons and is quantitatively expressed in millivolts relative to the standard electrode reaction ($H_2/2H^+ + 2e^-$) (92). The reducing force available from an electron donor/acceptor couple is expressed using the Nernst equation:

$$E_h = E_o + RT/nF \ln ([\text{electron acceptor}] / [\text{electron donor}]^2)$$

where E_o is the standard potential relative to a standard hydrogen electrode, R is the gas constant (8.314 J K^{-1} mol^{-1}), T is the absolute temperature (in Kelvin), n is the number of electrons transferred, and F is Faraday's constant (9.6485×10^{-4} C mol^{-1}). The E_h for an oxidation/reduction couple such as glutathione/glutathione disulfide (GSH/GSSG) depends on the inherent tendency of each chemical species to accept or donate electrons (E_o) and the concentrations of respective acceptors and donors (83). For the GSH/GSSG redox couple, the E_o values that is generally accepted is -240 mV for pH 7.0 (83, 89). Because E_o is pH dependent, the Nernst equation must be adjusted for conditions where pH is not 7.0 (16, 92). Calculation of redox potentials provide a means to understand the direction of electron flow where a more negative redox potential within a cell, tissue, extracellular space indicates a more potent reducing force. The E_h values are half-cell potentials, meaning that they only express what is possible from electron transfer from that redox couple; if there is no kinetically important pathway for electron transfer between two couples, the electron transfer cannot occur. It should also be recognized that because redox couples are not in equilibrium in biologic systems, all estimates are subject to error due to artifacts created by extraction and measurement.

1.2. Intracellular Redox Environment

Two principal systems maintain cellular thiol/disulfide redox balance: GSH and thioredoxin (Trx). These 2 systems are complimentary in that both are important for the maintenance of protein thiol redox potential (98). GSH, the major low molecular weight thiol in cells, is an essential antioxidant which provides protection from oxidants and electrophiles. GSH is present at millimolar concentrations in cells. GSH and its disulfide, GSSG, constitute the predominant thiol/disulfide redox couple in the cell (52).

The synthesis of GSH from glutamate, cysteine, and glycine is catalyzed by 2 cytosolic enzymes: γ-glutamylcysteine synthetase (GCS) and GSH synthetase (95). Because the intracellular pool of Cys is relatively small, Cys is considered the rate limiting amino acid for GSH synthesis in humans. While GSH synthesis can occur in most cell types, the liver is the major producer and exporter of GSH. The breakdown of GSH is catalyzed by γ-glutamyl transpeptidase, γ-glutamyl cyclotransferase, 5-oxoprolinase, and an intracellular protease (95).

Most cellular GSH is present in the cytosol with the remainder found in organelles including the mitochondria, nuclear matrix, and peroxisomes (75). Because the plasma membrane and intracellular membranes restrict movement of biomolecules, multiple compartments with different redox potentials exist within the intracellular space. Previous studies have established that cellular GSH/GSSG redox potential varies in association with cycle responses (82). The results show that as cells progress from proliferation (52) through contact inhibition (38), differentiation (60), and apoptosis (44), there is a progression from a more reduced to a more oxidized environment.

1.3. Extracellular Redox Environment

Cysteine (Cys) and its disulfide, cystine (CySS), constitute the major low-molecular weight thiol/disulfide couple in mammalian plasma (35). The predominant form of Cys in the plasma is CySS. Cys is in the range of 8 to 10 μM in the plasma while CySS is typically present at concentrations greater than 40 μM (54). The redox value for the Cys/CySS pool is approximately -80 ± 9 mV in healthy adults, which is about 60 mV more oxidized than intracellular Cys/CySS E_h (54). While earlier studies determined that thiol/disulfide redox couples functioned in the regulation of biological processes such as cell proliferation, gene expression, and enzyme catalysis, most of the available data focused on the role of GSH. The primary function of GSH in the extracellular space is not as an antioxidant but as a substrate for γ-glutamyltransferase, the enzyme that functions to provide Cys from GSH degradation. Because GSH is present at considerably lower concentrations than Cys and CySS in the extracellular environment, several *in vitro* studies were designed to determine if perturbation of the Cys/CySS redox couple would affect processes such as cell proliferation.

Studies by Hwang and Sinskey showed that the redox potential of the extracellular environment, measured with a potentiometric electrode, affected the maximum density that cells achieved in culture (39). Studies by Jonas et al (49) showed that this was specifically related to the E_hCySS. Moreover, as cell density became maximal with contact inhibition, oxidation of the extracellular E_hCySS occurred (82).

The experimental model used by Jonas et al (49) involved a human colon carcinoma cell line (Caco-2) in which extracellular E_hCySS was varied. Variation of the extracellular redox environment was established with a constant overall pool size by

varying the concentrations of Cys and CySS added to the culture medium. Incorporation of 5-bromo-2-deoxyuridine (BrdU) was used to measure DNA synthesis and was lowest at the most oxidized extracellular redox condition (0 mV) (49). In contrast, incorporation of BrdU increased as a function of extracellular redox state and was highest at the most reduced condition (-150 mV). Cellular GSH/GSSG redox potential was unaffected by variation of the extracellular Cys/CySS redox environment suggesting that the observed regulation occurs independently of cellular GSH. Moreover, results showed that after being cultured for 24 h under oxidized conditions, Caco-2 cells had the ability to regulate the extracellular redox environment to a value comparable to that found *in vivo* in human plasma (-80 mV) (Fig 1.1) (47). These results provided evidence that maintenance of the extracellular Cys/CySS redox environment is important to growth of Caco-2 cells.

Additional studies confirmed that other human cell lines have the ability to regulate the extracellular Cys/CySS redox environment in culture medium to approximately -80 mV (1). However, the mechanism(s) by which the regulation occurs is unknown and represents an important part of the research in the present dissertation. Jonas et al suggested two possible mechanisms by which reduction of the extracellular redox environment may occur (Fig 1.2). The first mechanism involves GSH transport from cells into the extracellular space. GSH released from tissues can reduce CySS by thiol/disulfide exchange reactions thereby contributing to maintenance of the extracellular redox environment (47). However, depletion of GSH by L-buthionine-[S,R]-sulfoximine (BSO), an inhibitor of GSH synthesis, showed no effect on the ability of HT-29 cells to regulate their extracellular redox environment (1). These data suggest that extracellular Cys/CySS redox potential is independent of cellular GSH status.

Another mechanism proposed by Dahm and Jones in a study of redox control in the small intestine involves transport of Cys and CySS in a Cys/CySS shuttle mechanism (Fig 1.2) (21). Because Cys is released into the lumen at a substantial rate and CySS is rapidly transported across the brush border, the combination of these processes with intracellular reduction of CySS could explain the regulation of extracellular Cys/CySS redox environment as observed in cell culture and in the jejunum of the rat (21). Detection of increased intracellular CySS concentrations and an increase in the Cys/CySS ratio are consistent with activation of the Cys/CySS shuttle mechanism (47). Taken together, the *in vitro* studies discussed above show that the extracellular Cys/CySS redox couple regulates cell proliferation independently of the cellular GSH/GSSG redox couple and that human colon carcinoma cells and the jejunum of the small intestine have an inherent ability to regulate the extracellular redox environment.

In addition to its role in the regulation of the cell cycle, studies show that extracellular Cys/CySS redox potential has the ability to regulate early events related to atherosclerosis *in vitro* (33). Go and Jones (33) found that a more oxidized extracellular Cys/CySS redox environment stimulated H_2O_2 production, activated nuclear factor-kappaB, increased expression of adhesion molecules (intercellular adhesion molecule-1, platelet endothelial cell adhesion molecule-1, P-selectin), and stimulated monocyte binding to endothelial cells. They concluded that the extracellular E_hCySS plays a key role in regulating early events of atherosclerosis and could be useful as a potential marker for vascular disease risk *in vivo*.

Jiang, et al determined that extracellular Cys/CySS redox potential is also useful in predicting susceptibility to oxidant-induced apoptosis (45). Retinal pigment epithelial

(RPE) cells exposed to more oxidizing physiologic Cys/CySS redox potentials (E_h > -55 mV) were more susceptible to oxidant-induced apoptosis induced by *tert*-butylhydroperoxide compared to reduced conditions (E_h < -89 mV). Results show that variation of extracellular redox potential contributes to a decline in cell populations by enhancing sensitivity to oxidant-induced apoptosis (45). Enhanced sensitivity to apoptosis could therefore provide a mechanism by which a more oxidized redox environment could explain degenerative changes associated with aging (51).

These studies suggest that in addition to effects on cell proliferation, mechanisms are present that support antioxidant defenses and susceptibility to apoptosis through Cys. Thus, together with the above data, the results suggest that extracellular redox potential is important in regulation of the cell cycle and disease.

1.4. Factors Affecting Extracellular Cys/CySS Redox Homeostasis

Oxidative stress has been shown to be an integral component of diseases and degenerative processes associated with aging. Initial studies of aging and the extracellular thiol/disulfide redox environment showed that plasma GSH concentrations were lower and plasma GSSG concentrations were increased in older subjects compared to younger subjects (91). Jones et al conducted a study in healthy individuals aged 19 to 84 years to which also examined Cys and CySS changes with age (58). Results showed a linear oxidation of plasma Cys/CySS redox potential with age at a rate of 0.16 mV/year over the entire age span. In contrast, plasma GSH/GSSG redox potential was maintained until 45 years of age; however, GSH/GSSG was subsequently oxidized at a rate of 0.7 mV/year (58). These data show that oxidative events increase throughout adulthood, but

similar to the *in vitro* data discussed above, the data also show that the two redox pools are regulated independently *in vivo.* Given that extracellular Cys/CySS redox potential is important for the regulation of numerous growth processes, the results suggest that extracellular Cys/CySS redox couple may function as an indicator of growth and repair functions *in vivo*.

Evidence also shows that oxidative stress is associated with diseases and conditions such as cardiovascular disease (14), cancer (61), age-related macular degeneration (13), chemotherapy (48), and lung injury (41). Because experimental data suggested that intracellular proatherogenic events and cell adhesion are regulated by extracellular thiol/disulfide redox environment *in vitro* (33), human studies were conducted to determine if oxidative stress, as measured by the extracellular redox potential, could predict early atherosclerosis in healthy, non-smoking subjects who are free of cardiovascular disease. Results showed that the extracellular GSH pool was an independent predictor for the presence of early atherosclerosis in an otherwise healthy population (3). Ashfaq et al concluded that this finding supported a role for oxidative stress in the pathogenesis of premature atherosclerosis and that the measurement of plasma GSH/GSSG redox potential could possibly help in the early identification of subjects at risk of cardiovascular disease (3).

In addition to cardiovascular disease, oxidative stress has been implicated in age-related macular degeneration (AMD). Studies show that antioxidant supplementation significantly increased plasma concentration of Cys resulting in a more reduced plasma redox environment (73). Moreover, zinc supplementation significantly decreased plasma CySS concentration but had no effect on the extracellular redox potential (74).

Perturbations in the extracellular thiol/disulfide redox environment are also associated with the progression and severity of acute lung injury (ALI) in a mouse model. Cys homeostasis is adversely affected during the inflammatory response to infection and injury. Following administration of endotoxin, plasma Cys/CySS redox potential was selectively oxidized in response to endotoxin while plasma GSH/GSSG redox potential remained unchanged (41).

There is accumulating evidence that in addition to aging and disease, lifestyle choices such as smoking can also alter extracellular thiol/disulfide redox homeostasis. In a study of extracellular redox states of the Cys/CySS and GSH/GSSG couples in subjects between the ages of 44 and 85 years, the Cys/Cy/SS redox potential in smokers was more oxidized than non-smokers (-64 ± 16 mV and -76 ± 11 mV respectively) (Fig 1.3) (76). Moreover, the GSH/GSSG redox potential was also more oxidized in smokers as compared to non-smokers (-128 ± 18 mV and -137 ± 17 mV respectively). Oxidation of the extracellular GSH/GSSG pool was explained by the role of GSH in detoxification of reactive species in smoke; however, oxidation of the extracellular Cys/CySS pool indicated that smoking may have additional effects on sulfur amino acid metabolism (76).

Dietary choices can also affect extracellular redox homeostasis. In a study of twins, Dai et al determined that consumption of the Mediterranean diet increased plasma GSH concentration and decreased plasma GSSG concentration (23). The authors concluded that an inverse association between adherence to the Mediterranean diet and oxidative stress occurred primarily as a result of lower GSSG concentrations. These results support the hypothesis that the Mediterranean diet has cardioprotective effects by lowering oxidative stress. Other information suggests that intake of sulfur amino acids

(SAA) contribute to the maintenance of extracellular thiol concentrations and/or the redox potential (81). Because SAA are involved in the maintenance of thiol/disulfide redox homeostasis, a diet deficient in these amino acids could result in a decrease in plasma Cys and GSH concentration thereby resulting in an oxidation of the extracellular redox environment.

1.5. Sulfur Amino Acid Metabolism

The sulfur-containing amino acids (SAA), Met and Cys, are required for diverse, critical biologic functions including protein synthesis, methylation, fatty acid metabolism, osmotic regulation, and regulation of cell division and growth. Met is considered an essential amino acid because it cannot be synthesized in amounts sufficient to sustain normal growth in mammals. In contrast, Cys is considered a conditionally essential amino acid because under normal conditions, the body can synthesize Cys from its amino acid precursor Met (96). Met and Cys are normally consumed as components of dietary proteins. Most dietary SAA are consumed from animal protein including red meat, poultry, fish, and eggs. With the exception of some legumes and nuts, a gram of plant-derived protein contains only 10-20% of the SAA content of animal protein (56, 100).

In addition to the intake of dietary proteins, turnover of body protein and peptide pools releases free Met and Cys into body pools. A considerable amount of turnover can be contributed to hydrolysis of secreted proteins (12). These proteins are secreted into the gastrointestinal tract where they are subsequently digested to amino acids that can be absorbed with those from dietary proteins. Because GSH serves as a reservoir of Cys, its degradation can also contribute to the free amino acid flux (12). When Cys supply is low,

GSH is released into the circulation and is subsequently degraded by γ-glutamyltranspeptidase. γ-glutamyltranspeptidase, which is located on the external side of the plasma membrane, transfers the glutamyl residue from extracellular GSH and yields cysteinyl-glycine. Cysteinyl-glycine is then degraded to Cys and glycine by a dipeptidase on the cell surface, facilitating the release of Cys back into circulation (95).

SAA enter the plasma and circulate as free amino acids until they are removed by tissues. While many tissues of the body are capable of converting Met to Cys via the enzymatic processes of transmethylation and transulfuration (Fig 1.4), the liver removes a substantial portion of SAA from the portal circulation and is the most active site of Met metabolism (94).

Met metabolism begins with its activation by ATP to form S-adenosylmethionine (SAM) in a reaction catalyzed by Met adenosyl-transferase (MAT). SAM, a major biological methyl donor for molecules such as DNA and RNA, donates its methyl group to an acceptor to produce S-adenosylhomocysteine (SAH). SAH is then hydrolyzed to yield adenosine and homocysteine by SAH hydrolase. This sequence of reactions is collectively referred to as transmethylation and is ubiquitously present in cells (Fig 1.4) (96).

Homocysteine is considered the intersection of the Met cycle and the transulfuration pathway in that it can be methylated back to Met by Met synthase or condensed with serine to form the thioether cystathionine in a reaction catalyzed by cystathionine β-synthase (10). This step commits the Met molecule to degradation via the transsulfuration pathway. Once formed, cystathionine is then hydrolyzed by cystathionine γ-lyase to form Cys and α-ketobutyrate and ammonia (Fig 1.4). L-Cys is further

metabolized in the liver to yield GSH and sulfate, which function in detoxification and elimination of xenobiotics. Because SAA are irreversibly modified in the aforementioned processes, there is a requirement for adequate sulfur amino acid intake that extends beyond the need for adequate amounts to maintain normal protein synthesis and turnover.

1.6. Recommended Dietary Allowance for Sulfur Amino Acids

The Recommended Dietary Allowance (RDA) for SAA is based upon nitrogen and sulfur amino acid balance studies. For an adult male, the RDA for SAA is about 1.9 g/day (29). While the mean SAA intake in the population is about 2.4 g/day, a large variation in SAA intake exists and can ranging from < 0.3 g to > 5 g/day (28) . While the number of Americans with low dietary intake of SAA is relatively small, most people undergo intermittent periods of SAA insufficiency due to food selection, dieting, fasting and illness. Individuals who restrict their diet to plant foods may be at risk of not getting adequate amounts of SAA.

1.7. Sulfur Amino Acid Availability and Thiol/Disulfide Redox Homeostasis

Of interest to SAA nutrition, previous studies of human colon carcinoma (HT-29) cells have shown that Cys deficiency limits cell growth, decreases intracellular GSH concentration, and is sufficient to result in a marked oxidation of intracellular GSH/GSSG redox potential (68). The readdition of Cys or CySS into the cell culture media produced a rapid recovery from the oxidized conditions associated with Cys deficiency (Fig 1.5) (68). The observed oxidation indicates that dietary availability of SAA may directly determine thiol/disulfide balance *in vitro*; however, specific effects of

altered dietary intake of SAA on plasma redox potential were not determined. A study in adult rats showed that 7 day SAA deficiency caused a significant decrease in plasma Cys and CySS concentrations, leading to an oxidation of Cys/CySS redox potential (Fig 1.6) (81). Moreover, decreased dietary SAA intake did not change plasma GSH concentrations but resulted in a significant increase in GSSG concentrations, leading to oxidation of plasma GSH/GSSG redox potential (81). This study confirmed that dietary SAA deficiency is sufficient to cause an oxidation of the extracellular Cys/CySS and GSH/GSSG redox potentials *in vivo*. Oxidation of the extracellular redox environment is associated with disease processes and aging and increases susceptibility of cells to injury (45). Therefore, taken together, these studies suggest that inadequate dietary SAA intake may also contribute to the onset or progression of diseases influenced by redox status or increased susceptibility of cells and tissues to toxicity and injury.

1.8. Acetaminophen Pharmacokinetics and Metabolism

In addition to the effects of inadequate dietary SAA, enhanced utilization of SAA due to drug metabolism could also affect redox states. Acetaminophen (APAP or Tylenol©), is one of the most widely used antipyretic and analgesic drugs in the world and utilizes cysteine in its metabolism. Because APAP is generally safe at therapeutic doses in healthy individuals, it has been used in many studies to evaluate physiologic effects on drug metabolism.

Following oral administration, APAP is rapidly absorbed by passive diffusion with first order kinetics in the gastrointestinal tract. Therefore, the rate of absorption depends on the individual gastric emptying rate (17). APAP is also uniformly and rapidly

distributed throughout body fluids due to a low degree of plasma protein binding at therapeutic doses. First pass metabolism is low, therefore APAP is highly bioavailable (62). In healthy individuals, plasma APAP concentrations reach a peak between 30 and 60 minutes. The half-life of APAP is approximately 2 hours at therapeutic doses and the apparent volume of distribution of APAP is approximately 1 L/kg. The volume of distribution is similar in healthy subjects, the elderly, and various patient groups (17).

With administration of recommended therapeutic doses, approximately 90% of APAP metabolism occurs in the liver by two main conjugation pathways, glucuronidation and sulfation (Fig 1.7). Sixty percent of the original dose is excreted in the urine as glucuronide conjugates and 30% as sulfate conjugates (97). An additional 2% is excreted in the urine as the parent compound. The remaining 5% to 8% undergoes oxidative conversion by human cytochrome P450 2E1, 2A6, and 3A4 to its reactive intermediate, N-acetyl-p-benzoquinonimine (NAPQI) (62) . In the presence of GSH, this metabolite is rapidly detoxified and excreted in the urine as Cys and mercapturic acid conjugates (17).

While well tolerated at therapeutic dosages, APAP is an established dose-dependent hepatotoxin. The most common method of toxicity is overdose, which is defined as the ingestion of a single dose of 10 to 15 g APAP in adults and 150 mg/kg APAP in children (90) . Excessive doses of APAP lead to the saturation of the glucuronidation and sulfation conjugation pathways. Dysfunction of the glucuronide and sulfate conjugation systems causes more APAP to undergo oxidative conversion by cytochrome P450 which in turn increases the amount of NAPQI produced. GSH concentration within the liver is depleted in the detoxification of the increased amount of NAPQI produced. Because GSH is an important factor in antioxidant defense,

hepatocytes are rendered highly susceptible to oxidant injury. Depletion of GSH allows binding to hepatic proteins and macromolecules and subsequent hepatic injury (32). Specific events that produce hepatocellular death following the formation of APAP protein adducts are poorly understood. Other processes including loss of mitochondrial or nuclear ion balance, macrophage activation, and pro-inflammatory signaling have also been suggested to be toxic mechanisms involved in APAP-mediated cell death. Accumulation of NAPQI can also occur in alcoholics with therapeutic doses of APAP due to depletion of GSH and induction of cytochrome P450 2E1 (85, 93).

1.9. Chemical detoxification and SAA Availability

Sulfation and detoxification by GSH are important conjugation pathways in the elimination of APAP and require Cys as a substrate precursor. Sulfation is the major pathway of elimination of low doses of APAP, and GSH is essential for detoxification of the reactive metabolite of APAP, NAPQI (46). A diet deficient in SAA would therefore be expected to suppress the conjugation of APAP by sulfation and GSH conjugation and increase the susceptibility of tissues to APAP hepatotoxicity.

To determine whether SAA deficiency potentiates APAP hepatoxicity, rats were fed diets containing varying concentrations of methionine. Results showed that prolonged SAA deficiency decreased hepatic GSH concentrations and increased the incidence and severity of APAP –induced hepatic necrosis (86). Moreover, SAA deficient animals metabolized APAP at a slower rate than control animals. A similar study showed that liver Cys concentrations were significantly lower in SAA deficient animals and that SAA limitation increased APAP-GSH conjugate excretion in the urine (88). Taken together, these studies indicate that SAA deficiency may increase the

requirement for sulfur containing amino acids and limit the availability of Met and Cys that can be used for protein synthesis and methylation reactions. Considering that a molar equivalent of about 0.2 g of Cys is used for metabolism of a maximal therapeutic dose of 1 g APAP; 4 doses per day would consume 0.8 g of Cys, or more than half of the RDA. Because of variation of intake in the population, the total equivalents of SAA needed for metabolism of the maximum daily dose of APAP and other critical biological processes can exceed individual intake of SAA.

1.10. Research Questions and Hypotheses

The available evidence indicates that the plasma E_h CySS is important in human health but relatively little is known about factors which affect extracellular E_h CySS or the mechanisms involved. Consequently, the purpose of this dissertation is to identify potential mechanisms for regulation of the plasma E_h CySS by using a model cell culture system and to conduct a small human study to test whether nutritional or drug treatment can alter plasma E_h CySS. In the latter, the study was designed to also test whether a change in Cys availability and subsequently E_h CySS was sufficient to alter APAP metabolism in humans *in vivo*.

Previous work in the area of extracellular redox regulation has provided information only on the response of the apical domain of polarized cells to an oxidative challenge. Because intestinal cells are polarized, it becomes necessary to mimic intestinal conditions to determine if the basal domain regulates the extracellular redox environment in a manner similar to or different from that observed on the apical domain. Therefore, the purpose of the cell culture study was to determine whether the basal

domain of Caco-2 cells can regulate the extracellular environment following exposure to CySS and if so, to determine whether the regulation occurs through function of known transport systems or involves cell membrane associated thiol/disulfide exchange, copper- or flavin-dependent oxidoreductases. The hypotheses were that the basal domain is capable of regulating the extracellular redox environment and that Cys/CySS transport mechanisms function in the reduction of the extracellular E_h CySS in Caco-2 cells.

As stated, Met and Cys are involved in the maintenance of thiol/disulfide redox homeostasis. Based on studies discussed earlier, one can anticipate that use of APAP could alter plasma Cys and GSH pools. The purpose of the human study was to determine whether APAP alters plasma Cys or GSH pools in healthy humans consuming adequate or inadequate dietary SAA intakes and whether short term SAA insufficiency alters APAP metabolism in healthy individuals. The experiment was designed with 4 study periods for each individual to allow direct comparisons of APAP and SAA insufficiency and APAP metabolism. The hypotheses were 1) that therapeutic doses of APAP alone or 2 days of SAA-insufficient diet alone would be insufficient to elicit changes in plasma Cys or GSH pools; 2) that the combination of SAA insufficiency and APAP administration would result in decreased Cys and GSH and more oxidized E_hCySS and E_hGSSG; and 3) that 2 days of a SAA-free diet would cause changes in the metabolic pattern and pharmacokinetics of APAP in the plasma and urine.

The present studies show that Cys and CySS transport mechanisms are important in regulation of plasma E_h CySS and that E_h is dependent upon dietary and drug exposures. However, the results also show that changes in plasma Cys and E_h CySS *in*

vivo due to dietary manipulation are not sufficient to cause a change in the pharmacokinetics or disposition of APAP.

FIGURE 1.1

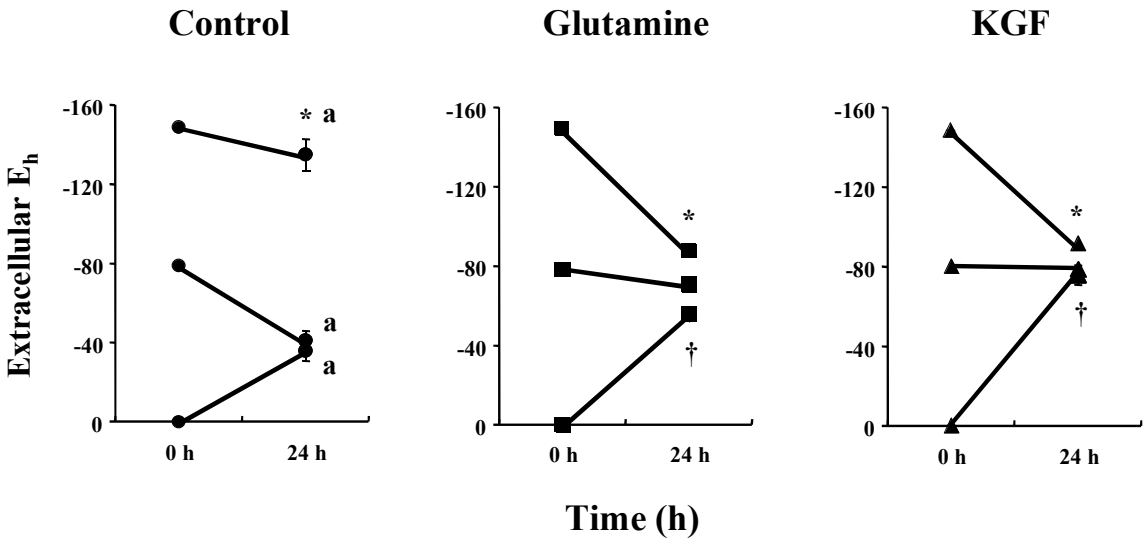

Control
Glutamine
KGF
Extracellular E_h
-160
-120
-80
-40
0
0 h
24 h
* a
a
a
*
†
*
†
Time (h)

Figure 1.1. Caco-2 cells regulate CySS/Cys E_h in the extracellular medium with either glutamine (Gln) supplementation or keratinocyte growth factor (KGF) treatment. Cells were serum starved and Gln starved for 24 h and then exposed to a range of thiol/disulfide redox potentials (E_h: 0, -80, and -150 mV) in the presence of no additives [control (Con)], Gln (10 mmol/l), or KGF (10 μg/l) for 24 h. Initial E_h conditions were obtained by varying medium Cys and CySS concentrations at pH 7.4. The measured E_h of the Cys/CySS pool in Caco-2 cell medium was calculated using the Nernst equation and HPLC-derived Cys and CySS concentrations. Data are shown as means ± SE. Error bars are smaller than symbol size when not apparent. Results represent data from 3 separate experiments, each done in triplicate. *$P < 0.05$ vs. the 0 mV condition and †$P < 0.05$ vs. the -150 mV condition within individual treatments (control, Gln, or KGF). $^aP < 0.05$ vs. both Gln and KGF-treated cells cultured at the same initial E_h. **Used with permission. Jonas, C. R. et al. Am J Physiol Regul Integr Comp Physiol 2003; 285: R1426.**

FIGURE 1.2

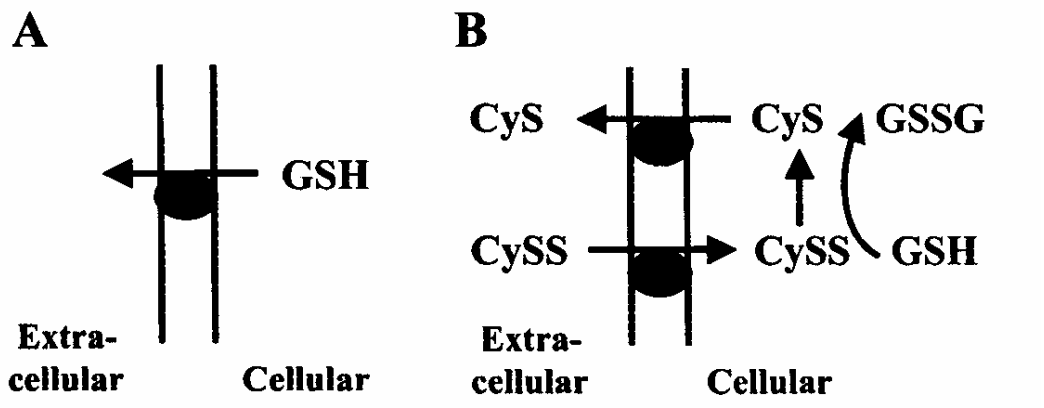

A
GSH
Extra-
cellular
Cellular
B
CyS
CyS
GSSG
CySS
CySS
GSH
Extra-
cellular
Cellular

Figure 1.2. Possible mechanisms for reduction of oxidizing extracellular E_h in Caco-2 cells. Used with permission. Jonas, CR et al. Am J Physiol Regul Integr Comp Physiol 2003; 285: R1427.

FIGURE 1.3

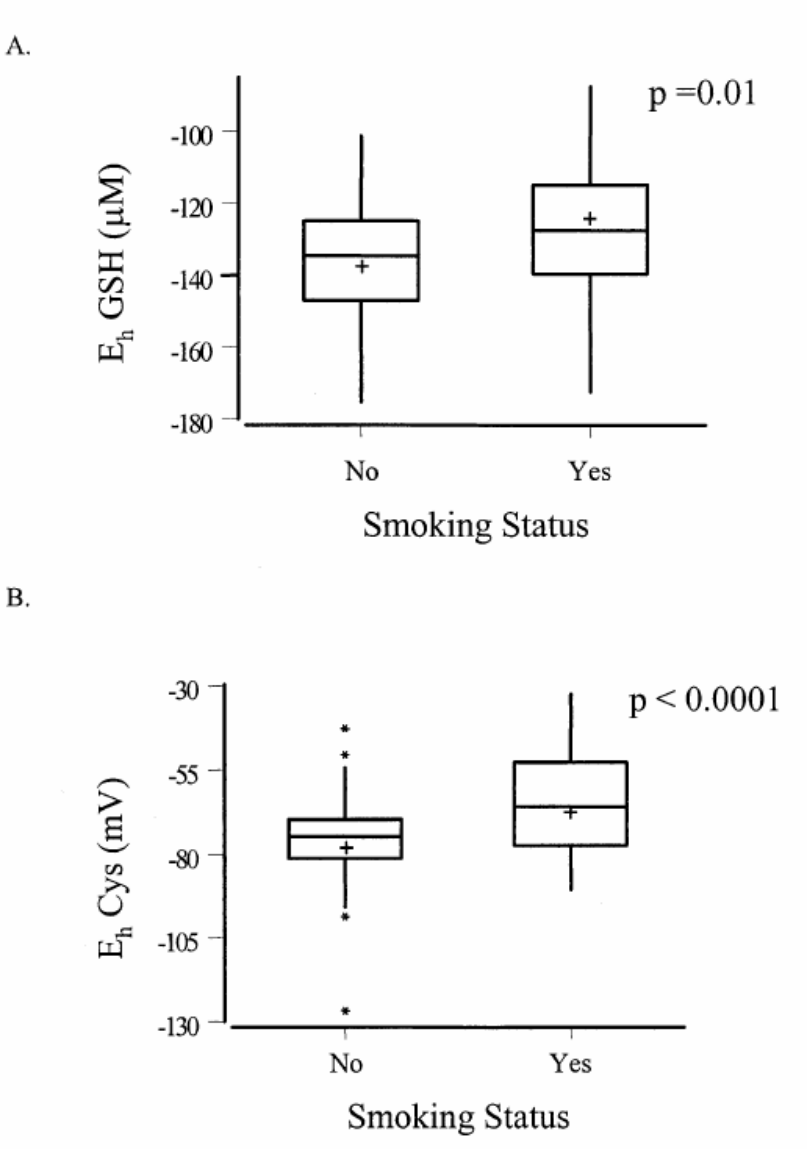

A.
p =0.01
-100
-120
-140
-160
-180
E_h GSH (μM)
No
Yes
Smoking Status
B.
p < 0.0001
-30
-55
-80
-105
-130
E_h Cys (mV)
No
Yes
Smoking Status

Figure 1.3. Comparison of redox states in plasma of smokers and nonsmokers. (A) GSH/GSSG redox couple was oxidized in smokers (−128 ± 18 mV) compared to nonsmokers (−137 ± 17 mV). (B) Cys/CySS redox couple was oxidized in smokers (−64 ± 16 mV) compared to nonsmokers (−76 ± 11 mV). **Used with permission. Moriarty, SE et al. Free Radic Biol Med. 2003 Dec 15; 35(12):1586.**

FIGURE 1.4

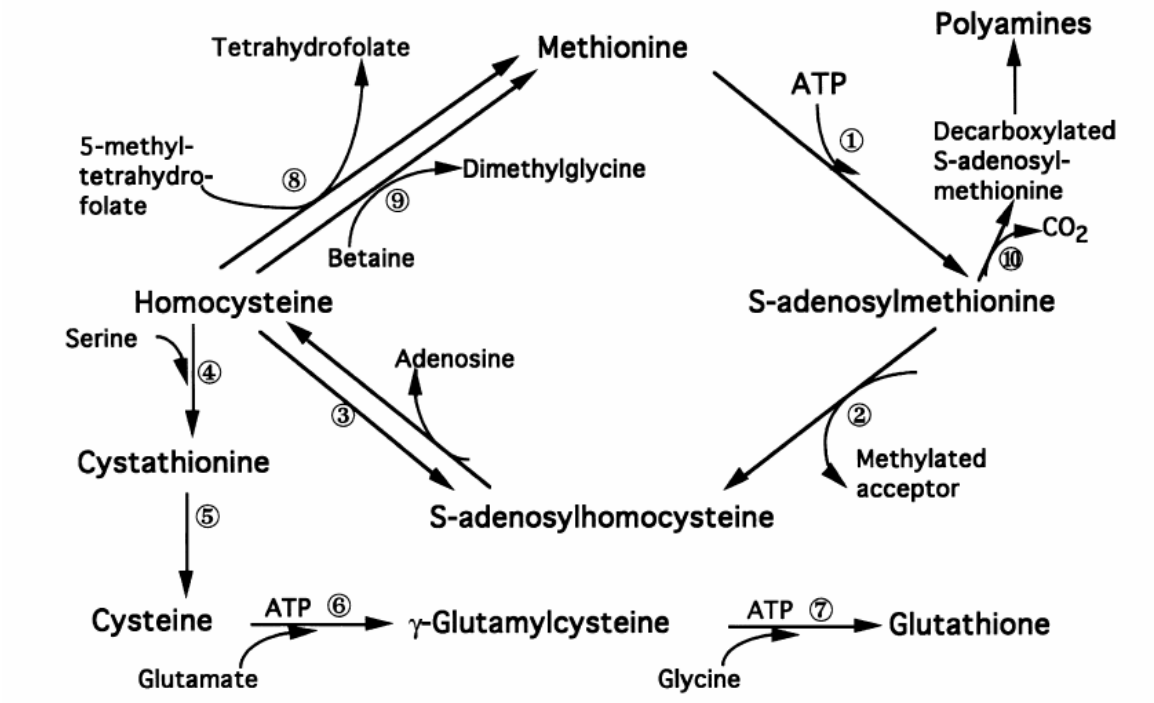

Tetrahydrofolate
Methionine
Polyamines
ATP
①
Decarboxylated
S-adenosyl-
methionine
5-methyl-
tetrahydro-
folate
⑧
Dimethylglycine
⑨
Betaine
CO_2
⑩
Homocysteine
S-adenosylmethionine
Serine
④
Adenosine
③
②
Methylated
acceptor
Cystathionine
S-adenosylhomocysteine
⑤
Cysteine
ATP ⑥
γ-Glutamylcysteine
ATP ⑦
Glutathione
Glutamate
Glycine

Figure 1.4. Transmethylation and transsulfuration pathways. (1) Methionine adenosyltransferase catalyzes the synthesis of SAM from methionine and ATP; (2) transmethylation reactions; (3) S-adenosylhomocysteine hydrolase catalyzes the reversible hydrolysis of S-adenosylhomocysteine to yield homocysteine and adenosine; (4) cystathionine β-synthase catalyzes the formation of cystathionine from homocysteine and serine; (5) γ-cystathionase catalyzes the formation of cysteine from cystathionine; (6) γ-glutamylcysteine synthetase, the rate-limiting enzyme in GSH synthesis, catalyzes the formation of γ-glutamylcysteine from cysteine and glutamate; (7) GSH synthetase catalyzes the formation of GSH from γ-glutamylcysteine and glycine; (8) methionine synthase regenerates methionine from homocysteine in a reaction that requires normal levels of folate and vitamin B_{12}; (9) betaine-homocysteine methyltransferase regenerates methionine from homocysteine in a reaction that requires betaine; (10) *S*-adenosylmethionine decarboxylase catalyzes the removal of the carboxyl group from SAM and channels it into the polyamine synthesis pathway. **Used with permission. Lu, SC. Int J Biochem Cell Biol. 2000 Apr; 32(4):393.**

FIGURE 1.5

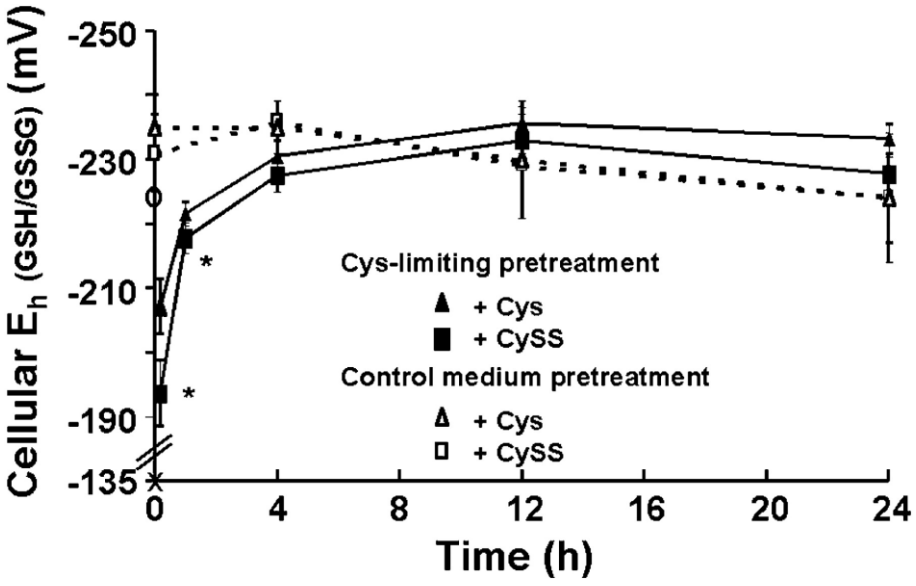

Cellular E_h (GSH/GSSG) (mV)
-250
-230
-210
-190
-135
0
4
8
12
16
20
24
Time (h)
*
*
Cys-limiting pretreatment
+ Cys
+ CySS
Control medium pretreatment
+ Cys
+ CySS

Figure 1.5. Recovery of redox state (E_h) of cellular glutathione/glutathione disulfide (GSH/GSSG) after the addition of cysteine (Cys) or cystine (CySS) to Cys-deficient human HT29 cells. Cells pretreated for 48 h with Cys-limiting medium are represented by a solid line, whereas control cells are represented by a broken line. The redox states of cellular GSH/GSSG for Cys-deficient cells and control cells before medium change are denoted by an "X" and an "O," respectively. Data are expressed as means ± SEM; $n = 6$ for deficient groups and $n = 4$ for control groups. Two-way ANOVA showed a significant effect of time ($P = 0.0001$) and group-by-time ($P = 0.0001$). The overall effect of group was not significant ($P = 0.759$). *Values for Cys and CySS treatments were significantly different from controls only for 10-min and 1-h time points. Within a group by time, only the 10-min and 1-h points differed from other times ($P < 0.05$). **Used with permission. Miller, LT et al. J Nutr 2002 Aug; 132(8):2305.**

FIGURE 1.6

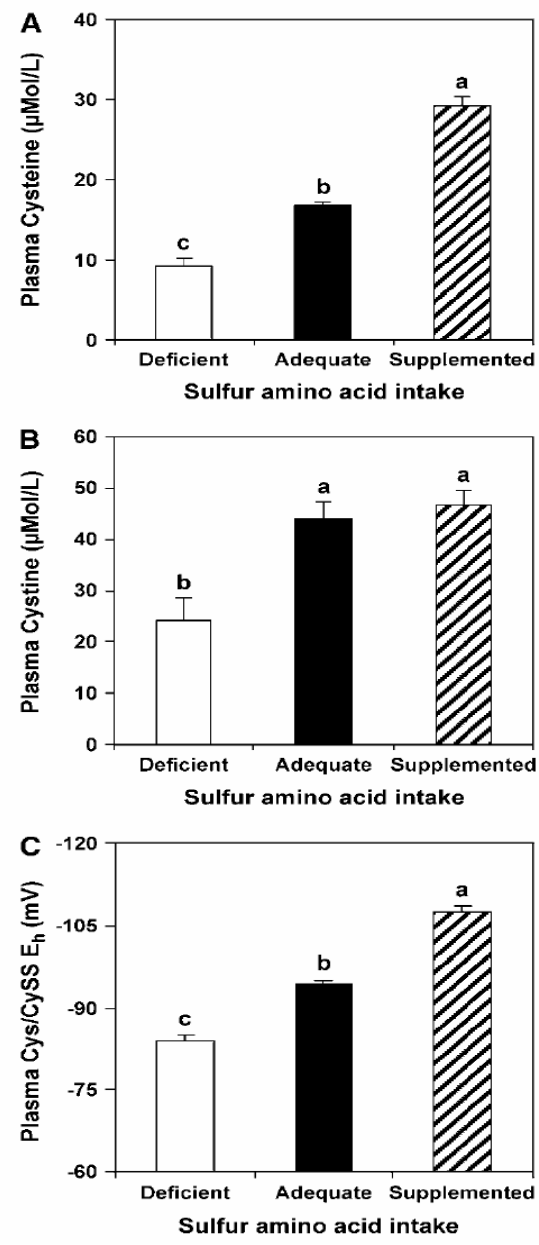

A
Plasma Cysteine (µMol/L)
40
30
20
10
0
a
b
c
Deficient
Adequate
Supplemented
Sulfur amino acid intake
B
Plasma Cystine (µMol/L)
60
50
40
30
20
10
0
a
a
b
Deficient
Adequate
Supplemented
Sulfur amino acid intake
C
Plasma Cys/CySS E_h (mV)
-120
-105
-90
-75
-60
a
b
c
Deficient
Adequate
Supplemented
Sulfur amino acid intake

Figure 1.6. The effect of dietary SAA content on rat plasma Cys (A) and CySS concentrations (B) and Cys/CySS redox potential (C). Means without a common letter differ, $P < 0.05$. Values are means ± SE, $n = 6$. For the reference group (standard rat chow ad libitum), plasma concentrations of Cys and CySS were 23 ± 3 and 53 ± 3 μmol/L, respectively, and plasma Cys/CySS redox potential was –100 ± 4 mV. **Used with permission. Nkabyo, YS et al. J Nutr 2006 May; 136(5):1244.**

FIGURE 1.7

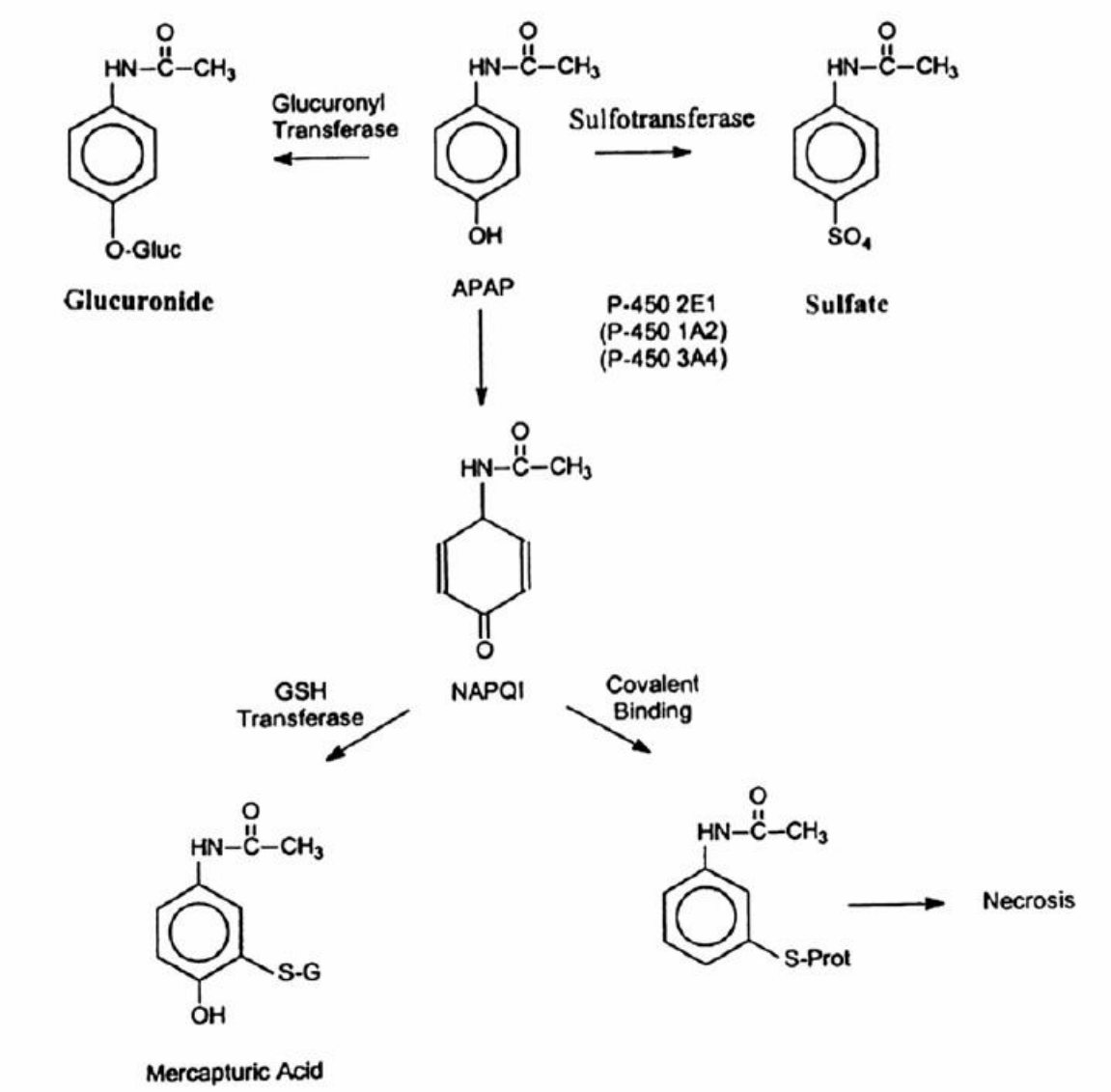

HN–C–CH3
O
O-Gluc
Glucuronide
Glucuronyl Transferase
OH
APAP
Sulfotransferase
SO4
Sulfate
P-450 2E1
(P-450 1A2)
(P-450 3A4)
NAPQI
GSH Transferase
Covalent Binding
S-G
Mercapturic Acid
S-Prot
Necrosis

Figure 1.7. Metabolism of acetaminophen. Most APAP is conjugated to either glucuronide or sulfate. The portion which is oxidized to NAPQI is further detoxified by glutathione transferase. If this system is overwhelmed, NAPQI binds to cellular targets leading to hepatocellular necrosis. **Used with permission. Zimmerman, HJ. Acetaminophen hepatotoxicity. Clin Liver Dis 1998; 2:527.**

Chapter 2: Characterization of Apical and Basal Thiol-Disulfide Redox Regulation in Human Colonic Epithelial Cells

Yanci O. Mannery, Thomas R. Ziegler, Li Hao, Yvonne Shyntum, Dean P. Jones

2.1 ABSTRACT

Control of extracellular thiol-disulfide redox potential is necessary to protect cell surface proteins from external oxidative and reductive stresses. Previous studies show that human colonic epithelial Caco2 cells, which grow in cell culture with the apical surface exposed to the medium, regulate extracellular cysteine/cystine redox potential (E_h) to physiologic values (~ -80 mV) observed *in vivo*. The present study tested whether extracellular E_h regulation occurs on the basal surface of Caco2 cells and investigated relevant mechanisms. Experiments were performed with confluent, differentiated cells grown on a permeable membrane surface. Cells were exposed to an oxidizing potential (0 mV) using a fixed cysteine/cystine ratio, and culture medium was sampled over time for change in E_h. Regulation of extracellular thiol-disulfide E_h on the basal domain was faster and the extent of change at 24 h was greater than on the apical surface. Mechanistic studies showed that redox regulation on the basal surface was partially sodium-dependent and inhibited by extracellular lysine, a competitive inhibitor of cystine transport by the y^+L system and by quisqualic acid, an inhibitor of the x_c^- system. Studies using the thiol-reactive alkylating agent 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS) and the glutathione synthesis inhibitor buthionine sulfoximine (BSO) showed that extracellular redox regulation was not attributable to plasma membrane cysteine/cystine interconversion or intracellular glutathione, respectively. Thus, the data show that redox regulation occurs at different rates on the apical and basal surfaces of the polarized Caco2 epithelial cell line and that the y^+L and x_c^- systems function in extracellular cysteine/cystine redox regulation on the basal surface.

2.2 INTRODUCTION

The cysteine/cystine (Cys/CySS) redox couple is the major low molecular weight thiol/disulfide redox system in most extracellular fluids in humans (54, 58). In plasma, the predominant species is the oxidized disulfide form, CySS, which is usually present at >40 μM; Cys is typically present at values of about 8-10 μM in the fasting state (5). The average redox potential (E_h) of this couple in young healthy individuals is -80 ± 9 mV (18), being more reduced following ingestion of Cys and more oxidized in a variety of pathophysiologic states (50, 75). Systematic variation of E_h in culture media shows that the Cys/CySS redox couple regulates biological processes such as cell proliferation, proinflammatory signaling and apoptosis (35). However, major questions remain concerning how cells regulate extracellular redox potential.

Culture medium for mammalian cells often contains high physiologic concentration of CySS (200 to 400 μM) and no added Cys. The resulting E_h is relatively oxidizing (>0 mV). Jonas et al showed that Caco2 cells *in vitro* regulated extracellular redox conditions from 0 mV to the physiologic range of -80 mV observed *in vivo* (47). In these experiments, cells were grown on a solid surface so that the observed extracellular redox regulation probably reflected that occurring at the apical surface. Regulation of intraluminal thiol/disulfide redox state had been previously shown in a vascularly perfused rat small intestinal preparation (20), and related studies also showed redox regulation in the vascular perfusate (22). Importantly, these studies suggested that the extracellular redox regulation occurred via a Cys/CySS shuttle mechanism in which CySS was transported into cells, reduced to Cys and transported back into the extracellular fluid (47, 63).

In the intestine, numerous sodium-dependent and sodium-independent systems facilitate the transport of CySS and Cys, including $b^{0,+}$, X_{AG}^-, ASC (apical surface) and L, y^+L, Asc (basal surface) (Table 1) (4, 30, 31), but studies to identify specific transporters involved in redox regulation and studies to examine contributions of other redox control mechanism are not available.

In the present study, Caco-2 cells were used as a model of human intestinal epithelial cells to study the polarity and mechanisms of extracellular redox regulation. Caco-2 cells are suitable for these studies because they differentiate to form an ileal-like polarized monolayer with brush border microvilli, tight junctions, intestinal transporters, and specific small intestinal enzymes and gene products (77). When grown on a permeable support, the polarized nature of these Caco-2 cell monolayers allows them to be used to study physiologic functions on the apical and basal domains. Because no previous study has characterized basal, as opposed to apical, thiol-disulfide redox regulation in intestinal cells, the purpose of the current study was to determine whether the basal domain of Caco-2 cells can regulate the extracellular environment following exposure to CySS and if so, to determine whether the regulation occurs through function of known transport systems or involves cell membrane-associated thiol/disulfide exchange, copper- or flavin-dependent oxidoreductases. The hypotheses were that the basal domain is capable of regulating the extracellular redox environment and that Cys/CySS transport mechanisms function in the reduction of the extracellular E_h CySS in Caco-2 cells.

2.3 MATERIALS AND METHODS

Chemicals. 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid, disodium salt (AMS) was purchased from Molecular Probes (Eugene, OR). All other chemicals and reagents were purchased from Sigma (St. Louis, MO).

Cell culture. The human colon carcinoma cell line Caco-2 was used between passages 29 and 32. Stock cultures were grown to confluency at 37°C in 5% CO_2 using Dulbecco's modified Eagle's high glucose medium (DMEM) containing 4.5 g/L of L-glutamine. DMEM was supplemented with non-essential amino acids, penicillin (10 U/ml), streptomycin (10 μg/ml), L-glutamine, and 10% fetal bovine serum. Once flasks reached 80% confluence, cells were split, seeded onto coated, permeable tissue culture inserts in six-well culture plates, enabling treatment/sampling from both apical and basal domains of the monolayer. Experiments were performed after 21-24 days, with the criterion for use being that the transepithelial electrical resistance (TER) was between 200 and 400 Ω/cm^2 (see below).

Integrity of the permeability barrier. Epithelial barrier permeability is a dynamic process which is sensitive to oxidative stress and can affect measured rates and steady-state characteristics of redox regulation on the opposite poles of the epithelial cell layer. Because the purpose of the present research was to test for redox regulation on the basal surface and not to study effects on barrier permeability, we used TER between 200 and 400 Ω/cm^2 as a criterion for acceptability of monolayers for use in experiments. TER was measured using an EVOMX electrovoltmeter with an STX2 electrode (World Precision Instruments, Sarasota, FL, USA); as cells reached confluency, TER increased and provided an index of tight junction formation. As an independent test of barrier

integrity following change in culture media, a limited number of experiments were also performed with 10 mg/ml fluorescein isothiocyanate (FITC)-dextran (average molecular weight, 4000), with 1 mM H_2O_2 as a positive control. Following removal of serum-free medium and rinsing of cells, FITC-dextran was added to the apical side, and medium was sampled from the basal side for periods up to 24 h. FITC-dextran concentration was quantified by fluorimetry ($\lambda_{exitation}$= 492 nm, $\lambda_{emission}$= 525 nm).

Cell treatment protocol. Caco-2 cells were serum- and glutamine-starved in MEM for 24 h, washed once with 1 ml PBS and immediately exposed to the oxidizing condition (0 mV) on the apical and basal domains. Extracellular redox potential of 0 mV was obtained by addition of 99.75 μM CySS and 0.5 μM Cys to cysteine-free DMEM, as previously described (47, 80). These concentrations were derived from calculations using the Nernst equation; measured initial E_h values shown in results deviate from this calculated value due oxidation during filter-sterilization after addition and carryover of relatively reduced medium during medium change. Medium from apical and basal domains was collected and cells were harvested for E_h determination at time points as indicated in results.

*Redox determination by HPLC***.** Aliquots of cell culture medium (200 μl) from apical and basal domains were added to ice-cold 10% perchloric acid, containing 0.2 M boric acid and 10 M γ-glutamylglutamate (γ-Glu-Glu) as the internal standard. These samples were centrifuged to remove precipitates and used for extracellular E_h determinations. Cells were rinsed twice with 1 ml PBS, and ice-cold 5% perchloric acid, containing 0.2 M boric acid and 10 mM γ-Glu-Glu as the internal standard, was added directly to each well. Cells were scraped, placed in microcentrifuge tubes and centrifuged

to separate precipitated protein. Pellets were resuspended in 1 M NaOH, and protein concentrations were measured using the Bradford method with rabbit γ-globulin as the protein standard (Biorad Laboratories, Hercules, CA) (7). All samples were stored at -20°C until derivatization with iodoacetic acid and dansyl chloride (55). HPLC with fluorescence detection was used to quantify dansyl derivatives of Cys, CySS, glutathione (GSH), and GSSG. These concentrations were used with the Nernst equation to calculate E_h of each redox couple as described (16, 52, 60).

Sodium dependence and inhibitor studies. To test for sodium dependence, sodium chloride in the cell culture medium on both the basal and apical sides was replaced with isoosmotic choline chloride. To test for possible involvement of specific transport systems in redox regulation, cells were exposed to oxidizing extracellular Cys/CySS in the absence and presence of 2.5 mM lysine, 300 μM quisqualic acid (QA) or 0.5 mM threo-β-hydroxy-aspartate (THA), inhibitors of the y^+L, X_c^- and X_{AG}^- systems respectively. To test for possible involvement of thiol oxidation in intestinal basal membranes, the copper-specific chelator bathocuproine disulfonate (BCS) was used at 0.5 mM (18). To test for possible involvement of flavin-dependent reductases, diphenyleneiodonium chloride (DPI) was used at 0.5 μM, a concentration known to provide general inhibition of flavoproteins (33). To test for possible dependence upon GSH, cellular GSH was depleted by pretreatment with 1 mM buthionine sulfoxide (BSO), a concentration which causes extensive loss of GSH over 24 h (1). To test for possible involvement of thiol/disulfide exchange involving cell surface thiols, the cell impermeant alkylating agent, AMS, was added at 0.5 mM, a concentration known to inhibit redox-dependent cell signaling (33, 80). In these experiments, cells were pre-

treated for 1 h, except for BSO experiments, in which cells were treated with BSO in serum-free conditions for 32 hours.

Statistical Analysis. Differences were compared across groups and time using one-way analysis of variance (ANOVA). Specific differences between treatments or time and their interactions were compared post-hoc using the Fisher's protected least-significant difference test. Results are expressed as mean ± SE, and differences were considered significant when $P < 0.05$.

2.4 RESULTS

Caco-2 cells regulate extracellular Cys/CySS redox potential more efficiently on the basal domain.

Caco-2 cells under standard tissue culture conditions are oriented with the apical surface exposed and regulate Cys/CySS redox potential in the culture medium (47). Experiments were performed with cells grown on permeable support membranes to determine whether the basal and apical domains individually have the capacity to regulate extracellular redox potential. In cells with Cys/CySS added to give 0 mV, basal extracellular Cys/CySS E_h was altered from 7.0 ± 5.5 mV at 0 h to -116 ± 3 mV after 24 h in culture (Fig 2.1A). The apical domain also regulated E_h, but the change was less, from 5.0 ± 3.9 mV at 0 h to -34 ± 9 mV after 24 h (Fig 2.1A). Basal CySS concentration decreased from 91 ± 1 μM at 0 h to 7 ± 1 μM after 24 h (Fig 2.1B). The CySS concentration on the apical domain also decreased over time; however, the change was less, from 94 ± 2 μM at 0 h to 40 ± 3 μM at 24 h (Fig 2.1B). Cys concentration on the basal domain significantly increased over time from 0.5 ± 0.3 μM at 0 h to 15.9 ± 0.3 μM

at 24 h (Fig 2.1C). Apical Cys concentration also increased over time, but to a lesser extent, from 0.6 ± 0.3 μM at 0 h to 1.6 ± 0.3 μM at 24 h (Fig 2.1C). The difference between apical and basal CySS and Cys concentrations, as well as the maintenance of TER during these experiments, indicated that the epithelial barrier was not disrupted during these experiments. Control experiments with FITC-dextran also showed that barrier function of Caco-2 cells was not lost during 24 h following exposure to 0 mV conditions (data not shown). Consequently, the results show that Caco2 cells regulate extracellular redox potential on both the apical and basal domains and that the basal domain has a greater capacity for extracellular E_h regulation.

Redox regulation by Caco-2 cells occurs at physiologic plasma CySS concentrations.

Cell culture media often contains 200 to 400 μM CySS while plasma concentrations are usually in the range of about 50 μM to 100 μM in humans (58). To determine whether redox regulation on the basal surface is dependent upon extracellular CySS concentration in this range, studies were performed to compare redox regulation with total Cys equivalents at 100 μM instead of 200 μM (i.e., CySS concentrations of approximately 50 μM and 100 μM, respectively) and initial E_h at 0 mV. Results showed that at the lower CySS concentration, which is characteristic of that for young, healthy adults, the extent of correction of E_h by 4 h was equivalent to that at the higher concentration, which is characteristic of older individuals (Fig 2.2). After 24 h, the cells at 100 μM initial conditions showed a greater change, from 6 ± 3 mV at 0 h to -114 ± 1 mV compared to 50 μM, from -2 ± 1 mV at 0 h to -85 ± 3 mV (Fig 2.2). Moreover, the data showed that cells at 100 μM initial conditions showed a greater decrease in

extracellular CySS concentration and a greater increase in extracellular Cys concentration at 4 and 24 hours (Fig S2.1A-B). Thus, the results show that Caco2 cells can regulate E_h on the basal surface over a concentration range typical of values found in human plasma and that the effect is greater at 24 h with 100 μM than with 50 μM CySS.

Regulation of extracellular redox environment is Na^+*-dependent.*

Previous results suggest that regulation of extracellular E_h occurs by a Cys/CySS shuttle mechanism dependent upon Cys and CySS transport (21). By this mechanism, CySS, which is found in greater concentrations in the extracellular space, is transported into the intracellular space and undergoes reduction to Cys, which is then released (21). To test whether extracellular E_h regulation is Na^+ dependent, experiments were performed with Na^+-free medium in which NaCl was replaced with choline chloride. Results showed that cells treated with sodium-free medium at 4 h demonstrated a decreased capacity to regulate the extracellular environment (-35.0 ± 3.3 at 4 h) compared to sodium-containing medium (-63.5 ± 0.2 mV) (Fig 2.3A). While extracellular CySS concentrations were significantly higher in cells treated with sodium-free medium (Fig 2.3B), exposure of the basal domain to sodium-free medium significantly lowered extracellular Cys concentrations (Fig 2.3C). Together, these data suggest that either a system(s) participating in CySS influx and/or Cys efflux requires sodium for function.

To determine if CySS transport was specifically affected by the absence of sodium, the concentration of CySS in the intracellular space was measured. Results showed that the intracellular CySS concentration was significantly lower in cells treated

in a sodium-free culture medium (Fig 2.4). These data show that Na^+-dependent CySS uptake contributes to the mechanism for extracellular redox regulation.

Lysine decreases the concentration of extracellular Cys.

To test for involvement of transport systems, experiments were performed with selective inhibitors. Lysine (Lys), a cationic amino acid, was tested as a competitive inhibitor of the y^+L system for transport of CySS on the basal domain. In cells exposed to 2.5 mM Lys, extracellular Cys concentrations were lower as compared to controls at 60 min and 240 min (Fig 2.5A). Results also showed that at 240 min, cells exposed to lysine demonstrated less of a change in extracellular E_h (-87 ± 0.8 at 4 h) compared to control (-95 ± 0.6 mV) (Fig 2.5B).

Because redox regulation was not completely inhibited following exposure of the basal domain to lysine, experiments were performed to determine if other CySS transport systems contribute to the mechanism to normalize the extracellular redox environment on the basal domain. To test whether extracellular E_h regulation is affected by inhibition of sodium-independent transport mechanisms, experiments were performed with QA (300 μM), an inhibitor of system x_c^-. Results showed that cells treated with quisqualic acid demonstrated a diminished capacity to regulate the extracellular environment (-37.7 ± 4.4 at 240 min) compared to control (-75.8 ± 0.4 mV) (Fig 2.6). THA (0.5 mM), an inhibitor of the x_{AG}^- system, was used as a negative control and showed that extracellular redox regulation was not affected by inhibition of the x_{AG}^- system (data not shown). Although complete inhibition did not occur when cells were exposed to lysine or QA, the data

indicate that the y^+L and x_c^- transport systems function as components of the Cys/CySS shuttle mechanism regulating extracellular E_h (21, 47).

Depletion of cellular GSH does not affect regulation of extracellular redox potential.

Additional experiments were performed to determine if mechanisms besides amino acid transport contribute to the ability of Caco-2 cells to reduce the extracellular redox environment. Glutathione (GSH) is a low-molecular weight thiol that is present in millimolar concentrations in the cell and has been previously shown to support CySS utilization by isolated hepatocytes through a mechanism involving release and reaction with CySS to generate Cys (67). In contrast, Anderson et al showed that the depletion of GSH in HT29 cells had no effect on capacity to regulate extracellular E_h (1). To determine whether regulation of E_h in Caco2 cells was dependent upon cellular GSH, cells were exposed to 1 mM BSO. A substantial decrease in cellular GSH concentration and oxidation of the cellular GSH/GSSG E_h occurred (Fig 2.7A and 2.7B). However, there was no significant effect on the ability of the basal domain to correct extracellular redox environment (Fig. 2.7C). Data are therefore consistent with previous evidence showing that GSH does not contribute to extracellular redox regulation in colonic epithelial cells.

Redox regulation is not dependent upon extracellular surface protein thiols, flavoproteins or cuproproteins.

To test other possible mechanisms contributing to regulation of the extracellular redox environment, experiments were performed with inhibitors of oxidoreductase

activities. AMS, a cell impermeant thiol-reacting reagent, was used at a concentration (0.5 mM) known to inhibit redox-dependent cell signaling in Caco2 cells (80). Results showed no significant effect on extracellular redox regulation (Fig 2.8A). Pretreatment with 5 μM DPI, an inhibitor of many flavoproteins, also resulted in no significant effect on redox regulation on the basal surface (Fig 2.8B). Similarly, treatment with 0.5 mM bathocuproine disulfonate, a nontoxic copper-specific chelator used to limit Cys oxidation in culture medium, had no effect on basal surface redox regulation (Fig S2.2). Thus, the data provide evidence that thiol-, flavin- and copper-dependent oxidoreductase mechanisms do not contribute significantly to the ability of the basal domain to regulate extracellular redox.

2.5 DISCUSSION

Previous research showed that the apical domain of Caco-2 cells regulates the extracellular redox environment towards physiologic values observed *in vivo* (47). Because Caco-2 cells are polarized, the present data extend this study to show that the polarized intestinal epithelial cells independently regulate the extracellular redox environment on the basal surface and that this occurs with a greater rate/capacity than on the apical surface. The data do not exclude the possibility that paracellular movement of Cys or CySS affect the absolute rates measured; however, measurements of TER and transepithelial FITC-dextran movement showed that barrier integrity did not substantially change during the experiments. Furthermore, the differences in concentrations of CySS and Cys on opposing sides under many conditions (e.g., Fig 2.1B and 2.1C) are inconsistent with the conclusion that regulation is an indirect consequence of paracellular

movement. On the other hand, data for CySS loss and Cys appearance in Fig 2.1 are consistent with a portion of CySS uptake from the apical side being released as Cys on the basal side. Such a transepithelial movement could explain an apparently lower rate of redox regulation on the apical side of cells grown on the semipermeable membrane compared to rates observed for cells grown on solid support (49), where the apical surface is exposed to the culture media.

In a study of luminal reduction of GSSG, Dahm and Jones (21) proposed a Cys/CySS shuttle mechanism for luminal GSSG reduction based upon the detection of luminal CySSG in association with regulation of luminal GSH/GSSG redox potential. Jonas et al provided additional support for the function of a Cys/CySS shuttle in regulation of extracellular Cys/CySS redox potential in Caco2 cells (47). They used radiolabeled CySS uptake in Caco2 cells to provide evidence for this shuttle mechanism; however, specific intestinal systems that function in transport were not studied and possible function of other mechanisms was not studied.

The present research confirms that redox regulation on the basal surface involves transport mechanisms by showing that this regulation is dependent upon extracellular Na^+ (Fig. 3A). The absence of sodium in the cell culture medium increased extracellular CySS concentrations (Fig 2.3B) and decreased extracellular Cys concentrations (Fig 2.3C) as compared to control. While it is possible that proteolysis could alter intracellular Cys concentrations, previous studies have shown that cellular protein content in Caco-2 cells is stable after day 16 in culture (82). Therefore, changes in proteolysis are not likely to have a large effect on intracellular Cys concentrations under the

conditions of the experiments. Taken together, the results show that either a system participating in CySS influx and/or Cys efflux require sodium for function.

We determined that CySS transport was selectively affected by measuring the concentration of cellular CySS following treatment with sodium-free medium. Figure 4 showed that cellular CySS content was dramatically decreased in cells treated with sodium-free medium as compared to controls (Fig 2.4). Further, transport-inhibitor studies showed a partial effect of lysine on redox regulation (Fig 2.5B). Partial inhibition of redox regulation by lysine suggested that other transporters may function in the Cys/CySS shuttle mechanism. Examination of sodium-independent transport systems showed that treatment with quisqualic acid (Fig 2.6) but not threo-β-hydroxyacetate (data not shown), markedly decreased the ability of the basal domain to normalize the extracellular redox environment. Therefore, the results provide evidence that the y^+L and the x_c^- systems contribute to extracellular Cys/CySS redox regulation. The results do not support a contribution of x_{AG}^- to this regulation. Other inhibitor studies showed that this regulation is not dependent upon cellular GSH (Fig 2.7A-C), surface thiols (Fig 2.8A), membranal flavoproteins (Fig. 8B) or cuproproteins (Fig S2.2). While these results do not exclude other mechanisms, they support the interpretation that redox regulation on the basal surface depends upon transport of redox-active molecules rather than transmembranal electron transfer mechanisms.

Available data suggests that there are at least six amino acid transport systems on the basal domain of intestinal epithelial cells, with three that participate in the transport of Cys or CySS (30, 31). The molecular identities of these have been extensively studied and the expression of the mRNA for some, such as SLC7 (y^+L), has been confirmed in

Caco-2 cells. However, we did not perform molecular characterization in the present study so we have retained functional rather than molecular descriptions. The Asc and L systems transport Cys and other neutral amino acids such as glycine, alanine, serine, and threonine. The transport function of these systems is sodium-independent (30, 31). In the Cys/CySS shuttle mechanism, the function of these transporters could be indirectly sodium-dependent if the uptake of CySS is sodium-dependent (See Fig 2.9). System y^+L is an amino acid transporter that mediates the sodium-dependent transport of neutral amino acids, including CySS as well as sodium-independent transport of cationic amino acids (9, 31). Previous studies show that transport of CySS by system y^+L occurs through the binding of sodium as a co-substrate, converting CySS to a suitable charge for transport (4). Cationic amino acids inhibit the uptake of the neutral amino acids by y^+L. Thus, the characteristics of Na^+ dependence, inhibition by lysine and appearance of Cys in the culture medium are consistent with a redox regulatory mechanism in which Na^+-dependent uptake of CySS occurs by y^+L, CySS reduction occurs intracellularly, and Cys is released by the Asc or L systems. These findings are consistent with the earlier findings of Fernandez et al (27) in a renal proximal tubule cell line, where CySS uptake on the apical surface occurred by y^+L, resulted in increased cellular Cys and efflux of cysteine by system L. The substantial dependence of redox regulation upon Na^+ (Fig 2.3) but only minor inhibition by lysine (Fig 2.5) in the present study may indicate that concentrations of lysine greater than 2.5 mM are needed for effective inhibition of y^+L.

The redox regulation was only about 50% Na^+-dependent (Fig 2.3); the Na^+-independent component of the Cys/CySS shuttle could involve x_c^-. System x_c^- is an amino acid antiporter that mediates the sodium-independent transport of cystine via 1:1

exchange with glutamate (11). Burdo et al provided evidence that the x_c^- transport system is present at the brush border in the intestine but did not examine basal localization (11). Because cells treated with QA demonstrated a decreased capacity to regulate the extracellular environment at 20, 60, and 240 min (Fig 2.6), the data suggest that system x_c^- is also present at the basal domain and participates in the Cys/CySS shuttle mechanism (Fig 2.9).

The physiologic relevance of extracellular redox regulation is rapidly being clarified by studies using a redox-clamp model in which extracellular E_h is systematically varied and cell signaling and related cell functions are measured (34) and by human studies associating E_h with health outcomes (57). The redox clamp studies show that cell surface and extracellular proteins, including growth factor receptors, integrins, and metalloproteinases, contain thiols which are sensitive to oxidation and alter cell proliferation, sensitivity to apoptosis and proinflammatory and profibrotic signaling. Human studies have shown associations of oxidation with cardiovascular disease risk, age-related macular degeneration and persistent atrial fibrillation. The present studies provide the most convincing data available to date that the regulation of extracellular redox potentials, which are known to differ in different body fluids, are controlled by transport mechanisms with different characteristics on opposite poles of epithelial cells.

In the intestines, this polarity could allow extracellular E_h appropriate for absorption on the apical surface independently of cell signaling on the basal surface. Our data show that the basal domain regulates extracellular redox potential to a greater extent than the apical domain (Fig 2.1), which could indicate a greater redox sensitivity of receptors or transporters on the basal surface. Alternatively, the overcorrection to -116 ±

3.4 mV by 24 h, which is beyond the physiologic range observed in human plasma (-80 ± 9 mV), may reflect the function of basal surface transport in amino acid absorption. Other responses to variations in extracellular redox potential, such as stimulation of cell proliferation (39, 49) and enhanced sensitivity to apoptosis (45), have been observed and suggest that different responses in the intestines could also be important during injury or growth signaling.

The *in vitro* cell culture model used for the current experiments has been widely used in many studies concerning intestinal absorption and transport. Growth of Caco-2 cells onto tissue culture plate inserts allows separate access to the apical and basolateral domain. While artificial in nature, this system more effectively mimics the physiology of the gut *in vivo* than does culture of cells on a non-permeable surface. A potential limitation of the study is that inhibitors, such as lysine and quisqualic acid, are not absolutely specific to single transport mechanisms and do not exclude the function of other systems. Thus, more specific interpretations will require availability of more specific inhibitors and/or use of knockout mice with individual transport deficiencies.

In summary, the basal domain of Caco-2 cells regulates the extracellular redox environment toward the physiologic range observed *in vivo* and does this more rapidly than the apical domain. This capability was sodium-dependent and inhibited by lysine and quisqualic acid, indicating that the y^+L and x_c^- systems function in the Cys/CySS shuttle mechanism regulating extracellular E_h. Inhibitor studies provided no evidence for function of other transport systems or oxidoreductase mechanisms. Together, the data show that the y^+L and x_c^- systems provide important components of the Cys/CySS shuttle

mechanism for maintenance of the extracellular redox environment on the basal surface of this epithelial cell line.

ACKNOWLEDGEMENTS

The authors thank Vadivel Ganapathy, Ph.D., for helpful discussion of intestinal amino acid transport systems and Susan H. Voss, Ph.D., for supply of confluent cell monolayers on permeable support.

TABLE 2.1. CySS and Cys transport mechanisms on the apical and basal surface of intestinal epithelial cells. Based upon Ganapathy (31)

Apical Surface

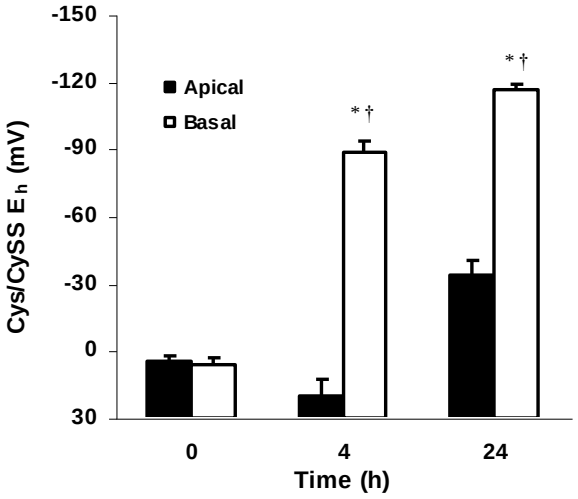
Transport System	Substrate	Sodium-Dependent
$b^{0,+}$	Neutral and cationic L-amino acids; CySS	No
X_{AG}^-	Anionic amino acids; CySS	Yes
ASC	Cys and other neutral amino acids	Yes

Basal Surface

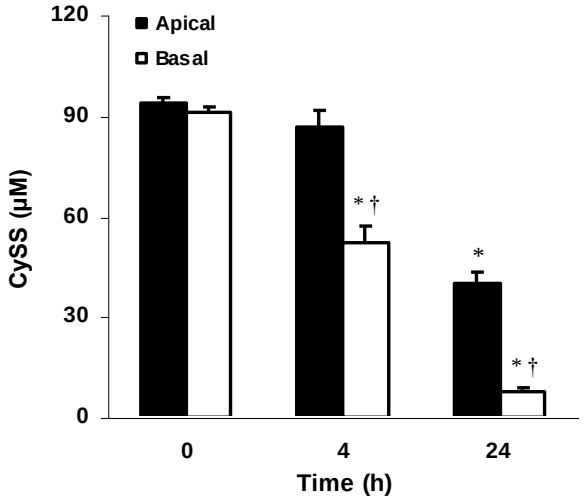
Transport System	Substrate	Sodium-Dependent
y^+L	Neutral L-amino acids; cationic amino acids; CySS	Yes
L	Cys and other neutral amino acids	No
Asc	Small neutral L- and D-amino acids; Cys	No

FIGURE 2.1

A.



B.



C.

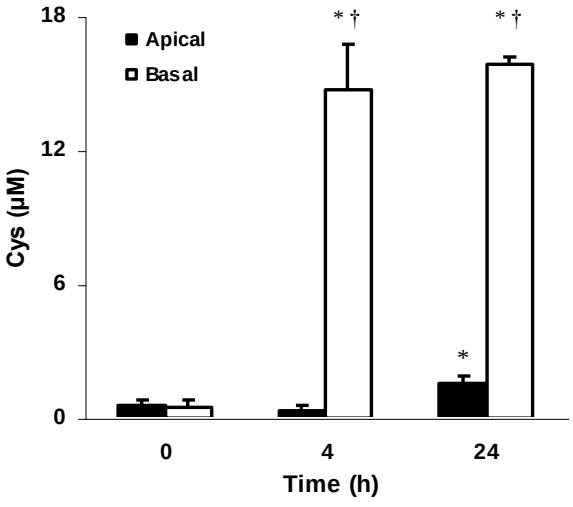


Figure 2.1. Extracellular redox regulation occurs on both apical and basal domains of Caco-2 cells. Caco-2 cells were grown on permeable membranes for 21-24 days to achieve a transcellular resistance > 200 Ω/cm^2 and treated under an oxidizing extracellular Cys/CySS E_h condition obtained by medium Cys and CySS concentrations calculated for 0 mV at pH 7.4 with a total of 200 μM in Cys equivalents. Media from the apical and basal domains were collected at 0, 4 and 24 h. Cys and CySS concentrations were determined by HPLC analysis and used with the Nernst equation to calculate respective E_h values shown in Panel A. Corresponding extracellular CySS and Cys concentrations are shown in Panels B and C, respectively. Data are expressed as mean ± SEM with n = 4. *P< 0.05 for apical versus basal domain; †P< 0.05 versus time 0 for apical and basal domains.

FIGURE 2.2

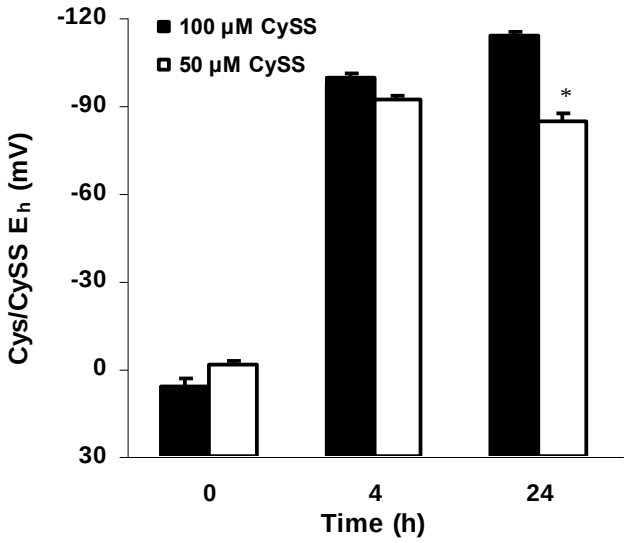
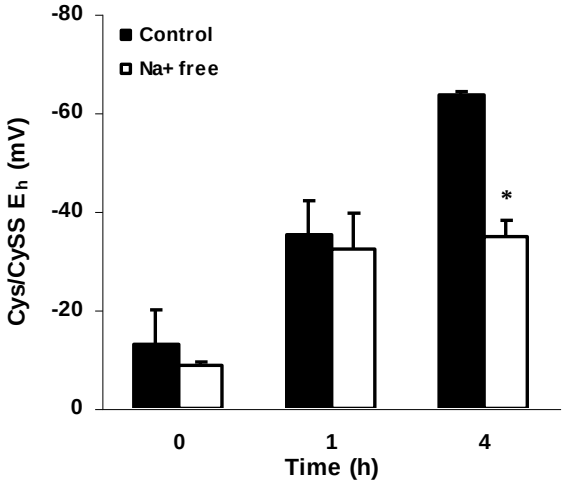


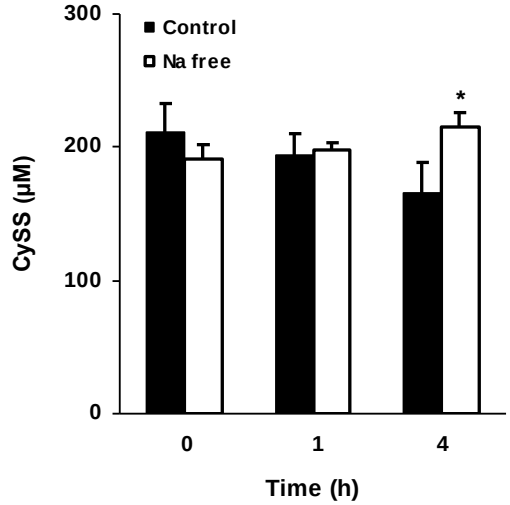
Figure 2.2. Effect of cystine (CySS) concentration on basal redox regulation. Concentrations of Cys and CySS needed to provide Cys/CySS E_h conditions (0 mV, pH 7.4) with total equivalents of 100 and 200 μM Cys were calculated using the Nernst equation; under these conditions, initial CySS concentration was approximately 50 and 100 M, μrespectively. After 0, 4, and 24 h, media was harvested at respective time points and E_h was calculated using the measured Cys and CySS concentrations and the Nernst equation. Data are expressed as mean ± SEM with n = 4. *P< 0.05 for 100 μM CySS versus 50 μM CySS. Measured concentrations for Cys and CySS are available in Fig S2.2A and S2.2B.

FIGURE 2.3

A.



B.



C.

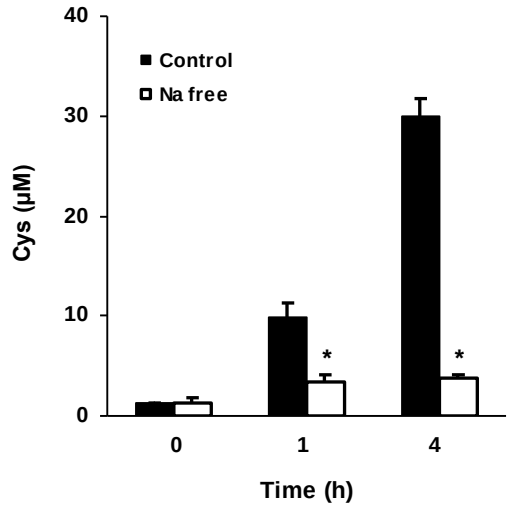


Figure 2.3. Effect of sodium-free medium on basal regulation of (A) extracellular Cys/CySS redox potential (E_h), (B) CySS and (C) Cys concentrations. Caco-2 cells were exposed to Cys/CySS E_h at 0 mV in the presence of culture medium containing sodium chloride (control) or with culture medium in which iso-osmolar choline chloride was used to replace sodium chloride (Na^+-free). Medium was harvested at 0, 1, and 4 h, and CySS and Cys were measured by HPLC. E_h was calculated using the measured Cys and CySS concentrations and the Nernst equation. Data are expressed as mean ± SEM with n=4. *P< 0.05 for control versus sodium-free treatment.

FIGURE 2.4

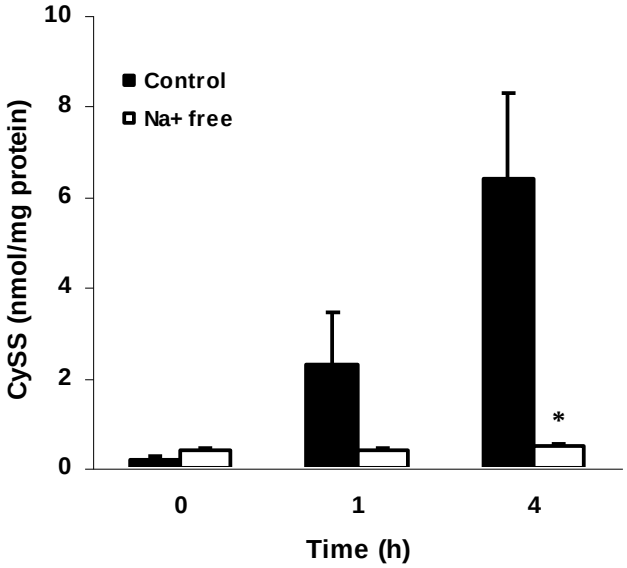
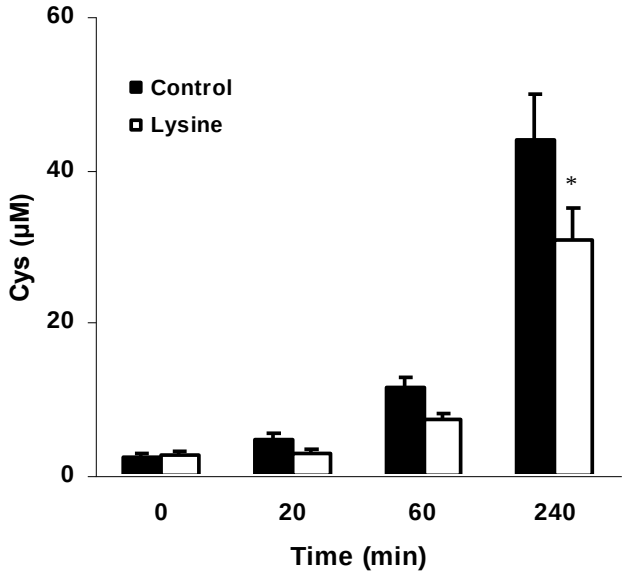


Figure 2.4. Effect of sodium-free medium on cellular CySS concentration in Caco-2 cells. Caco-2 cells were exposed to E_h at 0 mV in the presence or absence of sodium as described in Fig 2.3. Cells were harvested at 0, 1, and 4 h. Intracellular CySS concentration was determined following derivatization and HPLC analysis, and expressed relative to cellular protein content. Data are expressed as mean ± SEM with n = 4. *P< 0.05 for control versus sodium-free treatment.

FIGURE 2.5

A.


60
40
20
0
Cys (µM)
Control
Lysine
*
0
20
60
240
Time (min)

B.

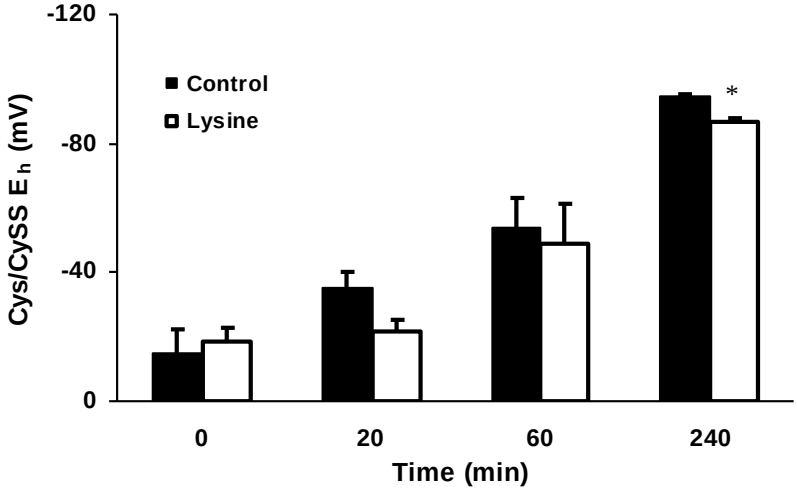

-120
-80
-40
0
Cys/CySS Eh (mV)
Control
Lysine
*
0
20
60
240
Time (min)

Figure 2.5. Effect of lysine on extracellular Cys concentration and Cys/CySS redox potential. Lysine (2.5 mM) was added to the cell culture medium as a competitive inhibitor of CySS transport in Caco-2 cells. Cells were exposed to lysine for 0, 20, 60, and 240 minutes and harvested. **A**. Extracellular Cys concentrations were determined following derivatization of samples and quantification using HPLC. **B**. Extracellular Cys/CySS E_h was calculated using the estimated extracellular Cys and CySS concentrations and the Nernst equation. The data are an average of 3 experiments, each performed in duplicate. Data are expressed as mean ± SEM. *P< 0.05 for control versus lysine-treated cells.

FIGURE 2.6

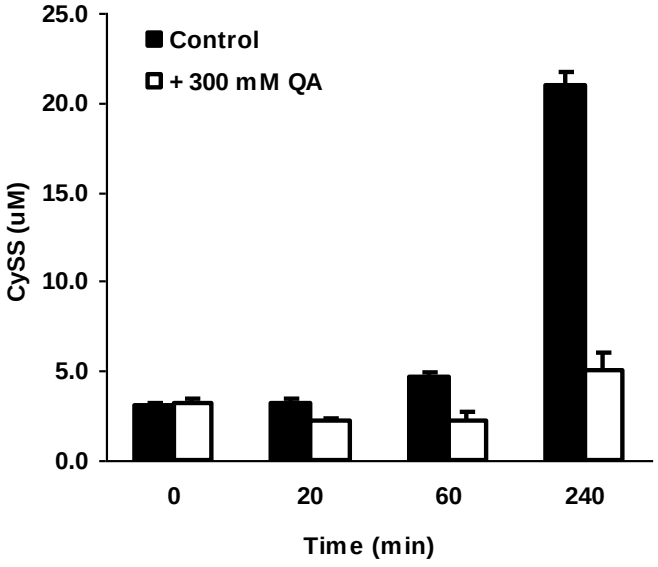

25.0
20.0
15.0
10.0
5.0
0.0
CySS (uM)
Control
+ 300 mM QA
0
20
60
240
Time (min)

Fig 2.6. Effect of quisqualic acid on extracellular Cys/CySS redox potential. Quisqualic acid (0.3 μM) was added to the cell culture medium as a competitive inhibitor of CySS transport in Caco-2 cells. Cells were exposed to quisqualic acid for 0, 20, 60, and 240 minutes and harvested. Extracellular Cys/CySS E_h was calculated using the estimated extracellular Cys and CySS concentrations and the Nernst equation. The data are an average of 3 experiments, each performed in duplicate. Data are expressed as mean ± SEM. *P< 0.05 for control versus quisqualic acid-treated cells.

FIGURE 2.7

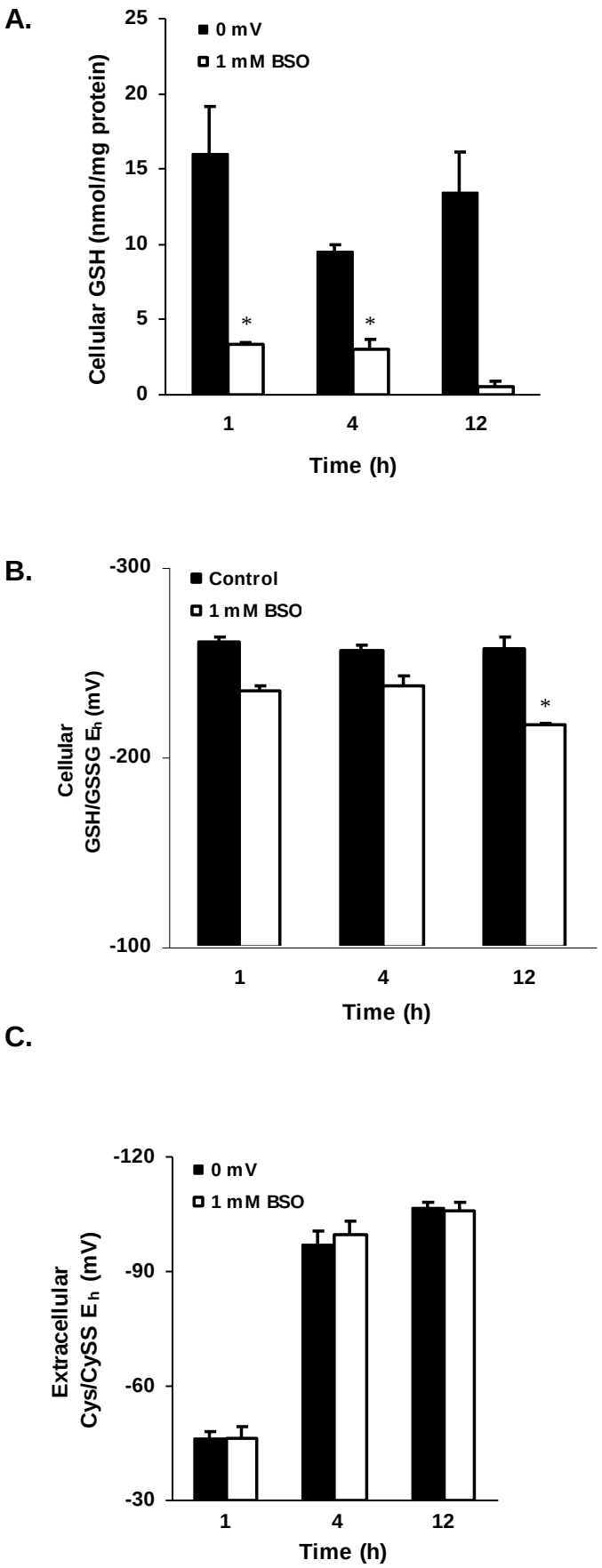
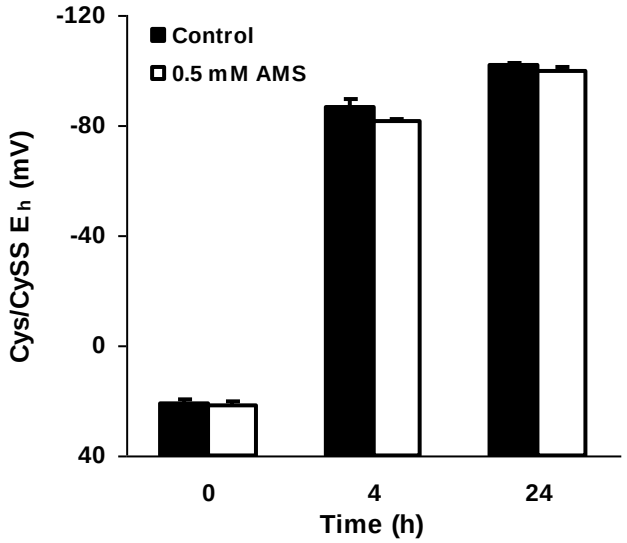

A.
0 mV
1 mM BSO
Cellular GSH (nmol/mg protein)
25
20
15
10
5
0
*
*
1
4
12
Time (h)
B.
Control
1 mM BSO
Cellular GSH/GSSG E_h (mV)
-300
-200
-100
*
1
4
12
Time (h)
C.
0 mV
1 mM BSO
Extracellular Cys/CySS E_h (mV)
-120
-90
-60
-30
1
4
12
Time (h)

Figure 2.7. Effect of BSO pretreatment on intracellular GSH concentration intracellular GSH/GSSG redox potential (GSH/GSSG E_h) and extracellular Cys/CySS redox regulation (Cys/CySS E_h). Cells were treated with or without 1 mM BSO for 32 h. Following washing, cells were exposed to 0 mV extracellular E_h with or without 1 mM BSO for 1, 4, or 12 h. Medium and cells were harvested at respective time points. **A**. GSH content was measured by HPLC and is expressed relative to cell protein, measured using the Bradford method. **B**. Intracellular GSH/GSSG E_h was calculated using the estimated cellular GSH and GSSG concentrations and the Nernst equation. To estimate cellular concentrations, 1 mg of cell protein was assumed to be associated with 5 μl of cell volume. **C**. Extracellular Cys/CySS E_h was calculated using the Cys and CySS concentrations measured by HPLC and the Nernst equation. Data are expressed as mean ± SEM with n = 4. *P< 0.05 for comparisons of control versus BSO treatment. No significant effects of BSO treatment were observed on extracellular Cys/CySS E_h.

FIGURE 2.8

A.


-120
-80
-40
0
40
Control
0.5 mM AMS
Cys/CySS E h (mV)
0
4
24
Time (h)

B.

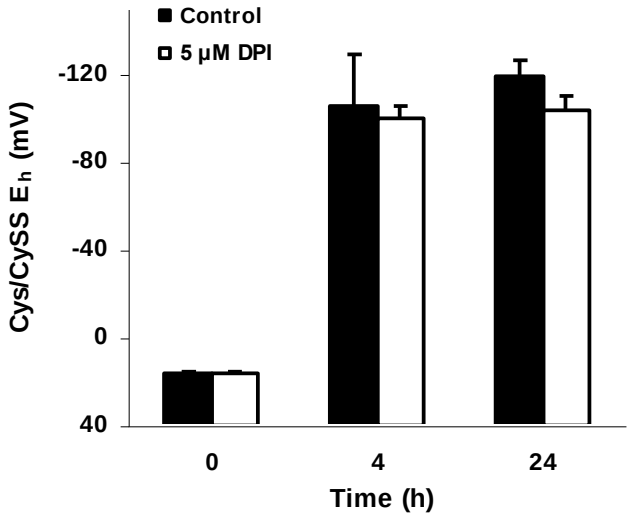

Control
5 µM DPI
-120
-80
-40
0
40
Cys/CySS E h (mV)
0
4
24
Time (h)

Figure 2.8. Effect of thiol inhibitor AMS or flavoprotein inhibitor DPI pre-treatment on extracellular redox regulation in Caco-2 cells. **A**. To test whether regulation of extracellular Cys/CySS E_h was dependent upon cell surface thiols, cells were pre-treated for 1 h with 0.5 mM AMS (4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid, disodium salt), a concentration known to inhibit redox-sensitive cell signaling by EGF receptor in Caco2 cells. Medium was removed, cells were washed and cells were exposed to 0 mV extracellular E_h. The extracellular Cys/CySS E_h at indicated times was determined using the measured Cys and CySS concentrations and the Nernst equation. Results show no significant effect of AMS on redox regulation. **B**. To test for possible function of plasma membrane flavin-dependent oxidoreductases, cells were pre-treated for 1 h with 5 μM DPI (diphenylphosphonium), a general flavoprotein inhibitor, and analyzed as in A. Data are expressed as mean ± SEM with n = 4. No significant effects were observed in comparisons of control versus DPI treatment. Additional data showing no significant effect of the copper chelator bathocuproine disulfonate on extracellular redox regulation are provided in Fig S2.2.

FIGURE 2.9

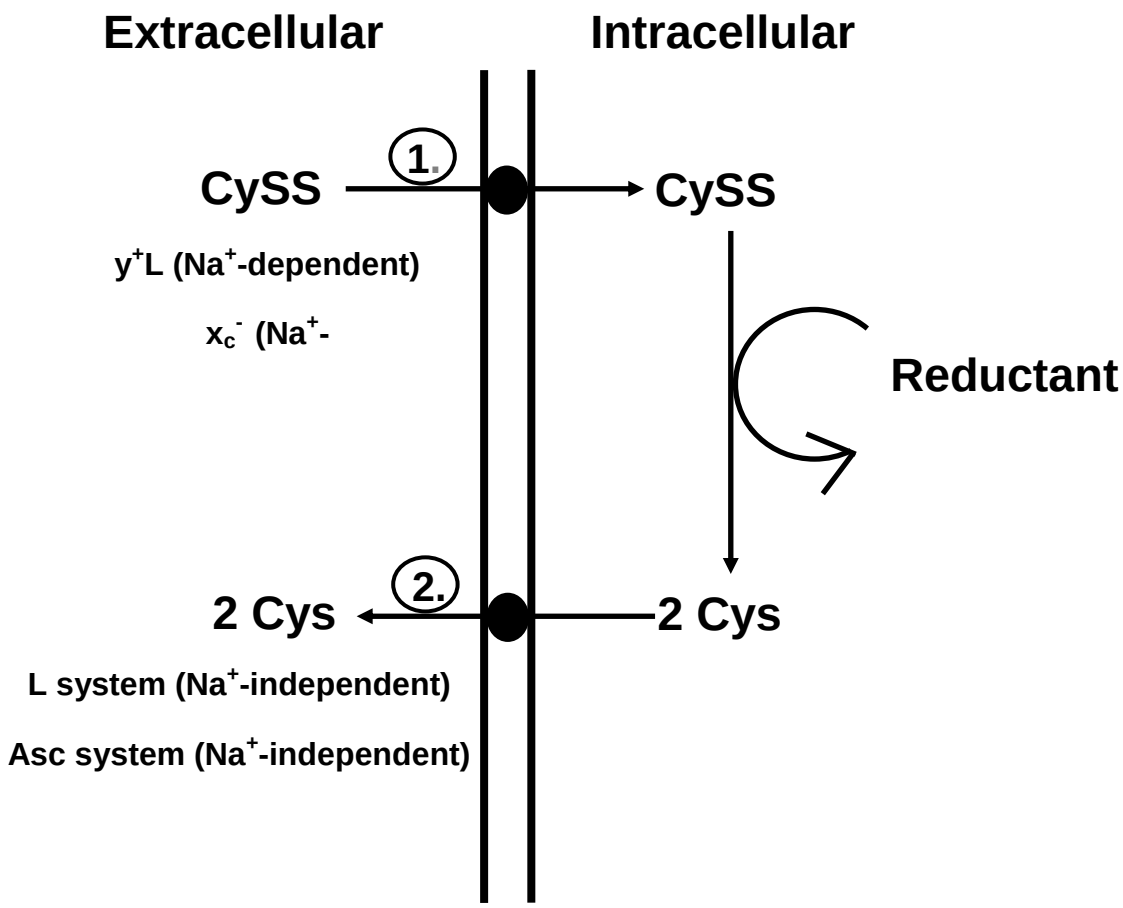
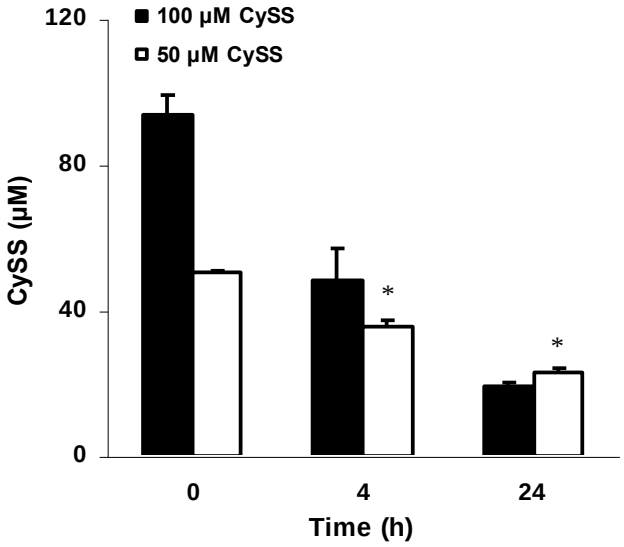

Extracellular
Intracellular
1.
CySS
CySS
y+L (Na+-dependent)
xc- (Na+-
Reductant
2.
2 Cys
2 Cys
L system (Na+-independent)
Asc system (Na+-independent)

Figure 2.9. Transport systems functioning in Cys/CySS shuttle mechanism for regulation of extracellular E_h on the basal surface of Caco-2 cells. A. Results from studies with sodium-free medium show that redox regulation is about 50% sodium dependent; inhibitor studies with lysine indicate that the sodium-dependent activity involves CySS uptake by y^+L. Inhibitor studies with quisqualic acid indicate that the sodium-independent activity involves CySS uptake by x_c^-. The mechanism for reduction of CySS to Cys is not known. B. Basal systems for Cys efflux include the L and Asc systems.

FIGURE S2.1

A.



B.

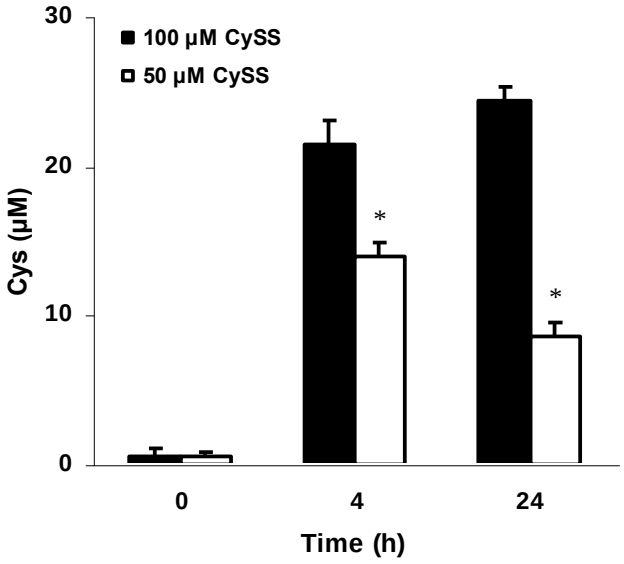


Figure S2.1. Effect of decreased cystine (CySS) in cell culture medium on basal extracellular cystine (CySS) and cysteine (Cys) concentrations. The concentration of CySS contained in cell culture media (200 to 400 µM) is greater than the typical range found in human plasma (50 to 100 µM). To determine whether redox regulation on the basal domain is dependent upon CySS concentrations similar to those found in plasma, experiments were conducted to compare redox regulation with total Cys equivalents at 50 µM and 100 µM. Cells were treated under oxidizing extracellular E_h conditions (0 mV, pH 7.4) which provided approximately 50 and 100 µM CySS concentrations. Media was harvested at 0, 4, and 24 h. Cys and CySS concentrations were determined by HPLC analysis. A. Results showed that cells at 100 µM initial conditions showed a greater decrease in extracellular CySS concentration, from 94 ± 6 µM at 0 h to 19 ± 1 µM at 24 h compared to 50 µM, from 50 ± 1 µM at 0 h to 23 ± 1 µM at 24 h. B. At 24 h, cells at 100 µM initial conditions showed a greater increase in extracellular Cys concentration, from 0.5 ± 0.5 µM at 0 h to 24 ± 1 µM at 24 h compared to 50 µM, from 0.5 ± 0.3 µM at 0 h to 8 ± 1.0 µM at 24 h. Taken together, the results show that Caco-2 cells regulate extracellular CySS and Cys concentrations on the basal domain over the range found in human plasma and that the effect is greater at 24 h with 100 µM than with 50 µM. Data are expressed as mean ± SEM with n = 4. *$P < 0.05$ for 100 µM CySS versus 50 µM CySS.

FIGURE S2.2

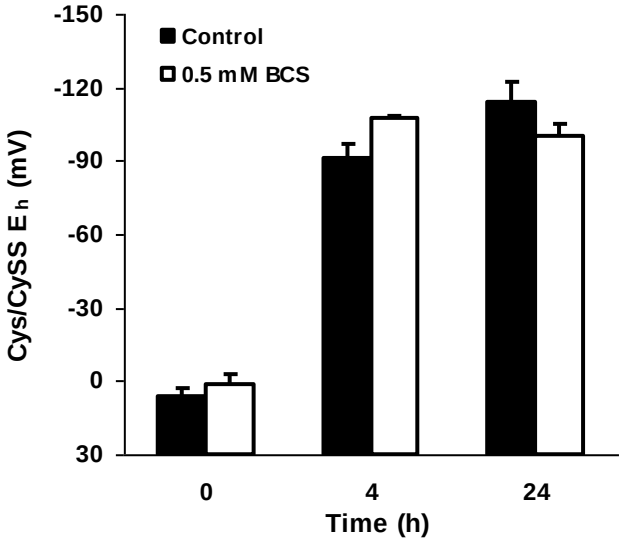


Figure S2.2. Effect of copper-dependent oxidoreductase inhibitor bathocuproine disulfonate (BCS) on extracellular redox regulation in Caco-2 cells. To test for possible involvement of thiol oxidation in intestinal basal membranes, experiments were performed with 0.5 mM BCS, a copper-specific chelator that has been used to limit cysteine autoxidation in cell culture medium. Medium from the basal domain was collected at 0, 4, and 24 h. Cys and CySS concentrations were determined by HPLC analysis and used with the Nernst equation to calculate respective E_h values. Results showed no significant effect on extracellular redox regulation. The data suggests that copper-dependent thiol oxidation does not contribute significantly to the ability of the basal domain to regulate extracellular redox. Data are expressed as mean ± SEM with n = 4. No significant differences were present in comparisons of control versus BCS treatment.

Chapter 3: Oxidation of Plasma Cysteine/Cystine and GSH/GSSG Redox Potentials by Acetaminophen and Sulfur Amino Acid Insufficiency in Humans

Yanci O. Mannery, Thomas R. Ziegler, Youngja Park, Dean P. Jones

3.1 ABSTRACT

Variations in plasma sulfur amino acid (SAA) pools are associated with disease risks, but little information is available about the factors affecting plasma SAA pools. Drug metabolism by glutathione (GSH) and sulfate conjugation can, in principle, represent a quantitatively important burden upon SAA supply. The present study was designed to determine whether therapeutic doses of acetaminophen (APAP) alter SAA metabolism in healthy human adults. A double-blind, crossover study incorporating 4 treatment periods with diets providing 100% of the Recommended Dietary Allowance (RDA) for SAA without or with APAP (15 mg/kg) and 0% RDA for SAA without or with APAP was performed. Following a 3-day equilibration period, chemically defined diets with 100% or 0% RDA for SAA were given for 2 complete days. On day 3, APAP or placebo was given in 2 successive doses (6 h interval) and timed plasma samples were collected. With SAA intake at 100% RDA, APAP administration oxidized the plasma cysteine/cystine redox potential (E_hCySS) but not the plasma GSH/GSSG redox potential (E_hGSSG). The extent of oxidation due to APAP was similar to that seen with 0% SAA and no APAP. However, APAP administration with 0% SAA did not cause further oxidation beyond APAP or 0% SAA alone. In contrast, an oxidation of the plasma E_hGSSG was apparent for SAA insufficiency only with APAP. The results suggest a need to evaluate possible effects of APAP in association with SAA insufficiency as a contributing factor in disease risk.

3.2 INTRODUCTION

The sulfur-containing amino acids (SAA), methionine (Met) and cysteine (Cys), are required to maintain protein synthesis (6, 99) and are linked to drug metabolism through use of Cys for biosynthesis of glutathione (GSH) and sulfate (53, 66). Met is nutritionally essential and is a precursor for Cys (96) so that both are important to Cys supply. Because Cys is irreversibly lost through use of GSH and sulfate for drug metabolism, the requirement for SAA intake during drug metabolism is greater than that normally needed for protein synthesis and turnover (88).

Previous studies of SAA availability and drug metabolism in rodents show that prolonged dietary deficiency of Met and ingestion of APAP significantly decreased body weight and hepatic GSH and Cys concentrations (86, 88). *In vitro* studies of SAA insufficiency alone showed that severe Cys deficiency inhibited proliferation, decreased GSH concentration, and caused a more oxidized intracellular GSH/GSSG redox potential (E_hGSSG) in human colonic epithelial cells (68). Dietary deficiency of SAA also caused a marked decrease in plasma Cys and CySS concentrations and a significant oxidation of E_hCySS values in rats, and this was associated with an increase in plasma GSSG concentration and more oxidized E_hGSSG (81). Together, these data suggest that an interaction between limited dietary SAA supply and drug metabolism could occur and be reflected in changes in the plasma Cys and GSH pools.

Such effects on plasma Cys and GSH could be important because decreased and/or oxidized plasma GSH and Cys pools have been associated with aging, chronic illness, and disease risk factors, including smoking and alcohol abuse (75). A diurnal variation study in humans showed that plasma Cys and GSH concentrations and redox

potentials vary in association with food intake (5). Comparison of SAA intake and GSH and sulfate conjugation suggest that drug metabolism could impact these variations. For instance, the Recommended Dietary Allowance (RDA) for SAA for an adult male is about 1.9 g/day (29). About 25% of APAP is metabolized through sulfation and GSH conjugation. With a maximal therapeutic dose of 1 g APAP, a molar equivalent of about 0.2 g of Cys would be used for APAP metabolism; 4 doses per day would consume 0.8 g, or more than half of the RDA. The mean SAA intake in Americans is considerably greater than the RDA so that the effects of use for drug metabolism in the general population are expected to be relatively small. However, SAA intake ranges from < 0.3 g to > 5 g per day (28) and APAP metabolism occurs principally in the liver, which also supplies GSH to the plasma to maintain Cys supply. Consequently, one can anticipate that use of APAP could alter plasma Cys and GSH pools.

The present study was designed to determine whether APAP alters plasma Cys or GSH pools in healthy humans consuming adequate or inadequate dietary SAA intakes. APAP was selected because it is a generally safe and widely used non-prescription medication (79) with metabolism dependent upon SAA. The experiment was designed with 4 study periods for each individual, 2 with adequate SAA intake in which APAP was compared to placebo and 2 with SAA free diet in which APAP was compared to placebo. Young healthy individuals were studied under conditions where there are no known risks for either APAP use or short-term SAA insufficiency. The specific hypotheses were that therapeutic doses of APAP would result in decreased plasma Cys and GSH and more oxidized E_hCySS and E_hGSSG under conditions of adequate and insufficient SAA intake. As a control, the effect of 2 days of SAA-insufficient diet on

plasma pools was also examined. SAA-insufficient diet and APAP treatment each caused decreases in plasma Cys and an oxidation of E_hCySS. Unexpectedly, APAP did not cause a decrease in Cys or an oxidation of E_hCySS under SAA-insufficient conditions.

3.3 MATERIALS, AND METHODS

Materials. Bathophenanthroline disulfonate sodium salt (BPDS), sodium heparin, sodium iodoacetate, dansyl chloride, L-serine, GSH, GSSG, Cys, CySS, and sodium acetate trihydrate were obtained from Sigma Chemical Corp (St. Louis, MO). γ-Glutamylglutamate (γ-Glu-Glu) was obtained from MP Biomedicals Corp. (Irvine, CA). Boric acid, sodium tetraborate, potassium tetraborate, perchloric acid, and acetic acid were reagent grade and purchased locally. Methanol, acetone, and chloroform were HPLC grade.

Human Subjects. This study was reviewed and approved by the Emory Investigational Review Board (501-2004). A total of 12 volunteers, self-described as healthy, were recruited by posting flyers in public locations in the Atlanta/Emory University community. Following written informed consent, volunteers were admitted to the outpatient unit of the Emory University Hospital General Clinical Research Center (GCRC), and screened by a physician (TRZ) via medical history, physical examination, fasting standard blood chemistry, urinalysis, and hematology tests. Serum pregnancy tests were performed in females to exclude pregnant individuals. Resting energy expenditure (REE) was determined using indirect calorimetry. Eligibility was established by a body mass index (BMI) < 27, the absence of acute and/or chronic illness, no current

smoking history, and age (18-40). Subjects taking nutritional supplements (with the exception of once-daily multivitamin-mineral supplements), antioxidants, or acetaminophen were asked to discontinue use 2 weeks prior to beginning each study period. With the exception that all females in the study were taking birth control pills, subjects were not taking prescription medications.

Experimental Design. The experimental model was a 2 × 2 factorial design in which subjects were studied under 4 different conditions with APAP (0 or 15 mg/kg) and SAA intake (0% or 100% of RDA) (Table 3.1). Prior to the each inpatient study period, subjects were provided balanced meals of conventional food items containing the RDA for SAA prepared by the GCRC Bionutrition Unit for 3 days (breakfast, lunch, dinner, and snack). Subjects were admitted into the GCRC at 19:00 on Day 3, and a baseline blood sample was drawn at 08:15 on Day 4. On Day 4 and 5, subjects were given a chemically defined diet containing either 100% SAA or 0% SAA at 08:30, 12:30, 17:30, and 21:30 (snack), with a respective distribution of total calories as 30%, 30%, 30% and 10%. All meals and snacks were consumed over no longer than a 20-min period. On Day 6 of each study period, plasma samples were collected hourly for 12 h following administration of APAP (15 mg/kg) or placebo at 08:15 and 14:15. On Day 6, no morning meal was given to avoid interference with the absorptive phase of acetaminophen. Subsequently, meals were given at 12:30 and 17:30. Study subjects served as their own controls to decrease intra-individual variation. The 4 study periods described in Table 3.1 were randomized for order and conducted at least one week apart.

Diet and nutrient intake. The protein equivalents of all diets were supplied in the form of specific L-amino acid mixtures (Ajinomoto USA, Teaneck, NJ), providing 1.0

g/kg per day as outlined in detail (64, 87). The standard mixture was patterned after hen's egg protein and provided all 9 indispensable (essential) amino acids, including Met, in amounts sufficient for the mean requirements of healthy young adults (64, 87), but which are higher than the requirements proposed by the World Health Organization (24). The standard amino acid mixture also contained 8 dispensable (non-essential) amino acids, including Cys and glutamate, and was glutamine- and taurine-free. To compensate for the difference in Met + Cys between the 0% and 100% SAA diets, the amount of all non-essential amino acids were proportionally changed to maintain a constant dietary nitrogen content. The dietary energy was mainly derived from lipid and carbohydrate sources provided in the form of protein-free wheat starch and butter/safflower oil cookies and a sherbet-based drink, as outlined previously (64, 87). The Cys was added to the 100% SAA sherbet-like drink immediately prior to consumption to minimize Cys oxidation to CySS. All subjects were highly compliant with research meals as verified by the GCRC Bionutrition Unit staff.

Adequate hydration and vitamin, mineral and electrolyte requirements were provided to meet or exceed recommended allowances (87). Water intake was ad libitum. All supplements were administered on a regular schedule by the GCRC research nurses. Body weight was determined daily and vital signs were obtained every 8 h. Low-level activity was allowed and restricted to walking on the GCRC.

Sampling and redox analysis. Baseline blood samples were drawn at 08:15 on Days 4-6 of each study period. In addition to the baseline blood draw at 08:15, samples were collected hourly through 20:15 on Day 6 of each study period (Table 4.1). On Day 6, a heparin-lock catheter was placed in a forearm vein for blood sampling. Blood (1.5

mL) was collected and immediately transferred to a microcentrifuge tube containing 0.15 ml of a preservative solution consisting of 0.5 mol L-serine/L, 9.3 mmol BPDS/L, 0.165 mol γ-glutamylglutamate, 0.4 mol boric acid, 0.1 mol sodium borate, 0.144 mol sodium iodoacetate, and 2.5 mg sodium heparin/mL (55). Following centrifugation to pellet cells, supernatant (200 µL) was transferred to microcentrifuge tubes containing 10% ice-cold perchloric acid and 0.2 M boric acid solution. All samples were stored at -80°C until derivatization with dansyl chloride (55). High-performance liquid chromatography (HPLC) with fluorescence detection was used to quantify dansyl derivatives of Cys, CySS, GSH, and GSSG. These concentrations were used with the Nernst equation to calculate E_h of each redox couple as described (16, 52, 60).

Statistics. Power analysis based on previous studies (24, 48, 58, 64, 87) indicated that 9 subjects would provide >96% power to detect a relevant SAA-free diet- or APAP-induced 4 mV difference in E_hGSSG and >80% to detect a difference of 4 mV in E_hCySS. The study was designed to determine effects of SAA and APAP independently. Not all subjects completed all study periods, so paired t-tests with Bonferroni correction were used for appropriate comparisons with the number of subjects for each comparison given in the figure legends. We used SPSS software (version 17; SPSS Inc, Chicago, IL) for all analyses. Area under the plasma concentration time curve (AUC) values were calculated using the trapezoidal rule. Results were considered significant at $p \leq 0.05$.

3.4 RESULTS

Subject characteristics

Demographic characteristics of the 12 subjects are summarized in Table 3.2. The study population was 50% male and the mean age (± SD) was 25 ± 4 y for women and 25 ± 5 y for men. Half of the subjects (n = 6) were African American, 4 were White, 1 was Hispanic, and 1 was Native American. All subjects were healthy, having no acute or chronic illness none were taking regular prescription medication related to illness. There was no significant difference in ethnicity, age or BMI between male and female subjects.

Effect of SAA insufficiency and APAP administration on plasma Cys and CySS concentrations. The details of effects of SAA insufficiency and APAP administration are shown for Cys in Fig 3.1, with corresponding AUC data in Fig 3.2. The overall conclusion from these complex data is that SAA insufficiency alone results in decreased plasma Cys but that APAP has no effect alone and does not exacerbate the effect of SAA insufficiency. While significant effects were observed for a few time points with APAP, AUC data (3.2A) showed that APAP administration had no signficant effect on plasma Cys concentration in the SAA replete state (Fig 3.1D).

The similarly extensive dataset for CySS is shown in Fig 3.3. Despite significant effects at a few time points, AUC data (Fig 3.2B) show that an SAA insufficient diet has, at most, a small effect on plasma CySS. However, a significant increase in plasma CySS concentration was observed with APAP in the SAA insufficient state (Fig 3.3C; Fig 3.2B). APAP administration did not change plasma CySS concentration in the SAA replete state (Fig 3.3D; Fig 3.2B).

Together, the data show that there are effects of SAA insufficiency and APAP on plasma Cys and CySS concentrations, but these effects are not common to all conditions and are not extensive. Most obvious from the complex data, SAA insufficiency decreases Cys without further effect due to APAP, and APAP increases CySS under the condition of SAA insufficiency.

Effect of SAA insufficiency and APAP administration on plasma E_hCySS. Although Cys and CySS concentrations in plasma appear to be determined by transport systems, cell culture studies indicate that mechanisms also exist to regulate the balance of the extracellular concentrations to maintain E_hCySS at approximately -80 mV (33, 47, 49). Calculated values for plasma E_hCySS are shown in Fig 3.4 with corresponding AUC data in Fig 3.2. Two important observations are apparent from these data: 1) SAA insufficiency alone caused a more oxidized E_hCySS compared to the SAA adequate diet (Fig 3.4A: Fig 3.2C); and 2) APAP administration alone caused a more oxidized plasma E_hCySS value compared to placebo (Fig 3.4D; Fig 3.2C). Unexpectedly, APAP administration did not cause oxidation of the plasma Cys/CySS pool under SAA insufficient conditions. Note that the experimental design was to address the effects of SAA and APAP independently and was not powered to address interaction effects.

Effect of SAA insufficiency and APAP administration on plasma GSH and GSSG concentration. The effects of dietary SAA and APAP on GSH concentration are shown in Fig 3.5, with corresponding AUC data in Fig 3.6. The two important features which emerge from these data are that plasma GSH was lower with SAA insufficient diet only in the presence of APAP, and that APAP resulted in lower plasma GSH only with SAA insufficient diet. Measurements of GSSG (Fig 3.7) showed a few time points were

significantly different; however, there were no overall effects on GSSG concentrations apparent from the AUC data (Fig 3.6).

Effect of SAA insufficiency and APAP administration on plasma E_hGSSG. The data for E_hGSSG are shown in Fig 3.8 with corresponding AUC data in Fig 3.6. A few individual comparisons were significantly different (Fig 3.8A-3.8D), but the only overall effect apparent from the AUC data was an oxidation of E_hGSSG by the SAA insufficient diet in the presence of APAP (Fig 3.6C). Thus, in contrast to the data for E_hCySS where no interaction between SAA insufficiency and APAP was apparent, the data for E_hGSSG show effects only for SAA insufficiency in the presence of APAP.

3.5 DISCUSSION

Previous research in rodents showed that consumption of a diet insufficient in SAA and/or prolonged ingestion of APAP causes a decrease in tissue Cys and GSH concentrations (86, 88). Plasma GSH is largely derived from hepatic release in a process thought to maintain supply of Cys throughout the body (75). However, the effects of altered SAA intake and therapeutic doses of APAP on extracellular redox potential have not previously been studied in humans. The present data show that mild SAA insufficiency significantly decreases plasma Cys concentration and caused more oxidized plasma E_h CySS value in humans. Under SAA insufficient conditions, therapeutic doses of APAP significantly increased plasma CySS concentration and decreased plasma GSH concentration. However, despite the changes due to SAA insufficiency, therapeutic doses of APAP did not further oxidize plasma E_h CySS or E_h GSSG.

The observed decrease in plasma GSH due to APAP suggests that APAP decreases hepatic GSH available for release into plasma. In a study of SAA insufficiency in human colon carcinoma (HT29) cells, Miller et al showed that severe Cys deficiency decreaseed intracellular GSH concentration, oxidized E_h GSSG values, and inhibited cell proliferation (68). Addition of Cys resulted in a nearly complete recovery of intracellular GSH concentration and produced a rapid recovery from the oxidized conditions associated with Cys deficiency (68). This earlier study provided a link between limited SAA intake and oxidation of intracellular E_h GSSG *in vitro*. Nkabyo et al provided evidence that redox changes occur *in vivo* in response to SAA insufficiency in both the cellular and the extracellular redox compartments (81). Results showed that in adult rats, seven days of dietary SAA insufficiency caused a decrease in tissue and plasma GSH concentrations, an increase in plasma GSSG, and oxidation of the plasma and intracellular E_h GSSG values. In addition, dietary SAA insufficiency caused a marked decrease in plasma Cys and CySS concentrations, leading to an oxidation of plasma E_h CySS values (81). Dietary supplementation of SAA resulted in an increase in plasma Cys concentration and more reduced plasma E_h CySS values (81). These studies in rats, coupled with the present data in humans, show that the level of dietary SAA intake can markedly alter the redox status of plasma Cys/CySS and intracellular GSH/GSSG. The present study adds to this knowledge by showing that acute therapeutic doses of APAP do not increase the extent of change due to SAA insufficiency in humans (Fig 3.4C; Fig 3.8C).

APAP metabolism is critically dependent upon SAA availability, and with toxic doses, the extent of toxicity is exacerbated by SAA limitation (86). APAP toxicity is

initiated by the metabolism of APAP to N-acetyl-p-benzoquinone imine (NAPQI) (25, 43). NAPQI depletes hepatic GSH and subsequently binds to cellular proteins to induce toxicity and oxidative stress (42, 70). Price and Jollow investigated whether limitation of SAA consumption would potentiate APAP hepatotoxicity in rats (86). The results showed that consumption of an SAA deficient diet and acute APAP administration decreased hepatic GSH concentrations and increased the incidence and severity of APAP-induced toxicity (86). The present data show that a therapeutic, non-toxic dose of APAP significantly decreases plasma GSH concentrations in humans consuming a SAA deficient diet (Fig 3.5B). These results are consistent with the rodent studies and further show that effects on the GSH system are apparent in humans even with normal therapeutic doses.

Effects on plasma Cys and GSH due to normal therapeutic doses of APAP are somewhat unexpected given the large pool of GSH in the liver. Reference values for hepatic GSH content are 3 to 10 mM, or 1.5 to 4.9 g GSH for a 1.6 kg liver. The respective content of Cys within GSH is 0.58 to 1.9 g. Assuming that all dietary SAA intake is available for conversion to Cys, the deficit due to 2 days without dietary SAA is twice the RDA, or 3.8 g Cys equivalents. Consequently, the potential deficit due to 2 days without SAA (3.8 g) is large relative to the Cys content in hepatic GSH (0.58 to 1.9 g). However, this comparison does not include the total body GSH, which is available due to interorgan GSH/cysteine cycling. Assuming 1 mM GSH in 50 L body water, the body contains about 15 g GSH, which contains 5.8 g Cys. In the present study, approximately 0.6 g of SAA would be needed to supply GSH and sulfate for conjugation

of 2 g of APAP. Consequently, the doses administered consume the equivalent of about 10% of the Cys contained in the total body pool of GSH.

Recent studies show that more oxidized values for plasma E_h CySS and E_h GSSG are associated with aging and risk factors for cardiovascular disease (35). In particular, an oxidation of 10 mV in plasma E_hCySS is associated with persistent atrial fibrillation (78) and increased pro-inflammatory signaling (40). Protein domains of receptors, transporters, and adhesion molecules on the surface of cells are exposed to the extracellular Cys/CySS pool, and a 10 mV oxidation is sufficient to cause a 2-fold change in the reduced:oxidized ratio of dithiol-disulfide couples. The present results show that APAP can cause a 10-mV oxidation of E_h CySS (Fig 3.4D), suggesting that APAP could affect redox signaling mediated via E_hCySS. No relevant in vivo data are available, but in vitro data show that an oxidized extracellular E_hCySS in cell culture activates proinflammatory signaling (Go and Jones 2005; Go et al FRBM 2009), profibrotic signaling (Ramirez et al 2007), stress signaling (Go et al, Tox Sci 2009), and apoptotic signaling (Jiang et al 2005). Although speculative, the present data suggest a number of unanticipated effects of therapeutic use of APAP could occur as a consequence of effects on plasma E_hCySS.

Under SAA insufficient conditions, APAP does not oxidize either Cys/CySS or GSH/GSSG (Fig 3.4C). This observation is potentially relevant to situations such as the anorexia associated with illness in which patients may consume diets with inadequate or no protein (and thus insufficient SAA) for several days. The results suggest that use of APAP under these conditions may not cause further oxidative changes, as measured in plasma. A limitation of the current study was the short duration of SAA depletion and

the limitation to only two doses of APAP in otherwise healthy adult subjects. Thus, studies of the effects of longer periods of APAP use, particularly in patients with illness consuming inadequate oral diets would be of interest, as would studies in children, in which APAP is commonly used during viral and other febrile illnesses. In contrast to the effects on the Cys couple, we found that APAP significantly decreased plasma GSH during the SAA insufficient period in our subjects (Fig 3.5C). Because the liver is a major source of plasma GSH, studies that evaluate the GSH response to APAP in patients with impaired hepatic function or with longer periods of SAA deficiency would also be of interest.

In conclusion, the present research shows that therapeutic doses of APAP result in a more oxidized plasma Cys/CySS pool. These changes occur in humans irrespective of short-term changes in SAA intake. SAA insufficiency or APAP administration decreased plasma GSH concentration but had no effect on E_h GSSG.

ACKNOWLEDGEMENTS

The authors thank the volunteers for their commitment and are grateful for the assistance of the nursing, bionutrition, and laboratory staff at the Emory University Hospital GCRC and the pharmacists and staff at the Research Pharmacy at Emory University Hospital.

FOOTNOTES

This work was supported by the National Institutes of Health National Institute of Environmental Health Sciences ES012929 (DPJ), ES009047 (DPJ) and National Center for Research Resources K24 RR023356 (TRZ), M01 RR00039/UL1 RR025008 (Emory University Hospital GCRC), and the Atlanta Clinical and Translational Science Institute TL1 RR025010 (YOM).

Presented in part at the Annual Meeting of the Federation of American Societies for Experimental Biology, Washington, D.C., May 2007. Mannery YO, Ziegler TR, and Jones DP (2007). A chemically defined diet with insufficient sulfur amino acids induces oxidation of plasma cysteine/cystine and glutathione/glutathione disulfide redox state in humans. *The FASEB Journal* 21(5): A697.

TABLE 3.1. Experimental Design

Study Period	Days 1-3, Outpatient Equilibration	Days 4-5, Inpatient ± SAA	Day 6, Inpatient ± APAP
1	3-days w/ 100% RDA for SAA	2-days in GCRC w/ 100% RDA for SAA	No APAP
2	3-days w/ 100% RDA for SAA	2-days in GCRC w/ 100% RDA for SAA	2-sequential doses of APAP
3	3-days w/ 100% RDA for SAA	2-days in GCRC w/ SAA-free diet	No APAP
4	3-days w/ 100% RDA for SAA	2-days in GCRC w/ SAA-free diet	2-sequential doses of APAP
Blood Sampling:		↑ ↑	↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑

[a.] Study periods described above were randomized for order and conducted at least one week apart

TABLE 3.2. Baseline characteristics of the total study population (n = 12)

	Males	**Females**	**p-value**
# of Subjects (%)	6 (50)	6 (50)	
Age	25 ± 5.2	25 ± 4.4	0.95
% Caucasian	33%	33%	
BMI	24.6 ± 0.78	23.8 ± 1.8	0.28

FIGURE 3.1

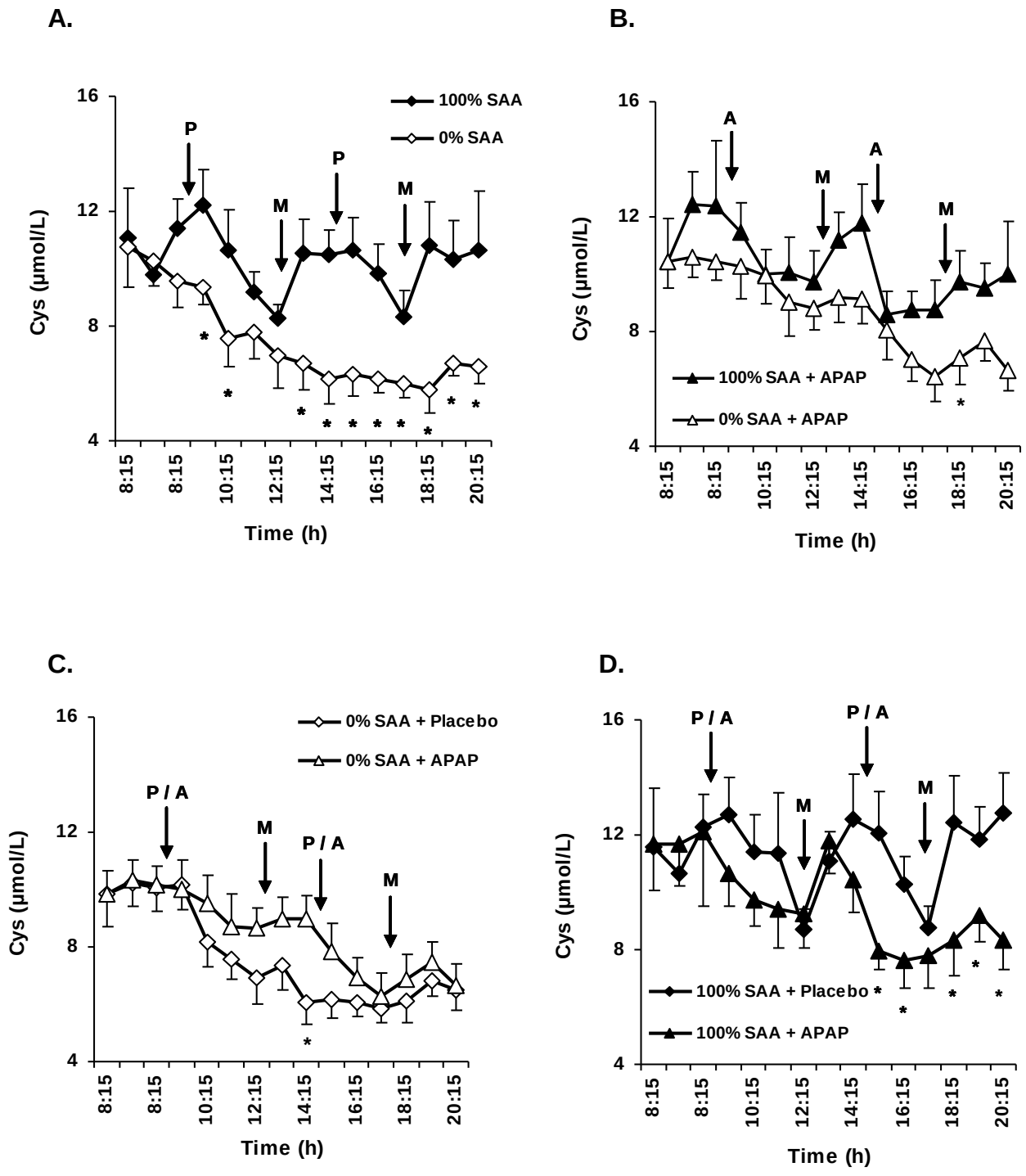

A.
100% SAA
0% SAA
P
M
P
M
Cys (µmol/L)
16
12
8
4
8:15
8:15
10:15
12:15
14:15
16:15
18:15
20:15
Time (h)
B.
A
M
A
M
100% SAA + APAP
0% SAA + APAP
Cys (µmol/L)
Time (h)
C.
0% SAA + Placebo
0% SAA + APAP
P / A
M
P / A
M
Cys (µmol/L)
Time (h)
D.
P / A
P / A
M
M
100% SAA + Placebo
100% SAA + APAP
Cys (µmol/L)
Time (h)

Figure 3.1. Effect of sulfur amino acid (SAA) insufficiency and acetaminophen (APAP) administration on plasma cysteine (Cys) concentration in healthy persons aged 18-40 y. The experiment was conducted with a 2 × 2 design to evaluate independent effects of SAA and APAP and determine whether there was an interactive effect. In each panel, the times of administration of placebo (P), APAP (A) and meals (M), are indicated by arrows. Data are expressed as means ± SE for all subjects at each respective time point. For paired statistical analyses, data were used only for appropriately pair as follows: **A**, 100% SAA versus 0% SAA (n = 8); **B,** 100% SAA + APAP versus 0% SAA + APAP (n = 8); **C,** 0% SAA + placebo versus 0% SAA + APAP (n = 9); and **D,** 100% SAA + placebo versus 100% SAA + APAP (n = 10). *Asterisk represents significance at $p < 0.05$.

FIGURE 3.2

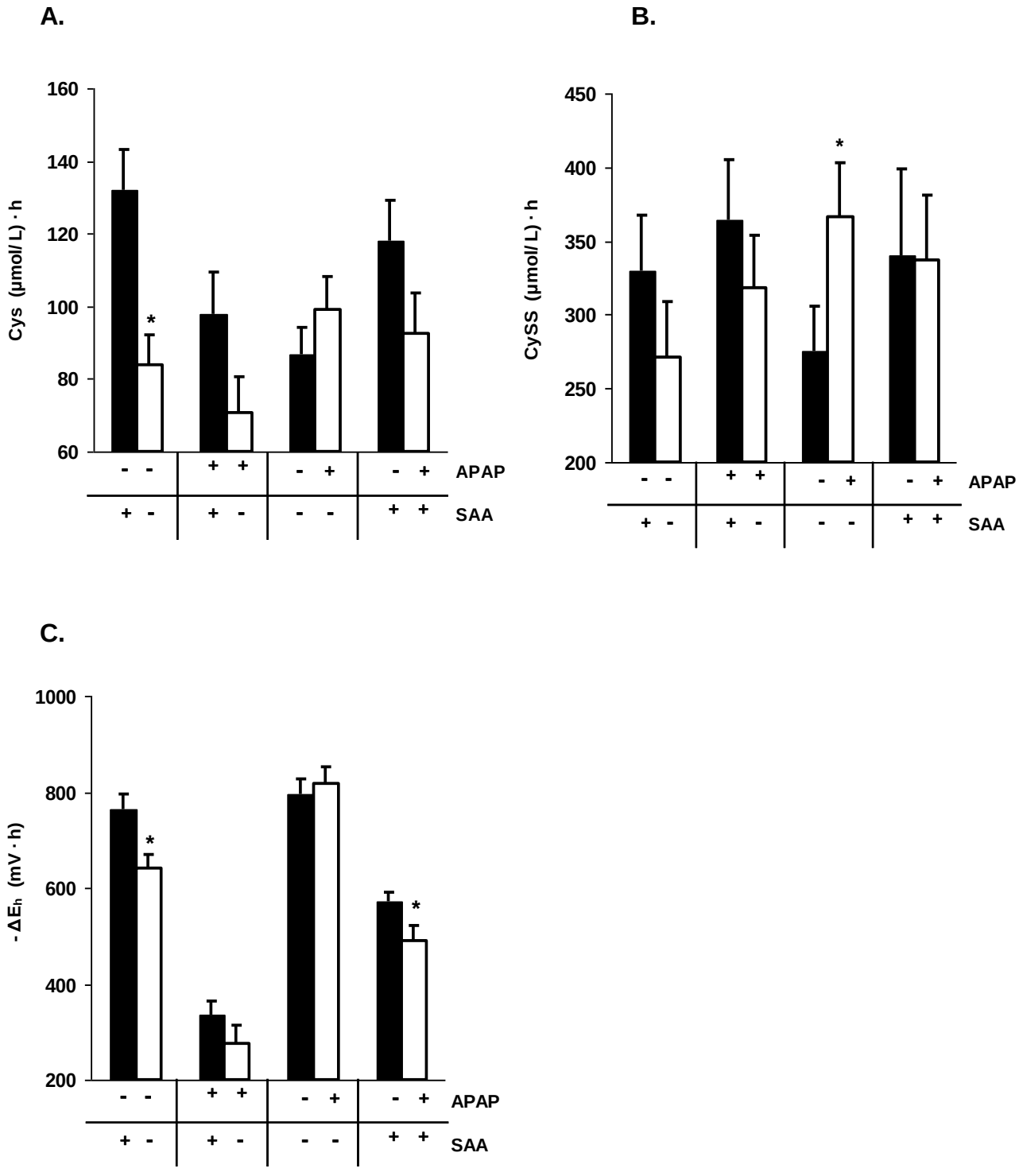


Figure 3.2. Area under the curve (AUC) for plasma cysteine (Cys) and cystine (CySS) concentrations and cysteine (Cys)/ cystine (CySS) redox potential (E_h CySS) in a 2 × 2 design to study effects of SAA insufficiency and therapeutic doses of acetaminophen (APAP). Area under the curve (AUC) values were calculated using the trapezoidal rule for the individual time courses used to create the summary data for A, Cys (Fig 1); B, CySS (Fig 3) and C, E_hCySS (Fig 4). Data are expressed as means ± SE for paired statistical analyses as follows: 100% SAA versus 0% SAA, n = 8; 100% SAA + APAP versus 0% SAA + APAP, n = 8; 0% SAA + placebo versus 0% SAA + APAP, n = 9; 100% SAA + placebo versus 100% SAA + APAP, n = 10. *Asterisk represents significance at $p < 0.05$.

FIGURE 3.3

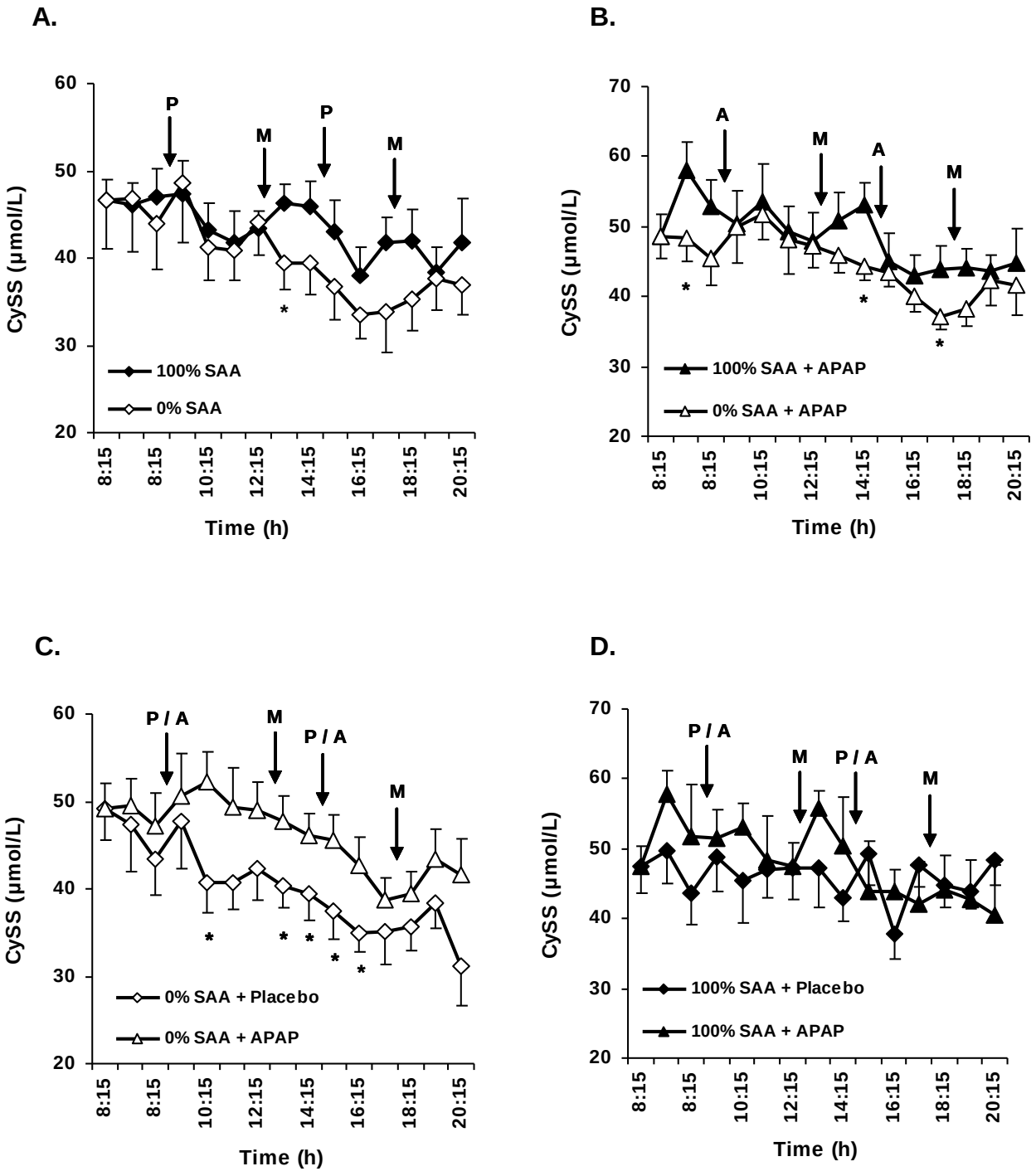

A.
B.
C.
D.
CySS (µmol/L)
Time (h)
P
M
A
P / A
100% SAA
0% SAA
100% SAA + APAP
0% SAA + APAP
0% SAA + Placebo
100% SAA + Placebo
8:15
10:15
12:15
14:15
16:15
18:15
20:15

Figure 3.3. Effect of sulfur amino acid deficiency and acetaminophen administration on plasma cystine (CySS) concentration in healthy persons aged 18-40 y. See legend to Fig 1 for details. *Asterisk represents significance at $p < 0.05$.

FIGURE 3.4

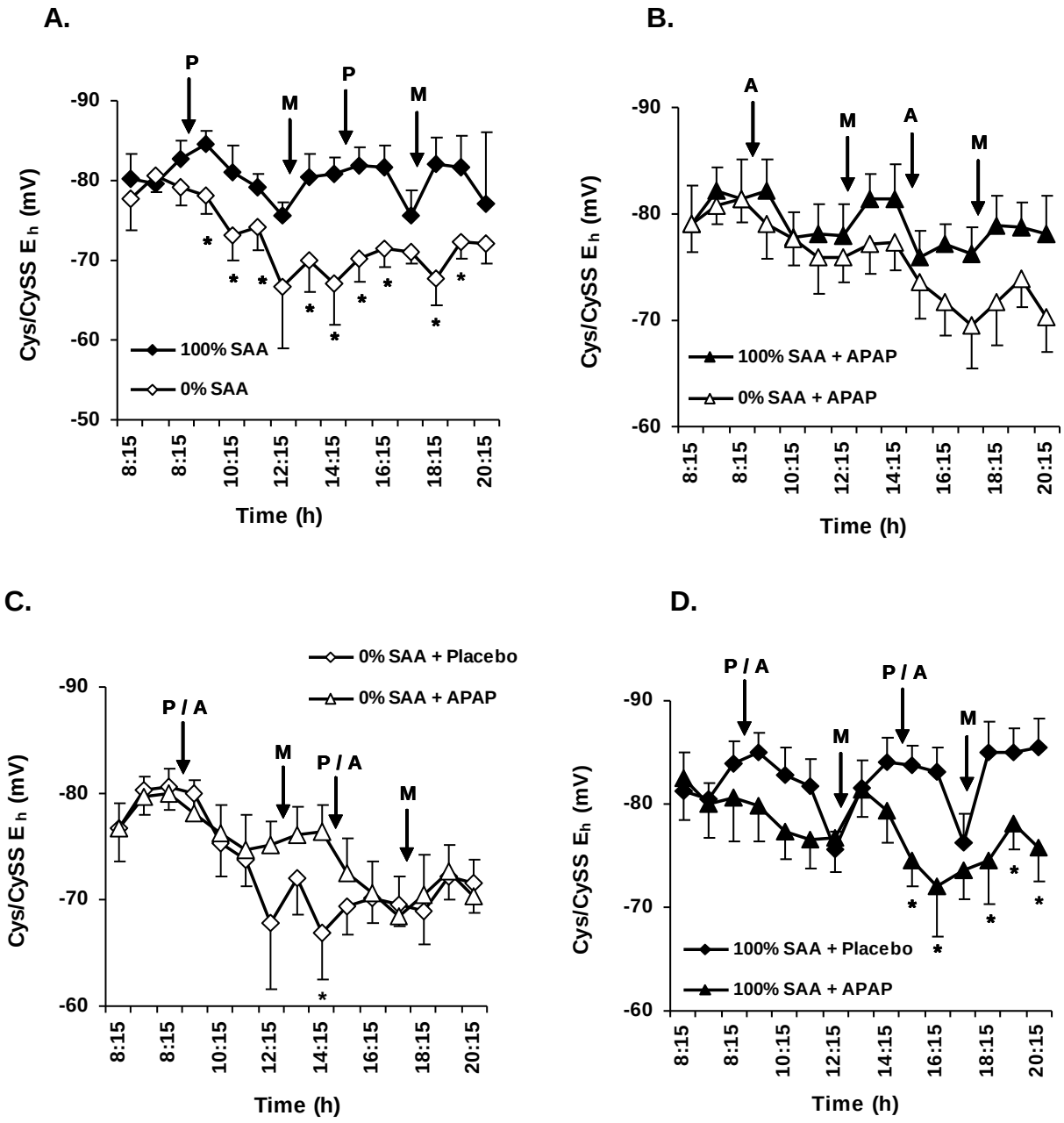

A.
P
M
P
M
-90
-80
-70
-60
-50
Cys/CySS E h (mV)
100% SAA
0% SAA
8:15
8:15
10:15
12:15
14:15
16:15
18:15
20:15
Time (h)
B.
A
M
A
M
-90
-80
-70
-60
Cys/CySS E h (mV)
100% SAA + APAP
0% SAA + APAP
8:15
8:15
10:15
12:15
14:15
16:15
18:15
20:15
Time (h)
C.
0% SAA + Placebo
0% SAA + APAP
P / A
M
P / A
M
-90
-80
-70
-60
Cys/CySS E h (mV)
8:15
8:15
10:15
12:15
14:15
16:15
18:15
20:15
Time (h)
D.
P / A
P / A
M
M
-90
-80
-70
-60
Cys/CySS Eh (mV)
100% SAA + Placebo
100% SAA + APAP
8:15
8:15
10:15
12:15
14:15
16:15
18:15
20:15
Time (h)

Figure 3.4. Effect of sulfur amino acid deficiency and acetaminophen administration on plasma cysteine (Cys)/ cystine (CySS) redox potential (E_h CySS) in healthy persons aged 18-40 y. Plasma E_h CySS was calculated using the Cys and CySS concentrations measured by HPLC and the Nernst equation. See legend to Fig 1 for details. *Asterisk represents significance at $p < 0.05$.

FIGURE 3.5

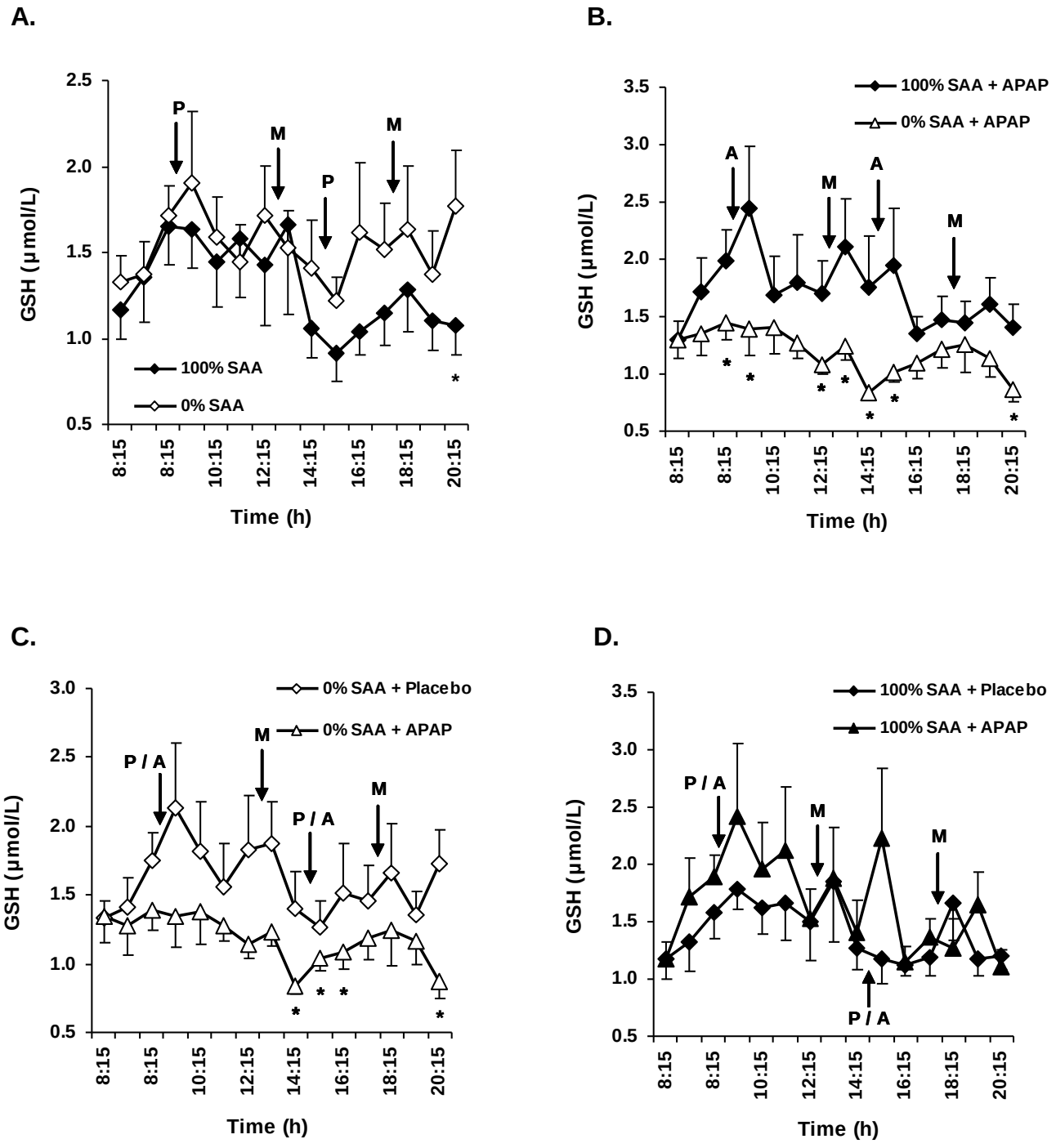

A.
GSH (µmol/L)
2.5
2.0
1.5
1.0
0.5
P
M
P
M
100% SAA
0% SAA
*
8:15
8:15
10:15
12:15
14:15
16:15
18:15
20:15
Time (h)
B.
3.5
3.0
2.5
2.0
1.5
1.0
0.5
100% SAA + APAP
0% SAA + APAP
A
M
A
M
*
Time (h)
C.
3.0
2.5
2.0
1.5
1.0
0.5
0% SAA + Placebo
0% SAA + APAP
M
P / A
P / A
M
*
Time (h)
D.
3.5
3.0
2.5
2.0
1.5
1.0
0.5
100% SAA + Placebo
100% SAA + APAP
P / A
M
M
P / A
Time (h)

Figure 3.5. Effect of sulfur amino acid deficiency and acetaminophen administration on plasma glutathione (GSH) concentration in healthy persons aged 18-40 y. See legend to Fig 1 for details. *Asterisk represents significance at $p < 0.05$.

FIGURE 3.6

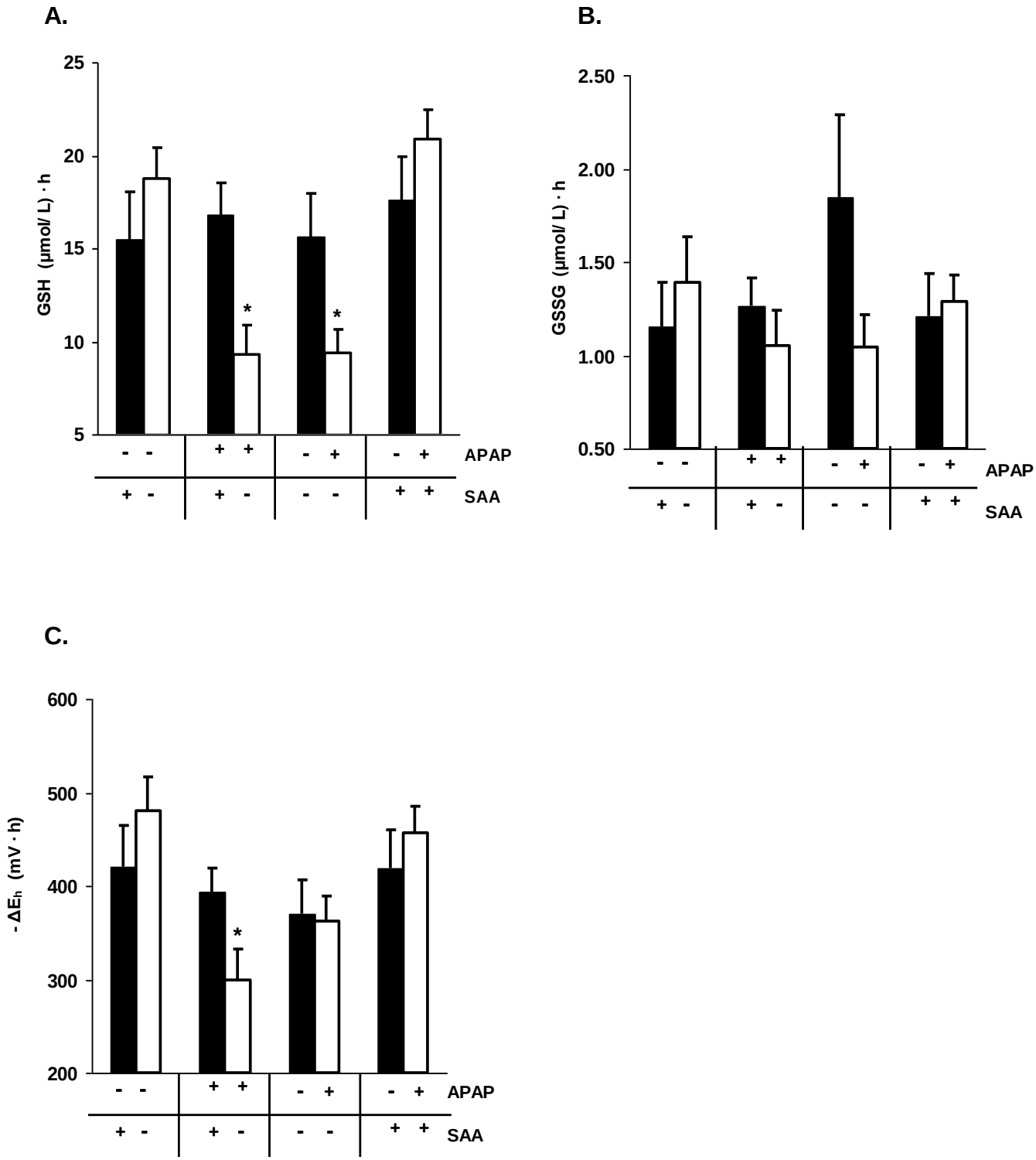

A.
GSH (µmol/ L) · h
25
20
15
10
5
*
*
- - + + - + - + APAP
+ - + - - - + + SAA
B.
GSSG (µmol/ L) · h
2.50
2.00
1.50
1.00
0.50
- - + + - + - + APAP
+ - + - - - + + SAA
C.
- ΔEh (mV · h)
600
500
400
300
200
*
- - + + - + - + APAP
+ - + - - - + + SAA

Figure 3.6. Area under the curve for plasma glutathione (GSH) and glutathione disulfide (GSSG) concentrations and glutathione (GSH)/ glutathione disulfide (GSSG) redox potential (E_h GSSG) in a 2 × 2 design to study effects of SAA insufficiency and therapeutic doses of acetaminophen (APAP). Area under the curve (AUC) values were calculated using the trapezoidal rule for the individual time courses used to create the summary data for A, GSH (Fig 5); B, GSSG (Fig 7) and C, E_hGSSG (Fig 8). See Fig 2 for additional details.

FIGURE 3.7

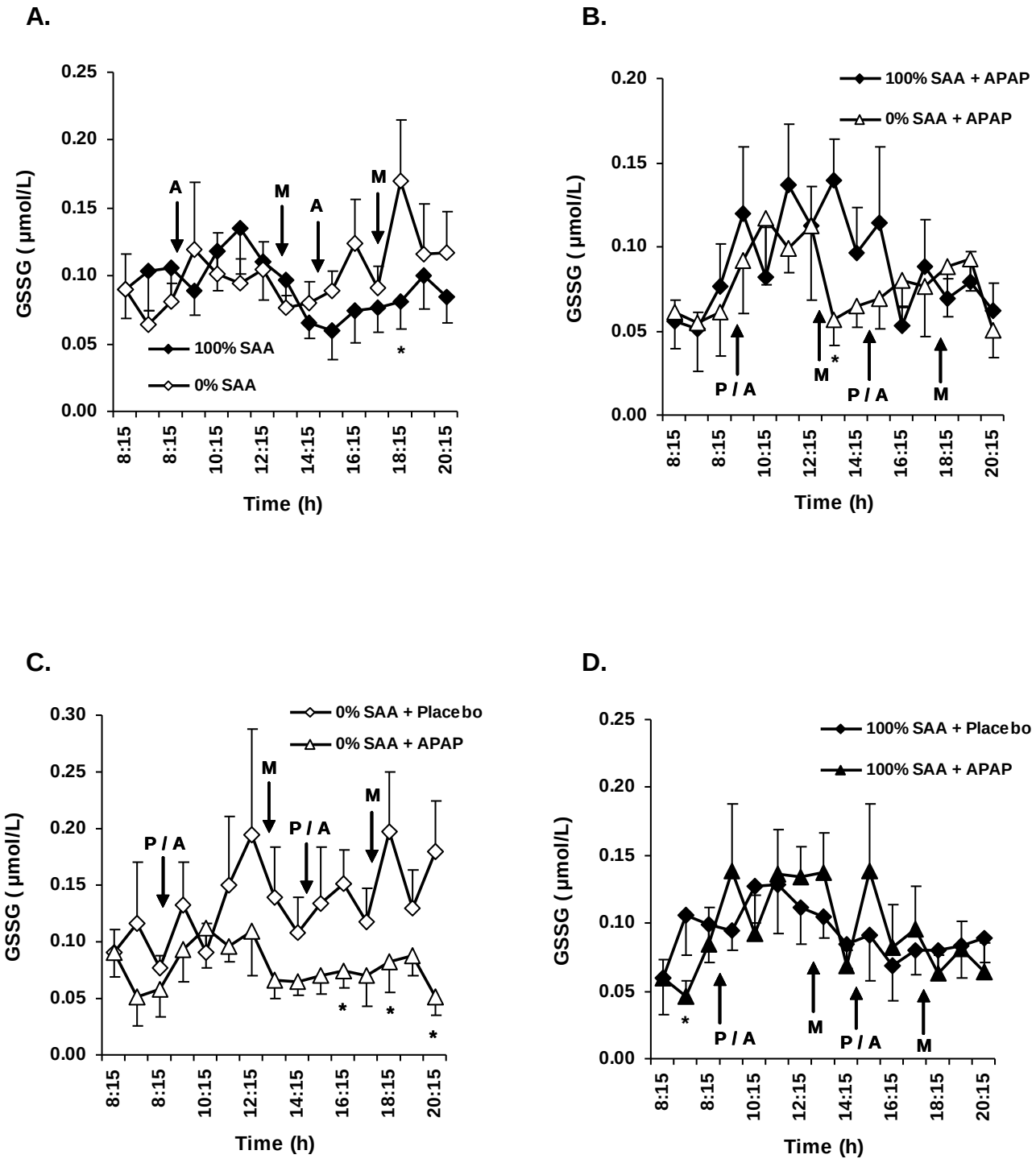

A.
GSSG (µmol/L)
0.25
0.20
0.15
0.10
0.05
0.00
A
M
A
M
*
100% SAA
0% SAA
8:15
8:15
10:15
12:15
14:15
16:15
18:15
20:15
Time (h)
B.
100% SAA + APAP
0% SAA + APAP
P / A
M
*
P / A
M
C.
0% SAA + Placebo
0% SAA + APAP
0.30
P / A
M
P / A
M
*
*
*
D.
100% SAA + Placebo
100% SAA + APAP
*
P / A
M
P / A
M

Figure 3.7. Effect of sulfur amino acid deficiency and acetaminophen administration on plasma glutathione disulfide (GSSG) concentration in healthy persons aged 18-40 y. See legend to Fig 1 for details. *Asterisk represents significance at $p < 0.05$.

FIGURE 3.8

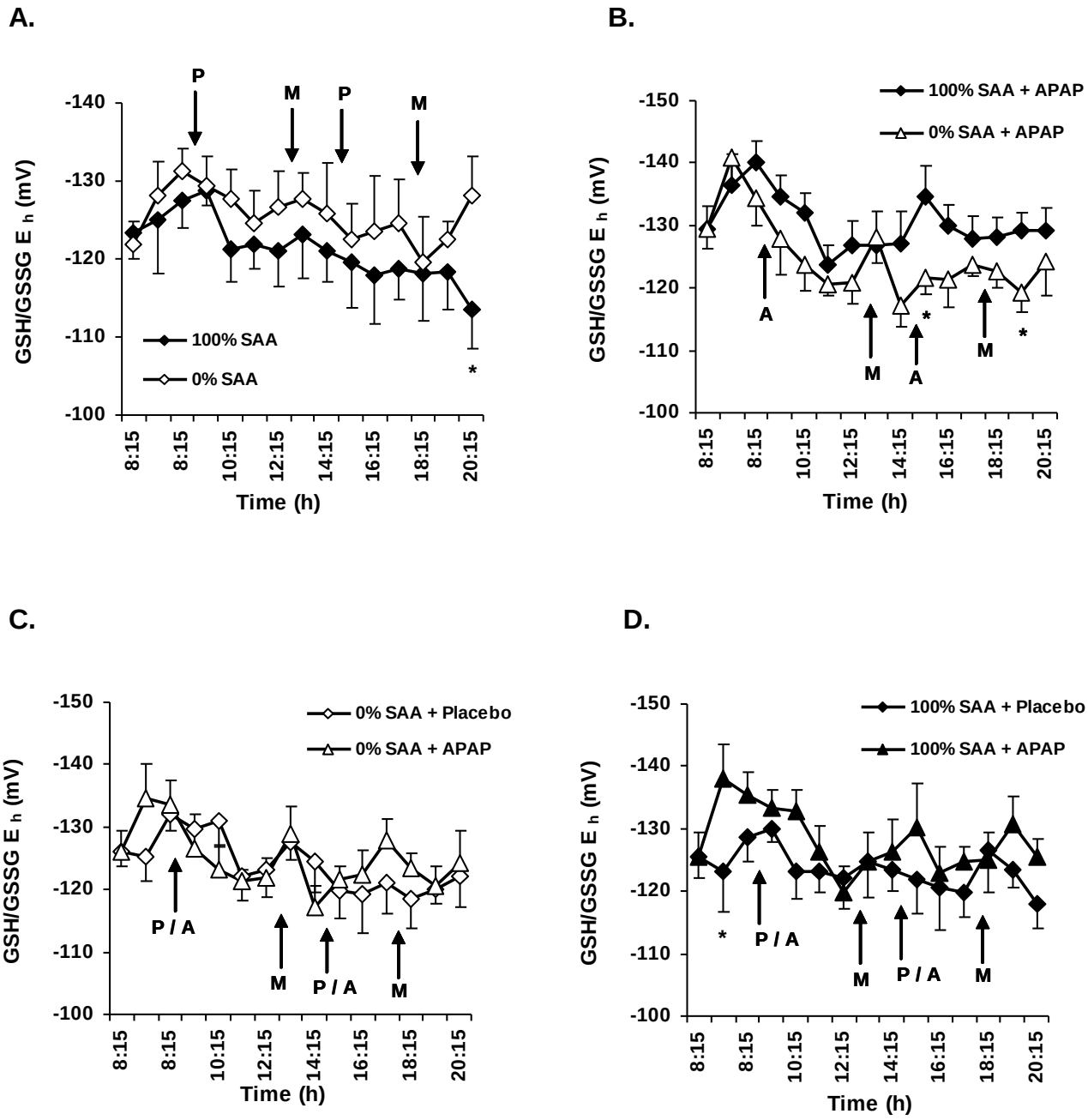

A.
B.
C.
D.
GSH/GSSG E h (mV)
Time (h)
8:15
10:15
12:15
14:15
16:15
18:15
20:15
-100
-110
-120
-130
-140
-150
P
M
P
M
100% SAA
0% SAA
*
100% SAA + APAP
0% SAA + APAP
A
M
A
*
M
*
0% SAA + Placebo
0% SAA + APAP
P / A
M
P / A
M
100% SAA + Placebo
100% SAA + APAP
*
P / A
M
P / A
M

Figure 3.8. Effect of sulfur amino acid deficiency and acetaminophen administration on plasma GSH/GSSG redox potential (E_hGSSG). Plasma E_h GSSG was calculated using the GSH and GSSG concentrations measured by HPLC and the Nernst equation. See legend to Fig 1 for additional details. *Asterisk represents significance at $p < 0.05$.

Chapter 4: Acetaminophen Elimination Half-life in Humans is Unaffected by Short-Term Consumption of Sulfur Amino Acid-Free Diet

Yanci O. Mannery, Thomas R. Ziegler, Youngja Park, Dean P. Jones

4.1 ABSTRACT

Sulfation and glutathione (GSH) conjugation are important pathways for elimination of acetaminophen (APAP). Previous studies in rodents show that limitation of dietary sulfur amino acids (SAA) reduces biosynthesis of 3´-phosphoadenosine-5´-phosphosulfate (PAPS), the precursor for sulfation, and GSH, the precursor for the mercapturatic acid pathway. The amount of SAA needed for the metabolism of 2 doses of APAP is equivalent to 62% of the Recommended Dietary Allowance (RDA) for SAA in humans. A decrease in the activity of these metabolic pathways could lead to decreased elimination of the reactive metabolite of APAP and possibly affect risk of APAP use. To determine whether intake of a sulfur amino acid (SAA) deficient diet alters APAP metabolism, a pilot clinical study with a double-blind, crossover design was performed. Subjects received the RDA for SAA for 3 days for equilibration. Following admission to the clinical research unit, subjects were given a chemically-defined diet with 100% or 0% of the RDA for SAA for 2 days. On day three, 2 doses of APAP (15 mg/kg) or placebo were given successively with 6 h interval. Plasma samples were collected at baseline and hourly for 12 h, and two 6-h urine aliquots were collected during this time course. The data show that SAA limitation a) did not change the pattern of APAP metabolites in plasma or urine and b) did not alter APAP pharmacokinetics. Thus, the results show that 2 days of diet completely devoid of SAA has no effect on acetaminophen metabolism or disposition in healthy humans.

4.2 INTRODUCTION

Acetaminophen (APAP) is one of the most widely used antipyretic and analgesic drugs in the world. After therapeutic doses, about 2% of APAP is excreted in the urine unchanged (8). Approximately 90% of APAP is metabolized by way of conjugation reactions where 60% is metabolized through glucuronidation and 30% through sulfation (8). The remaining 5% to 8% is converted by human cytochrome P450 2E1 to its reactive intermediate, N-acetyl-p-benzoquinonimine (NAPQI) (19). In the presence of glutathione (GSH), this metabolite is rapidly detoxified and excreted in the urine as GSH, cysteine (Cys) and mercapturic acid conjugates (2, 71).

Sulfur-containing amino acids (SAA), methionine (Met) and Cys, are required for drug metabolism through the use of Cys for biosynthesis of sulfate and GSH (53, 66). Previous studies of SAA availability and APAP metabolism showed that prolonged dietary deficiency of Cys and Met significantly lengthened the half-life of a low dose of APAP in rodents (86). The markedly slower elimination of APAP in SAA-deficient animals was due primarily to a depression of sulfate conjugation. Short term dietary deficiency of Cys and sulfate slowed the elimination of APAP from the blood and significantly decreased the urinary excretion of the APAP-sulfate conjugate (36). Moreover, rodents with diet-induced SAA deficiency converted more APAP into its toxic metabolite, as suggested by increased excretion of APAP-Cys and APAP-mercapturate conjugates (36). Taken together, these studies suggest that nutritional deficiency of SAA can affect the individual pathways of APAP metabolism by impairment of sulfate conjugation. This impairment could potentially enhance the susceptibility to APAP-induced liver injury.

While the mean SAA intake in Americans is considerably greater than the Recommended Daily Allowance (RDA) of 1.9 g/day (29), individual SAA intake is variable and can range from < 0.3 g to > 5 g per day (28). Considering that a molar equivalent of about 0.2 g of Cys is used for metabolism of a maximal therapeutic dose of 1 g APAP; 4 doses per day would consume 0.8 g, or more than half of the RDA. Because of variation of intake in the population, the total equivalents of SAA needed for metabolism of the maximum daily dose of APAP can exceed individual intake of SAA.

The present study was designed to determine whether short term SAA insufficiency alters APAP metabolism in healthy individuals. The experiment was designed with 2 study periods in which each individual was equilibrated to either the RDA or 0% of the RDA for SAA prior to APAP administration in 2 successive doses of 15 mg/kg, 6 h apart. Young healthy individuals were studied under conditions where there are no known risks for either APAP use or short-term SAA insufficiency. The specific hypothesis was that 2 days of a SAA-free diet would cause changes in the metabolic pattern and pharmacokinetics of APAP in the plasma and urine.

4.3 MATERIALS, AND METHODS

Materials. Authentic standards of APAP metabolites (APAP-glucuronide, APAP-sulfate, APAP-Cys, APAP-GSH, and APAP-mercapturate) were a generous gift from Dr. José Manautou (University of Connecticut, Storrs, CT). Methanol and acetic acid were HPLC grade.

Human Subjects. This study was reviewed and approved by the Emory Investigational Review Board. The study was designed to determine the effect of 2 days

of SAA deficient diet on APAP metabolism as part of a study which evaluated effect of APAP on plasma cysteine and glutathione pools. The latter is described along with experimental details in an accompanying paper (Mannery et al, 2010). Briefly, 12 healthy volunteers (18-40 y old) with a body mass index (BMI) < 27, no acute and/or chronic illness, and no current smoking history were studied in under 2 different conditions with acetaminophen (2 doses; 15 mg/kg) and SAA intake (0% or 100% of RDA) (Table 4.1). Prior to the each inpatient study period, subjects were given an equilibration diet for 3 days, admitted into the Emory General Clinical Research Center and given a chemically modified diet containing either 100% SAA or 0% SAA for 2 days. During these 5 days, the subjects consumed no alcohol to avoid induction of CYP 2E1. On Day 6 of each study period, APAP (15 mg/kg) or placebo was administered under fasting conditions at 08:15. Subsequently, a meal was given at 12:30 and a second dose was administered at 14:30. Plasma samples were collected hourly for 12 hours following APAP or placebo administration, and two 6-h urine samples were collected during this time. Meals were prepared by the Bionutrition unit, and adequate hydration and vitamin, mineral and electrolyte requirements were provided to meet or exceed recommended allowances (87). Water intake was ad libitum. Body weight was determined daily and vital signs were obtained every 8 h.

APAP analysis. APAP and its metabolites in urine were analyzed using an HPLC method as previously described (15, 37). Aliquots of urine samples (20 µl) were injected into a Zorbax SB 5µM C_{18} reverse phase column (4.6 mm × 250 mm). APAP and its metabolites were eluted using a mobile phase composed of 12.5% HPLC-grade methanol, 1% acetic acid, and 86.5% water, run at flow rate of 1.2 ml/min. The elution of

metabolites was monitored at a wavelength of 254 nm. Retention times of APAP and its metabolites were determined by comparison with authentic standards. Quantification was based on integrated peak areas. Concentration of APAP and its metabolites was calculated using an APAP standard curve (37).

APAP and its metabolites in plasma were analyzed using the HPLC method of Moldeus (72). Perchloric acid (3N) was added 2:1 to plasma samples to precipitate protein. Following centrifugation and filtration, the supernatant was used for analysis. A linear gradient was used to separate APAP and its metabolites with a flow rate of 1.7 ml/min. Solvent A consisted of 1% aqueous acetic acid. Solvent B was composed of 1% aqueous acetic acid/methanol/ethyl acetate (90:15:1). The mobile phase was kept at 75% A and 25% B for 7 minutes. This was followed by a 20 minute linear gradient that finished at 99% B. The composition of the mobile phase was restored to initial conditions using an 8 minute linear gradient. All other HPLC conditions were the same as those described above.

Statistics. Results are expressed as means ± S.E.M. We used SPSS software (version 17; SPSS Inc, Chicago, IL) for all analyses. Area under the curve (AUC) values were calculated using the trapezoidal rule. Paired t-tests with the Bonferroni correction were used to determine if time points for respective groups were significantly different. Results were considered significant at $p < 0.05$.

4.4 RESULTS

Subject Characteristics.

The study population is described in an accompanying manuscript (Mannery et al, 2010). Fifty percent of the subjects were male, the mean age was 25 ± 4 y and there was no significant difference between males and females. Half of the subjects (n = 6) were African American, 4 were White, 1 was Hispanic, and 1 was Native American. All were healthy, with no history or evidence of acute or chronic illness and none were taking prescription medications. There were no adverse reactions to SAA insufficiency or APAP dose. Because of uncontrollable scheduling difficulties, only 8 of 9 subjects completed APAP study periods with both SAA sufficiency and insufficiency.

Plasma APAP metabolite concentration was not affected by SAA insufficiency. The effects of SAA limitation on plasma APAP concentration are shown in Figure 4.1A, with corresponding AUC data in Figure 4.2A. Measurements of the plasma concentration of APAP showed that the peak concentration occurred at 09:15 under SAA insufficient conditions (Fig 4.1A). The peak plasma appearance of APAP was delayed to 10:15 under SAA sufficient conditions. The pattern of APAP concentration in the plasma was similar under both dietary conditions (Fig 4.1A). Despite observing a significant effect at the 15:15 time point, AUC data show that an SAA insufficient diet had no overall effect on plasma APAP concentration (Fig 4.2A).

Limitation of SAA did not alter the pattern of APAP-glucuronide concentration in the plasma (Fig 4.1B). Despite observing a significant increase in APAP-glucuronide at the 19:15 time point, AUC data confirm that SAA limitation had no overall effect on plasma APAP-glucuronide concentration (Fig 4.2A). Similarly, SAA insufficiency did

not alter the pattern of appearance or concentration of plasma APAP–sulfate (Fig 4.1C; Fig 4.2B). The pattern of appearance or concentration of APAP-Cys, APAP-GSH, and APAP–mercapturate were not significantly altered by SAA limitation (Fig 4.1D; Fig 4.2B). However, it should be noted that there was a relatively large variation among individuals for the time course data, so this conclusion is largely based upon the AUC data (Fig 4.2B).

APAP pharmacokinetics were not affected by SAA insufficiency. To determine whether SAA limitation altered APAP pharmacokinetics, the volume of distribution (V_d), half-life ($t_{1/2}$), clearance (CL), and rate of elimination (k_{elim}) were calculated following the first dose of APAP. The results showed that the $t_{1/2}$ for APAP under conditions of SAA insufficiency was not significantly different from the $t_{1/2}$ under control conditions (Table 4.2). The V_d was also similar to control values, indicating that the total aqueous space available for APAP distribution was not affected by SAA insufficiency (Table 4.2). There was no significant effect of SAA limitation on CL or AUC for APAP, which indicates that SAA insufficiency had no effect on the elimination of APAP (Table 4.2).

Urinary excretion of APAP metabolites was not affected by SAA limitation. Excretion of APAP in the urine was not affected by SAA limitation (Fig 4.3A). While the concentration of APAP excreted into the urine was higher during the 14:00 to 20:00 period, this increase was not significant (Fig 4.3A). SAA insufficiency did not cause significant changes in the excretion of the glucuronide conjugate (Fig 4.3B) or the sulfate conjugate (Fig 4.3C) in the urine. SAA limitation appeared to cause an increase in the concentration of APAP-Cys, -GSH, and –mercapturic acid conjugates excreted in the urine during the 14:00 to 20:00 period; however, the difference was not significant

overall (Fig 4.3C). Expression of APAP and metabolites as a percentage of total urinary recovery also showed no effect of SAA insufficiency on APAP metabolism (Fig 4.4A; Fig 4.4B).

4.5 DISCUSSION

The present research was a component of a 2 × 2 factorial human study to determine whether APAP alters plasma Cys or GSH pools in healthy individuals with adequate or inadequate SAA intake (65). APAP decreased plasma Cys with both 0% and 100% SAA intake, having the greatest effect after the 2nd dose of APAP with 100% SAA. These results suggest that metabolism adapts to insufficient SAA intake by mobilizing body stores, i.e., by protein and/or GSH breakdown. Consistant with this, plasma GSH was decreased in the SAA-insufficient after the 2nd dose, indicating that the combination of SAA insufficiency and APAP metabolism decreased hepatic GSH. Rodent studies show that hepatic GSH release is proportional to hepatic GSH concentration (84). Moreover, human studies using isotopic tracer methods showed that the fractional turnover of GSH is decreased in humans during SAA insufficiency. Together, the data show that APAP at normal therapeutic doses alter Cys metabolism but that homeostatic mechanisms largely preserve both Cys and GSH pools.

Accumulating evidence suggests that the redox balance of the plasma Cys and GSH pools can be important in disease mechanisms even without major changes in concentrations of Cys and GSH, *per se* (57, 75). Cell surface proteins in endothelial cells contain Cys residues which undergo redox changes in response to a more oxidized E_h CySS (33), and platelet activation is responsive to changes in extracellular E_h GSSG (26).

The SAA insufficiency caused a more oxidized plasma Cys/CySS redox potential value (E_h CySS) but had no effect on plasma GSH/GSSG redox potential value (E_h GSSG) (65). Whether the effect on E_h CySS has any health consequences is unknown, but it is worthy of study because the magnitude of effect is similar to changes in E_h CySS which have been associated with disease processes (65). It should be noted, however, that APAP administration resulted in the oxidation of plasma E_h CySS values only in the SAA replete state but not in an SAA insufficient state, suggesting that adaptive responses to SAA insufficiency probably function to protect against adverse effects.

Because the therapeutic, non-toxic doses of APAP significantly decreased plasma GSH concentrations in humans with SAA insufficiency (65), one may expect that this would also perturb APAP metabolism. However, the present data show that SAA limitation had no affect on the pattern of APAP metabolite concentrations in the plasma (Fig 4.1) or urine (Fig 4.3). These results are consistent with the interpretation that short-term SAA insufficiency does not compromise APAP metabolism. Pharmacokinetic parameters including half-life, volume of distribution, elimination rate, and clearance rate were unaffected by consumption of a SAA insufficient diet for 2 days (Table 4.2). Thus, even though APAP affects plasma GSH under SAA insufficient conditions, the data provide no evidence that APAP metabolism is altered or that risk of adverse effects would be increased by 2 days of SAA insufficiency.

These results are consistent with previous findings concerning APAP metabolism. Glucuronidation is the major pathway for APAP metabolism and is regulated independently of other pathways. Glucuronidation can be limited by a relative insufficiency of glucose, hypoxia, and alcohol consumption, but the pathway is not

known to be influenced by SAA intake. Sulfation is the second most important pathway and, in the short term, is resistant to SAA insufficiency because of the large sulfate pool in the body. In the present study, no measurements of sulfate were obtained, so it is not known whether the SAA insufficiency caused a significant decrease in sulfate availability. However, the effects on Cys and GSH were modest so that one can anticipate that adaptive changes protecting these pools also protected the sulfate pools. The lack of effect on the mercapturate pathway for APAP metabolism is also consistent with known characteristics which are largely determined by the extent of bioactivation of APAP (69).

The results of the present study in humans with 2 d of SAA-free food and APAP at 15 mg/kg are different from those obtained in rats given 75 mg/kg APAP following 3 days with Met but lacking inorganic sulfate or Cys (36). The latter showed that APAP metabolism and pharmacokinetic properties were altered due to a reduced capacity of rats to sulfate APAP. The decrease in sulfation was not compensated by an increase in glucuronidation (36). Instead, the decrease in sulfation resulted in increased conversion of APAP into its toxic metabolite, as suggested by increased excretion of APAP-Cys, -GSH, and –mercapturic acid in the urine. These findings indicate that an SAA deficient diet causes a decrease in detoxification by sulfation and an increase in toxicity by APAP which could be more severe after repeated doses of APAP. The current study shows that two 15 mg/kg doses of APAP after 2 days of SAA-free food in humans do not provide evidence for a corresponding shift in metabolism. It must be pointed out, however, that the present study was designed with a modest period of SAA insufficiency and only 2 doses of APAP to minimize risk of toxicity.

The present study does not completely exclude effects of SAA insufficiency on APAP toxicity in other select subpopulations. The participants in the present study were young, had no known acute or chronic disease, did not smoke, and did not abuse alcohol. Consequently, extrapolation of these present findings to at-risk populations is inappropriate. Similarly, individuals with malabsorption or prolonged periods of inadequate SAA intake may exhibit different responses.

In conclusion, the present research shows that APAP pharmacokinetics and distribution of metabolites are not affected by SAA deficiency. Although there are many unexplained cases of human toxicity following administration of therapeutic doses of APAP, results of the current study indicate that variation in short-term SAA availability is not likely to be a major factor.

ACKNOWLEDGEMENTS

The authors thank the volunteers for their commitment, and are grateful for the assistance of the nursing, bionutrition, and laboratory staff at the Emory University Hospital GCRC and the pharmacists and staff at the Research Pharmacy at Emory University Hospital.

TABLE 4.1. Experimental Design

Study Period	Days 1-3, Outpatient Equilibration	Days 4-5, Inpatient ± SAA	Day 6, Inpatient ± APAP
1	3-days w/ 100% RDA for SAA	2-days in GCRC w/ 100% RDA for SAA	2-sequential doses of APAP
2	3-days w/ 100% RDA for SAA	2-days in GCRC w/ SAA-free diet	2-sequential doses of APAP
Blood sampling:		↑ ↑	↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑

[a.] Study periods described above were randomized for order and conducted at least one week apart

TABLE 4.2. Pharmacokinetic constants for plasma APAP following short-term SAA insufficiency

	100% SAA	**0% SAA**	**p-value**
Max plasma concentration (nmol/ml)	65.4 ± 7.2	61.6 ± 8.9	
AUC ($mg \cdot L^{-1} \cdot h$)	15.6 ± 1.9	14.0 ± 0.97	0.41
V_D (L)	75.8 ± 9.3	79.4 ± 8.0	0.70
$t_{½}$ (h)	2.8 ± 0.3	2.8 ± 0.3	0.98
k_{elim} (h^{-1})	0.3 ± 0.03	0.3 ± 0.03	0.81
CL ($L \cdot h^{-1}$)	19.0 ± 1.2	20.67 ± 0.3	0.31

FIGURE 4.1

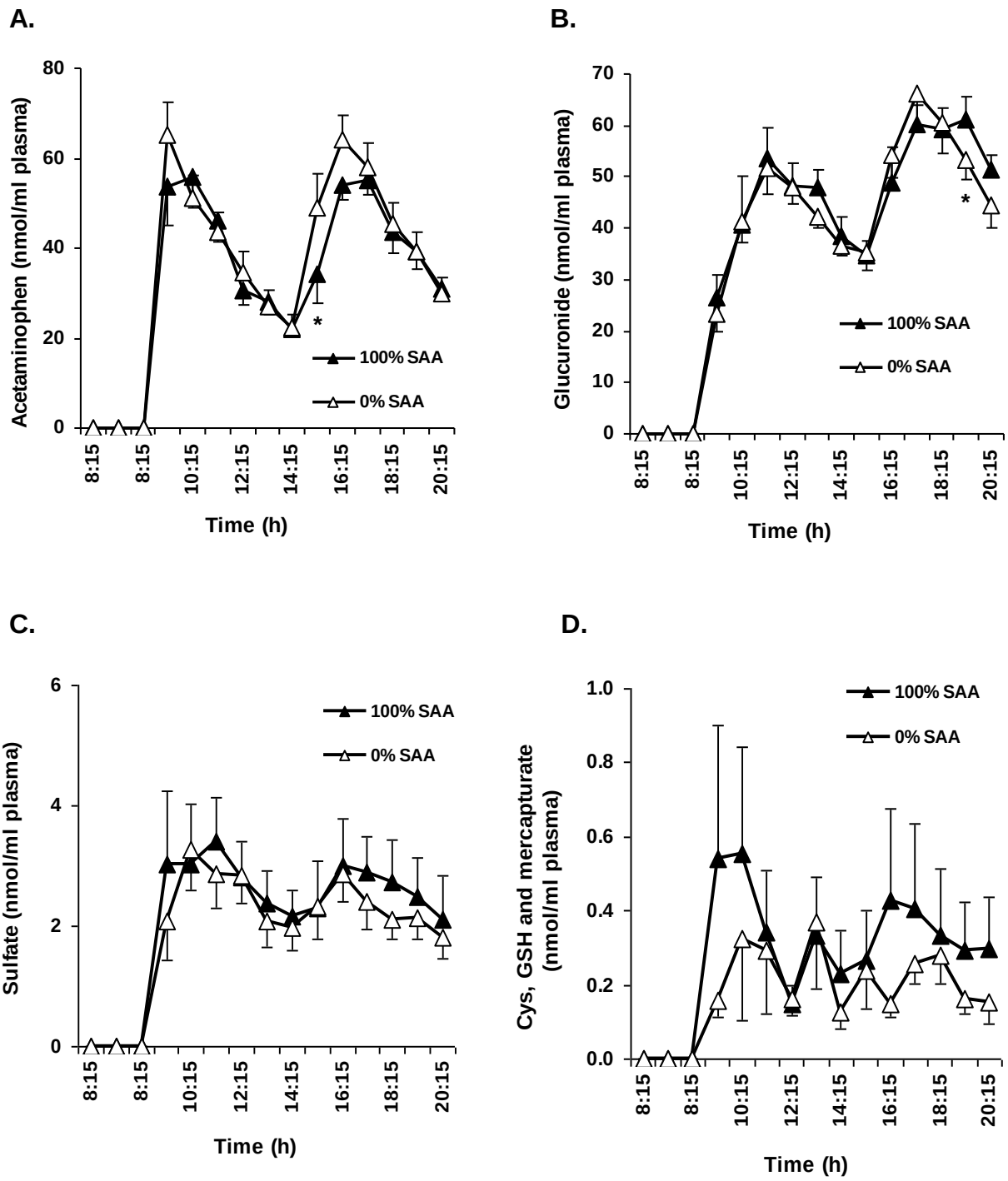
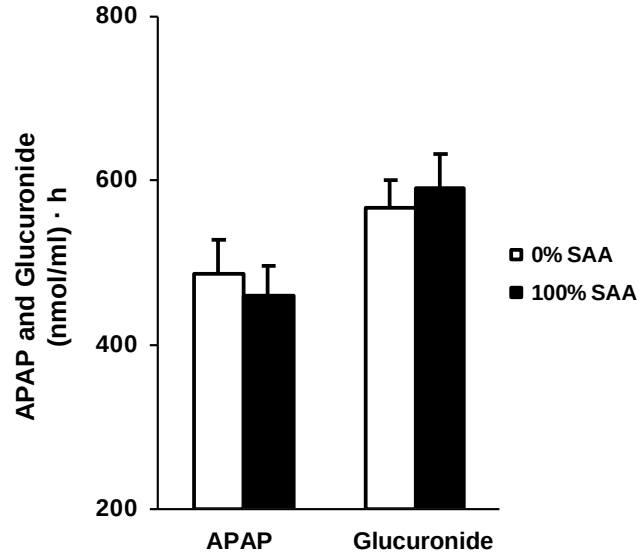

A.
Acetaminophen (nmol/ml plasma)
80
60
40
20
0
*
100% SAA
0% SAA
8:15
8:15
10:15
12:15
14:15
16:15
18:15
20:15
Time (h)
B.
Glucuronide (nmol/ml plasma)
70
60
50
40
30
20
10
0
*
100% SAA
0% SAA
8:15
8:15
10:15
12:15
14:15
16:15
18:15
20:15
Time (h)
C.
Sulfate (nmol/ml plasma)
6
4
2
0
100% SAA
0% SAA
8:15
8:15
10:15
12:15
14:15
16:15
18:15
20:15
Time (h)
D.
Cys, GSH and mercapturate (nmol/ml plasma)
1.0
0.8
0.6
0.4
0.2
0.0
100% SAA
0% SAA
8:15
8:15
10:15
12:15
14:15
16:15
18:15
20:15
Time (h)

Figure 4.1. Plasma concentrations of APAP and APAP metabolites following 2 days of an SAA adequate diet (100% of the RDA) or an SAA-deficient diet (0% of the RDA). APAP and metabolite concentrations were determined by HPLC. Data are expressed as means ± SE for each respective time point. *Significant at $p < 0.05$, $n = 8$.

FIGURE 4.2

A.


800
600
400
200
APAP and Glucuronide (nmol/ml) · h
0% SAA
100% SAA
APAP
Glucuronide

B.

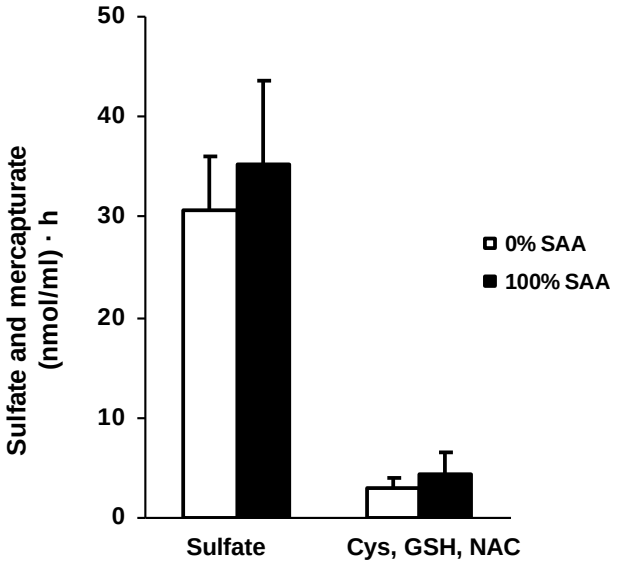

50
40
30
20
10
0
Sulfate and mercapturate (nmol/ml) · h
0% SAA
100% SAA
Sulfate
Cys, GSH, NAC

Figure 4.2. Area Under the Curve (AUC) analysis for plasma acetaminophen and metabolites following 2 days of an SAA adequate diet (100% of the RDA) or an SAA-deficient diet (0% of the RDA). Area Under the Curve (AUC) was calculated using the APAP metabolite concentrations measured by HPLC analysis (See Fig 1). *Significant at $p < 0.05$, $n = 8$.

FIGURE 4.3

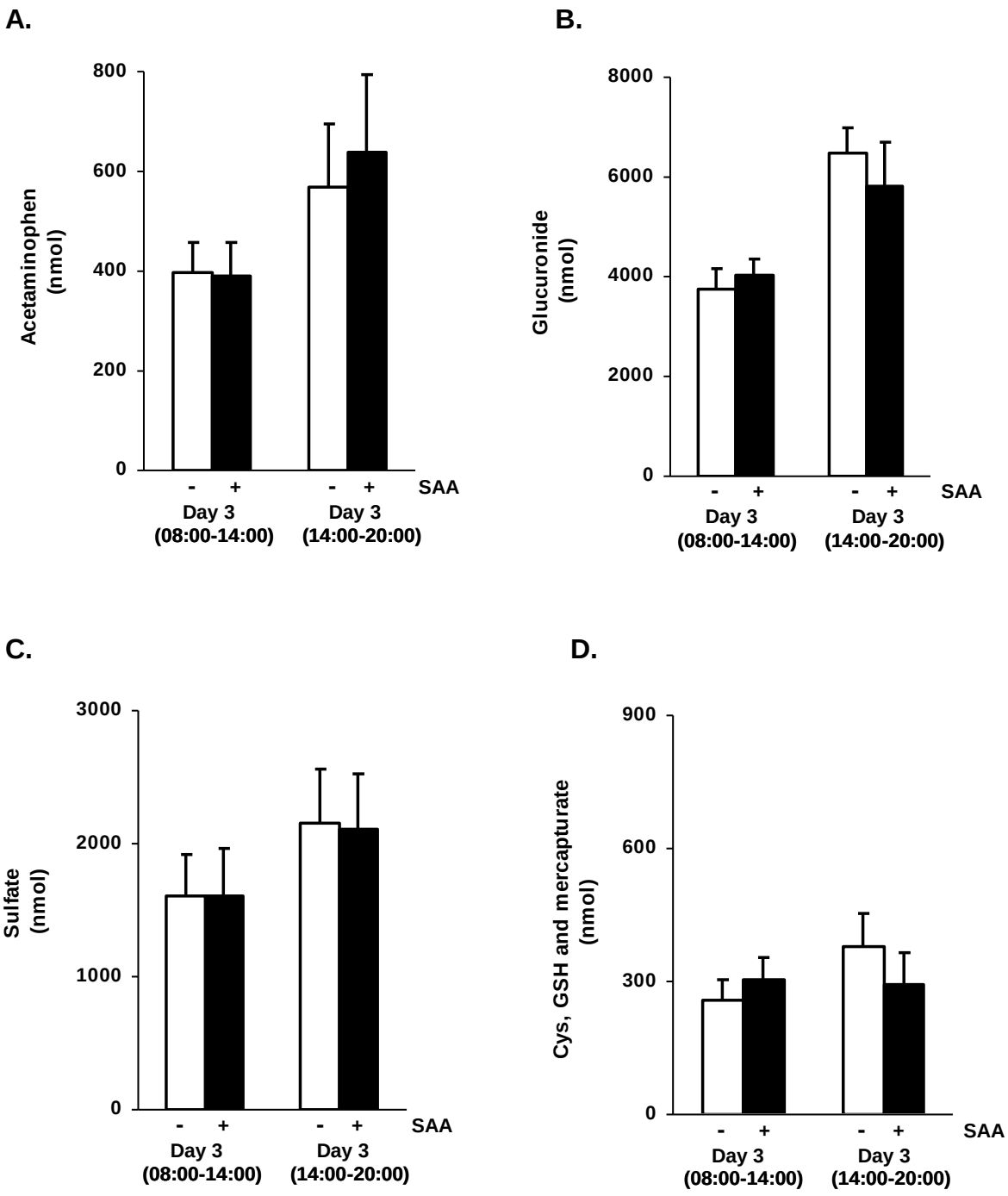
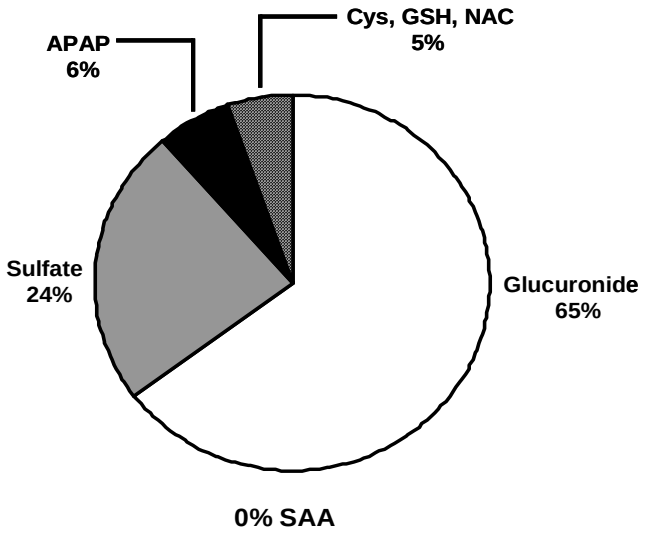

A.
Acetaminophen (nmol)
800
600
400
200
0
- + - +
SAA
Day 3 (08:00-14:00)
Day 3 (14:00-20:00)
B.
Glucuronide (nmol)
8000
6000
4000
2000
0
- + - +
SAA
Day 3 (08:00-14:00)
Day 3 (14:00-20:00)
C.
Sulfate (nmol)
3000
2000
1000
0
- + - +
SAA
Day 3 (08:00-14:00)
Day 3 (14:00-20:00)
D.
Cys, GSH and mercapturate (nmol)
900
600
300
0
- + - +
SAA
Day 3 (08:00-14:00)
Day 3 (14:00-20:00)

Figure 4.3. Effect of sulfur amino acid deficiency on urinary APAP and APAP metabolite recovery. Urine was collected in 6-h intervals following administration of APAP and analyzed by HPLC. Results showed no significant differences due to consumption of SAA deficient food for 2 days.

FIGURE 4.4

A.


Cys, GSH, NAC
5%
APAP
6%
Sulfate
24%
Glucuronide
65%
0% SAA

B.

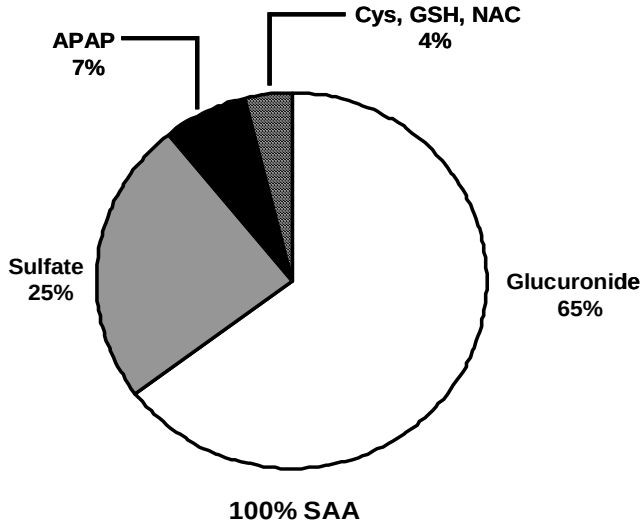

Cys, GSH, NAC
4%
APAP
7%
Sulfate
25%
Glucuronide
65%
100% SAA

Figure 4.4. Percentages of APAP and APAP metabolites recovered in urine while consuming diet adequate or insufficient in sulfur amino acids. Data are expressed as percentage of cumulative 12-h urinary excretion of APAP metabolites (APAP, APAP-glucuronide, APAP-sulfate, and APAP-Cys, -GSH, -mercapturate) following 2 doses of APAP (15 mg/kg) given 6 h apart.

Chapter 5: Conclusions and Future Directions

The redox potential of the extracellular Cys/CySS pool plays an important role in redox communication between cells and tissues. Several studies show that cell processes such as cell proliferation, differentiation, cell adhesion, and apoptosis vary as a function of extracellular redox potential over the physiologic range found *in* vivo. Studies in cell culture and intestinal models showed that intrinsic mechanisms are present within cells to regulate the extracellular redox environment. More specifically, when either the apical surface of Caco2 cells or the lumen of the gut, respectively, are exposed to an oxidative redox potential, the cells have the ability to correct the extracellular Cys/CySS redox potential toward physiologic range observed in vivo. The present research described in Chapter 2 added to this knowledge by showing that extracellular E_h CySS regulation occurs on the basal surface of Caco2 cells by mechanisms involving known Cys and CySS transport systems.

While both apical and basal surfaces of Caco-2 cells regulate the extracellular redox environment, the basal domain has a greater capacity for extracellular E_h regulation (Fig 2.1A). Preliminary mechanistic studies suggested the role of a Cys/CySS shuttle for maintenance of extracellular thiol/disulfide redox potential. The current study showed that regulation of redox potential on the basal surface was sodium-dependent and inhibited by extracellular lysine, a competitive inhibitor of cystine transport by the y^+L system. This research provides an important advance in knowledge because previous studied were contradictory in terms of extracellular redox regulation. Specifically, Reed et al (1978) concluded that GSH released from hepatocytes reduced CySS to allow Cys uptake. In HT29 cells, however, depletion of cellular GSH by pretreatment with BSO had no effect on the ability to reduce extracellular CySS and control E_hCySS.

Conversely, limitation of Cys in the cell culture media caused a marked decrease in intracellular GSH concentration and cellular E_hGSSG, but no effect on extracellular E_hCySS. Thus, the present evidence shows that the extracellular Cys/CySS redox couple can be regulated independently of the cellular GSH system because of the function of Cys and CySS transport systems.

Direct translation of these results into animals studies has not been done. However, rodent studies show that dietary availability of SAA impact the regulation of thiol/disulfide redox potential in the extracellular space. Relevant studies showed that nutritional insufficiency of SAA caused a marked oxidation of plasma Cys/CySS E_h and GSH/GSSG E_h in rodents.

In addition to the role of SAA in extracellular redox homeostasis, previous studies have shown that APAP metabolism is also dependent upon adequate SAA intake. Insufficient intake of SAA altered the individual pathways of APAP metabolism, lengthened the half-life and elimination rate, and increased susceptibility of rodents to APAP-induced hepatotoxicity.

Human studies have also shown that reductive mechanisms are important for absorption of redox-sensitive nutrients and the maintenance of conditions for digestion, failure of the mechanism could contribute to the oxidation of the extracellular space and related development of digestive disorders. Perturbation of the extracellular thiol/disulfide redox state occurs as a consequence of aging, chemotherapy, diseases such as macular degeneration and cardiovascular disease, and lifestyle consequences such as smoking and alcohol abuse. Taken together, the results suggest that control of the

extracellular thiol/disulfide redox environment may be an important mechanistic link between health status, disease, and nutritional availability.

The human study discussed in Chapters 3 and 4 extended by examining the effects of nutritional intake and drug metabolism on the extracellular thiol/disulfide redox environment. Results showed that a SAA-insufficient diet caused a more oxidized plasma E_hCySS in healthy subjects. With SAA intake at 100% RDA, APAP administration oxidized the plasma E_hCySS. Together, the data show that E_hCySS is dependent both upon dietary and drug exposures. Although SAA are necessary to maintain extracellular redox homeostasis and are required for drug metabolism, administration of therapeutic doses of APAP did not further oxidize plasma E_hCySS in a SAA insufficient state. The results suggest that use of APAP under these conditions may not cause further oxidative changes, as measured in plasma. SAA insufficiency also did not alter the pattern of APAP metabolites in the plasma or urine nor did it alter APAP pharmacokinetics. It is possible that we did not observe changes in the appearance of metabolites in the plasma and urine or the pharmacokinetic properties of APAP in our study population because the duration of SAA insufficiency was short, only two therapeutic doses of APAP were administered, and participating subjects were young and healthy. Therefore, studies of the effect of longer periods of SAA insufficiency on APAP metabolism would be of interest. However, studies of chronic consumption of a SAA-insufficient diet and APAP administration are not feasible to perform in humans because oxidation of the extracellular thiol/disulfide redox environment may increase susceptibility to toxicity even at therapeutic doses.

Future Directions

Previous studies in rodent models show that chronic consumption of a diet insufficient in sulfur amino acids decreased plasma and tissue concentrations of Cys and GSH and caused more oxidized plasma and tissue Cys/CySS and GSH/GSSG redox values. Based on this information, studies could be performed to determine whether long-term SAA insufficiency alters the pattern of APAP metabolites in the plasma and urine in rodents. Moreover, these studies could also examine whether SAA insufficiency increases sensitivity of the liver and kidney to toxicity following administration of an APAP dose of lowest observable effect level. Information from these studies could provide insight into potential causes of unexplained cases of human toxicity following administration of therapeutic doses of APAP.

Although long-term studies of SAA insufficiency and APAP administration are not possible in a human population without a threat of toxicity, metabolomic studies could be designed to provide data on the extent of normal variation in metabolic profiles within healthy individuals using high-resolution ^{1}H-NMR spectroscopy or liquid chromatography coupled to mass spectrometry. Metabolomic analyses such as ^{1}H-NMR, would provide valuable information on how complex biological systems change in response to stresses such as APAP exposure and dietary change and therefore stratify subjects based on individual biochemical profiles. Used in combination with key GSH and Cys redox measurements, studies with ^{1}H-NMR would show whether changes due to chemical exposure and diet can be discriminated.

Taken together, the studies discussed in this dissertation identified potential mechanisms for regulation of the extracellular redox environment and provided evidence

that extracellular E_hCySS is affected by nutritional limitation and drug exposure. These data indicate that plasma Cys/CySS redox potential may be an important biomarker of nutritional status and prove useful for evaluation of complex interactions of diet and drug exposure which could affect risk of adverse reactions and drug toxicities.

REFERENCES

1. **Anderson CL, Iyer SS, Ziegler TR, and Jones DP.** Control of extracellular cysteine/cystine redox state by HT-29 cells is independent of cellular glutathione. *Am J Physiol Regul Integr Comp Physiol* 293: R1069-1075, 2007.

2. **Andrews RS, Bond CC, Burnett J, Saunders A, and Watson K.** Isolation and identification of paracetamol metabolites. *J Int Med Res* 4: 34-39, 1976.

3. **Ashfaq S, Abramson JL, Jones DP, Rhodes SD, Weintraub WS, Hooper WC, Vaccarino V, Harrison DG, and Quyyumi AA.** The relationship between plasma levels of oxidized and reduced thiols and early atherosclerosis in healthy adults. *J Am Coll Cardiol* 47: 1005-1011, 2006.

4. **Bannai S.** Transport of cystine and cysteine in mammalian cells. *Biochim Biophys Acta* 779: 289-306, 1984.

5. **Blanco RA, Ziegler TR, Carlson BA, Cheng PY, Park Y, Cotsonis GA, Accardi CJ, and Jones DP.** Diurnal variation in glutathione and cysteine redox states in human plasma. *Am J Clin Nutr* 86: 1016-1023, 2007.

6. **Bottiglieri T.** S-Adenosyl-L-methionine (SAMe): from the bench to the bedside--molecular basis of a pleiotrophic molecule. *Am J Clin Nutr* 76: 1151S-1157S, 2002.

7. **Bradford MM.** A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248-254, 1976.

8. **Brodie BB and Axelrod J.** The fate of acetanilide in man. *J Pharmacol Exp Ther* 94: 29-38, 1948.

9. **Broer S.** Amino acid transport across mammalian intestinal and renal epithelia. *Physiol Rev* 88: 249-286, 2008.

10. **Brosnan JT and Brosnan ME.** The sulfur-containing amino acids: an overview. *J Nutr* 136: 1636S-1640S, 2006.

11. **Burdo J, Dargusch R, and Schubert D.** Distribution of the cystine/glutamate antiporter system xc- in the brain, kidney, and duodenum. *J Histochem Cytochem* 54: 549-557, 2006.

12. **Burrin DG and Stoll B.** Emerging aspects of gut sulfur amino acid metabolism. *Curr Opin Clin Nutr Metab Care* 10: 63-68, 2007.

13. **Cai J, Nelson KC, Wu M, Sternberg P, Jr., and Jones DP.** Oxidative damage and protection of the RPE. *Prog Retin Eye Res* 19: 205-221, 2000.

14. **Cantor EJ, Mancini EV, Seth R, Yao XH, and Netticadan T.** Oxidative stress and heart disease: cardiac dysfunction, nutrition, and gene therapy. *Curr Hypertens Rep* 5: 215-220, 2003.

15. **Chen C, Hennig GE, and Manautou JE.** Hepatobiliary excretion of acetaminophen glutathione conjugate and its derivatives in transport-deficient (TR-) hyperbilirubinemic rats. *Drug Metab Dispos* 31: 798-804, 2003.

16. **Clarke WM.** The standard hydrogen half-cell and the standardization of oxidation-reduction potentials and pH numbers. In: *Oxidation-Reduction Potentials of Organic Systems*. Baltimore, MD: Waverly, 1960, p. 248-272.

17. **Clissold SP.** Paracetamol and phenacetin. *Drugs* 32 Suppl 4: 46-59, 1986.

18. **Coloso RM, Drake MR, and Stipanuk MH.** Effect of bathocuproine disulfonate, a copper chelator, on cyst(e)ine metabolism by freshly isolated rat hepatocytes. *Am J Physiol* 259: E443-450, 1990.

19. **Dahlin DC, Miwa GT, Lu AY, and Nelson SD.** N-acetyl-p-benzoquinone imine: a cytochrome P-450-mediated oxidation product of acetaminophen. *Proc Natl Acad Sci U S A* 81: 1327-1331, 1984.

20. **Dahm LJ and Jones DP.** Clearance of glutathione disulfide from rat mesenteric vasculature. *Toxicol Appl Pharmacol* 129: 272-282, 1994.

21. **Dahm LJ and Jones DP.** Rat jejunum controls luminal thiol-disulfide redox. *J Nutr* 130: 2739-2745, 2000.

22. **Dahm LJ and Jones DP.** Secretion of cysteine and glutathione from mucosa to lumen in rat small intestine. *Am J Physiol* 267: G292-300, 1994.

23. **Dai J, Jones DP, Goldberg J, Ziegler TR, Bostick RM, Wilson PW, Manatunga AK, Shallenberger L, Jones L, and Vaccarino V.** Association between adherence to the Mediterranean diet and oxidative stress. *Am J Clin Nutr* 88: 1364-1370, 2008.

24. **Di Buono M, Wykes LJ, Ball RO, and Pencharz PB.** Dietary cysteine reduces the methionine requirement in men. *Am J Clin Nutr* 74: 761-766, 2001.

25. **Dong H, Haining RL, Thummel KE, Rettie AE, and Nelson SD.** Involvement of human cytochrome P450 2D6 in the bioactivation of acetaminophen. *Drug Metab Dispos* 28: 1397-1400, 2000.

26. **Essex DW and Li M.** Redox control of platelet aggregation. *Biochemistry* 42: 129-136, 2003.

27. **Fernandez E, Torrents D, Chillaron J, Martin Del Rio R, Zorzano A, and Palacin M.** Basolateral LAT-2 has a major role in the transepithelial flux of L-cystine in the renal proximal tubule cell line OK. *J Am Soc Nephrol* 14: 837-847, 2003.

28. **Flagg EW, Coates RJ, Eley JW, Jones DP, Gunter EW, Byers TE, Block GS, and Greenberg RS.** Dietary glutathione intake in humans and the relationship between intake and plasma total glutathione level. *Nutr Cancer* 21: 33-46, 1994.

29. **FNB.** Protein and Amino Acids. In: *Dietary Reference Intakes for Energy, Carbohydrate, Fiber, Fat, Fatty Acids, Cholesterol, Protein, and Amino Acid (Macronutrients)*. Washington, D.C.: The National Academies Press, 2005, p. 589-768.

30. **Ganapathy V, Ganapathy, M.E., Leibach, F.H.** Intestinal transport of peptides and amino acids. In: *Current Topics in Membranes*: Academic Press, 2001, p. 379-412.

31. **Ganapathy V, Gupta, N., Martindale, R.G.** Protein digestion and absorption. In: *Physiology of the gastrointestinal tract* (Fourth ed.), edited by Johnson LR: Elseiver Academic Press, 2006, p. 1667-1692.

32. **Gibson JD, Pumford NR, Samokyszyn VM, and Hinson JA.** Mechanism of acetaminophen-induced hepatotoxicity: covalent binding versus oxidative stress. *Chem Res Toxicol* 9: 580-585, 1996.

33. **Go YM and Jones DP.** Intracellular proatherogenic events and cell adhesion modulated by extracellular thiol/disulfide redox state. *Circulation* 111: 2973-2980, 2005.

34. **Go YM and Jones DP.** Redox clamp model for study of extracellular thiols and disulfides in redox signaling. *Methods in Enzymology* 474: In press, 2010.

35. **Go YM and Jones DP.** Redox compartmentalization in eukaryotic cells. *Biochim Biophys Acta* 1780: 1273-1290, 2008.

36. **Gregus Z, Kim HJ, Madhu C, Liu Y, Rozman P, and Klaassen CD.** Sulfation of acetaminophen and acetaminophen-induced alterations in sulfate and 3'-phosphoadenosine 5'-phosphosulfate homeostasis in rats with deficient dietary intake of sulfur. *Drug Metab Dispos* 22: 725-730, 1994.

37. **Howie D, Adriaenssens PI, and Prescott LF.** Paracetamol metabolism following overdosage: application of high performance liquid chromatography. *J Pharm Pharmacol* 29: 235-237, 1977.

38. **Hutter DE, Till BG, and Greene JJ.** Redox state changes in density-dependent regulation of proliferation. *Exp Cell Res* 232: 435-438, 1997.

39. **Hwang C and Swinskey A.** The role of oxidation-reduction potential in monitoring growth of cultured mammalian cells. In: *Production of Biologicals from Animal Cells in Culture*, edited by Spier RE GJ, Meignier B. Oxford, UK: Butterworth-Heinemann, 1991, p. 548-569.

40. **Iyer SS, Accardi CJ, Ziegler TR, Blanco RA, Ritzenthaler JD, Rojas M, Roman J, and Jones DP.** Cysteine redox potential determines pro-inflammatory IL-1beta levels. *PLoS One* 4: e5017, 2009.

41. **Iyer SS, Jones DP, Brigham KL, and Rojas M.** Oxidation of plasma cysteine/cystine redox state in endotoxin-induced lung injury. *Am J Respir Cell Mol Biol* 40: 90-98, 2009.

42. **Jaeschke H, Knight TR, and Bajt ML.** The role of oxidant stress and reactive nitrogen species in acetaminophen hepatotoxicity. *Toxicol Lett* 144: 279-288, 2003.

43. **James LP, Mayeux PR, and Hinson JA.** Acetaminophen-induced hepatotoxicity. *Drug Metab Dispos* 31: 1499-1506, 2003.

44. **Jiang S, Cai J, Wallace DC, and Jones DP.** Cytochrome c-mediated apoptosis in cells lacking mitochondrial DNA. Signaling pathway involving release and caspase 3 activation is conserved. *J Biol Chem* 274: 29905-29911, 1999.

45. **Jiang S, Moriarty-Craige SE, Orr M, Cai J, Sternberg P, Jr., and Jones DP.** Oxidant-induced apoptosis in human retinal pigment epithelial cells: dependence on extracellular redox state. *Invest Ophthalmol Vis Sci* 46: 1054-1061, 2005.

46. **Jollow DJ, Thorgeirsson SS, Potter WZ, Hashimoto M, and Mitchell JR.** Acetaminophen-induced hepatic necrosis. VI. Metabolic disposition of toxic and nontoxic doses of acetaminophen. *Pharmacology* 12: 251-271, 1974.

47. **Jonas CR, Gu LH, Nkabyo YS, Mannery YO, Avissar NE, Sax HC, Jones DP, and Ziegler TR.** Glutamine and KGF each regulate extracellular thiol/disulfide redox and enhance proliferation in Caco-2 cells. *Am J Physiol Regul Integr Comp Physiol* 285: R1421-1429, 2003.

48. **Jonas CR, Puckett AB, Jones DP, Griffith DP, Szeszycki EE, Bergman GF, Furr CE, Tyre C, Carlson JL, Galloway JR, Blumberg JB, and Ziegler TR.** Plasma antioxidant status after high-dose chemotherapy: a randomized trial of parenteral nutrition in bone marrow transplantation patients. *Am J Clin Nutr* 72: 181-189, 2000.

49. **Jonas CR, Ziegler TR, Gu LH, and Jones DP.** Extracellular thiol/disulfide redox state affects proliferation rate in a human colon carcinoma (Caco2) cell line. *Free Radic Biol Med* 33: 1499-1506, 2002.

50. **Jones DP.** Radical-free biology of oxidative stress. *Am J Physiol Cell Physiol* 295: C849-868, 2008.

51. **Jones DP.** Redefining oxidative stress. *Antioxid Redox Signal* 8: 1865-1879, 2006.

52. **Jones DP.** Redox potential of GSH/GSSG couple: assay and biological significance. *Methods Enzymol* 348: 93-112, 2002.

53. **Jones DP, Brown LA, and Sternberg P.** Variability in glutathione-dependent detoxication in vivo and its relevance to detoxication of chemical mixtures. *Toxicology* 105: 267-274, 1995.

54. **Jones DP, Carlson JL, Mody VC, Cai J, Lynn MJ, and Sternberg P.** Redox state of glutathione in human plasma. *Free Radic Biol Med* 28: 625-635, 2000.

55. **Jones DP, Carlson JL, Samiec PS, Sternberg P, Jr., Mody VC, Jr., Reed RL, and Brown LA.** Glutathione measurement in human plasma. Evaluation of sample collection, storage and derivatization conditions for analysis of dansyl derivatives by HPLC. *Clin Chim Acta* 275: 175-184, 1998.

56. **Jones DP, Coates RJ, Flagg EW, Eley JW, Block G, Greenberg RS, Gunter EW, and Jackson B.** Glutathione in foods listed in the National Cancer Institute's Health Habits and History Food Frequency Questionnaire. *Nutr Cancer* 17: 57-75, 1992.

57. **Jones DP and Liang Y.** Measuring the poise of thiol/disulfide couples in vivo. *Free Radic Biol Med* 47: 1329-1338, 2009.

58. **Jones DP, Mody VC, Jr., Carlson JL, Lynn MJ, and Sternberg P, Jr.** Redox analysis of human plasma allows separation of pro-oxidant events of aging from decline in antioxidant defenses. *Free Radic Biol Med* 33: 1290-1300, 2002.

59. **Kemp M, Go YM, and Jones DP.** Nonequilibrium thermodynamics of thiol/disulfide redox systems: a perspective on redox systems biology. *Free Radic Biol Med* 44: 921-937, 2008.

60. **Kirlin WG, Cai J, Thompson SA, Diaz D, Kavanagh TJ, and Jones DP.** Glutathione redox potential in response to differentiation and enzyme inducers. *Free Radic Biol Med* 27: 1208-1218, 1999.

61. **Kovacic P and Jacintho JD.** Mechanisms of carcinogenesis: focus on oxidative stress and electron transfer. *Curr Med Chem* 8: 773-796, 2001.

62. **Larson AM.** Acetaminophen hepatotoxicity. *Clin Liver Dis* 11: 525-548, vi, 2007.

63. **Lash LH and Jones DP.** Characteristics of cysteine uptake in intestinal basolateral membrane vesicles. *Am J Physiol* 247: G394-401, 1984.

64. **Lyons J, Rauh-Pfeiffer A, Yu YM, Lu XM, Zurakowski D, Tompkins RG, Ajami AM, Young VR, and Castillo L.** Blood glutathione synthesis rates in healthy adults receiving a sulfur amino acid-free diet. *Proc Natl Acad Sci U S A* 97: 5071-5076, 2000.

65. **Mannery YO, Ziegler TR, Park Y, and Jones DP.** Oxidation of Plasma Cysteine/Cystine and GSH/GSSG Redox Potentials by Acetaminophen and Sulfur Amino Acid Insufficiency. *J Pharmacol Exp Ther*, 2010.

66. **McCarver DG and Hines RN.** The ontogeny of human drug-metabolizing enzymes: phase II conjugation enzymes and regulatory mechanisms. *J Pharmacol Exp Ther* 300: 361-366, 2002.

67. **Meredith MJ and Reed DJ.** Status of the mitochondrial pool of glutathione in the isolated hepatocyte. *J Biol Chem* 257: 3747-3753, 1982.

68. **Miller LT, Watson WH, Kirlin WG, Ziegler TR, and Jones DP.** Oxidation of the glutathione/glutathione disulfide redox state is induced by cysteine deficiency in human colon carcinoma HT29 cells. *J Nutr* 132: 2303-2306, 2002.

69. **Mitchell JR, Jollow DJ, Potter WZ, Davis DC, Gillette JR, and Brodie BB.** Acetaminophen-induced hepatic necrosis. I. Role of drug metabolism. *J Pharmacol Exp Ther* 187: 185-194, 1973.

70. **Mitchell JR, Jollow DJ, Potter WZ, Gillette JR, and Brodie BB.** Acetaminophen-induced hepatic necrosis. IV. Protective role of glutathione. *J Pharmacol Exp Ther* 187: 211-217, 1973.

71. **Mitchell JR, Thorgeirsson SS, Potter WZ, Jollow DJ, and Keiser H.** Acetaminophen-induced hepatic injury: protective role of glutathione in man and rationale for therapy. *Clin Pharmacol Ther* 16: 676-684, 1974.

72. **Moldeus P.** Paracetamol metabolism and toxicity in isolated hepatocytes from rat and mouse. *Biochem Pharmacol* 27: 2859-2863, 1978.

73. **Moriarty-Craige SE, Adkison J, Lynn M, Gensler G, Bressler S, Jones DP, and Sternberg P, Jr.** Antioxidant supplements prevent oxidation of cysteine/cystine redox in patients with age-related macular degeneration. *Am J Ophthalmol* 140: 1020-1026, 2005.

74. **Moriarty-Craige SE, Ha KN, Sternberg P, Jr., Lynn M, Bressler S, Gensler G, and Jones DP.** Effects of long-term zinc supplementation on plasma thiol metabolites

and redox status in patients with age-related macular degeneration. *Am J Ophthalmol* 143: 206-211, 2007.

75. **Moriarty-Craige SE and Jones DP.** Extracellular thiols and thiol/disulfide redox in metabolism. *Annu Rev Nutr* 24: 481-509, 2004.

76. **Moriarty SE, Shah JH, Lynn M, Jiang S, Openo K, Jones DP, and Sternberg P.** Oxidation of glutathione and cysteine in human plasma associated with smoking. *Free Radic Biol Med* 35: 1582-1588, 2003.

77. **Napolitano M, Rainaldi G, Bravo E, and Rivabene R.** Influence of thiol balance on micellar cholesterol handling by polarized Caco-2 intestinal cells. *FEBS Lett* 551: 165-170, 2003.

78. **Neuman RB, Bloom HL, Shukrullah I, Darrow LA, Kleinbaum D, Jones DP, and Dudley SC, Jr.** Oxidative stress markers are associated with persistent atrial fibrillation. *Clin Chem* 53: 1652-1657, 2007.

79. **Nimni ME, Han B, and Cordoba F.** Are we getting enough sulfur in our diet? *Nutr Metab (Lond)* 4: 24, 2007.

80. **Nkabyo YS, Go YM, Ziegler TR, and Jones DP.** Extracellular cysteine/cystine redox regulates the p44/p42 MAPK pathway by metalloproteinase-dependent epidermal growth factor receptor signaling. *Am J Physiol Gastrointest Liver Physiol* 289: G70-78, 2005.

81. **Nkabyo YS, Gu LH, Jones DP, and Ziegler TR.** Thiol/disulfide redox status is oxidized in plasma and small intestinal and colonic mucosa of rats with inadequate sulfur amino acid intake. *J Nutr* 136: 1242-1248, 2006.

82. **Nkabyo YS, Ziegler TR, Gu LH, Watson WH, and Jones DP.** Glutathione and thioredoxin redox during differentiation in human colon epithelial (Caco-2) cells. *Am J Physiol Gastrointest Liver Physiol* 283: G1352-1359, 2002.

83. **Nkabyo YS, Ziegler TR, and Jones DP.** Composition and Regulation of Thiol-Disulfide Redox State. In: *Redox Genome Interactions in Health and Disease*, edited by Fuchs J, Podda M and Packer L. New York, NY: Marcel Dekker, 2004, p. 43-60.

84. **Ookhtens M, Hobdy K, Corvasce MC, Aw TY, and Kaplowitz N.** Sinusoidal efflux of glutathione in the perfused rat liver. Evidence for a carrier-mediated process. *J Clin Invest* 75: 258-265, 1985.

85. **Prescott LF.** Paracetamol, alcohol and the liver. *Br J Clin Pharmacol* 49: 291-301, 2000.

86. **Price VF and Jollow DJ.** Effects of sulfur-amino acid-deficient diets on acetaminophen metabolism and hepatotoxicity in rats. *Toxicol Appl Pharmacol* 101: 356-369, 1989.

87. **Raguso CA, Regan MM, and Young VR.** Cysteine kinetics and oxidation at different intakes of methionine and cystine in young adults. *Am J Clin Nutr* 71: 491-499, 2000.

88. **Reicks M, Calvert RJ, and Hathcock JN.** Effects of prolonged acetaminophen ingestion and dietary methionine on mouse liver glutathione. *Drug Nutr Interact* 5: 351-363, 1988.

89. **Rost J and Rapoport S.** Reduction-Potential of Glutathione. *Nature* 201: 185, 1964.

90. **Rumack BH and Matthew H.** Acetaminophen poisoning and toxicity. *Pediatrics* 55: 871-876, 1975.

91. **Samiec PS, Drews-Botsch C, Flagg EW, Kurtz JC, Sternberg P, Jr., Reed RL, and Jones DP.** Glutathione in human plasma: decline in association with aging, age-related macular degeneration, and diabetes. *Free Radic Biol Med* 24: 699-704, 1998.

92. **Schafer FQ and Buettner GR.** Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radic Biol Med* 30: 1191-1212, 2001.

93. **Schiodt FV, Lee WM, Bondesen S, Ott P, and Christensen E.** Influence of acute and chronic alcohol intake on the clinical course and outcome in acetaminophen overdose. *Aliment Pharmacol Ther* 16: 707-715, 2002.

94. **Shoveller AK, Stoll B, Ball RO, and Burrin DG.** Nutritional and functional importance of intestinal sulfur amino acid metabolism. *J Nutr* 135: 1609-1612, 2005.

95. **Sies H.** Glutathione and its role in cellular functions. *Free Radic Biol Med* 27: 916-921, 1999.

96. **Stipanuk MH and Watford M.** Amino acid metabolism. In: *Biochemical and Physiological Aspects of Human Nutrition*, edited by Stipanuk MH. Philadelphia, PA: W. B. Saunders, 2000, p. 233-286.

97. **Watkins PB and Seeff LB.** Drug-induced liver injury: summary of a single topic clinical research conference. *Hepatology* 43: 618-631, 2006.

98. **Watson WH, Chen Y, and Jones DP.** Redox state of glutathione and thioredoxin in differentiation and apoptosis. *Biofactors* 17: 307-314, 2003.

99. **Young VR.** Protein and amino acids. In: *Present Knowledge in Nutrition, 8th Edition* edited by Bowman BA and Russell RM. Washington, D.C.: ILSI Press, 2001, p. 43-58.

100. **Zezulka AY and Calloway DH.** Nitrogen retention in men fed isolated soybean protein supplemented with L-methionine, D-methionine, N-acetyl-L-methionine, or inorganic sulfate. *J Nutr* 106: 1286-1291, 1976.