



γ -Glutamylcysteine inhibits oxidative stress in human endothelial cells

Yukiko K. Nakamura ^a, Michael A. Dubick ^b, Stanley T. Omaye ^{a,*}

^a Department of Nutrition, University of Nevada, Reno 89557, USA

^b U.S. Army Institute of Surgical Research, San Antonio 78234, USA

ARTICLE INFO

Article history:

Received 12 July 2011

Accepted 16 October 2011

Keywords:

γ -Glutamylcysteine

Glutathione

Glutathione synthetase

Oxidative stress

ABSTRACT

Aims: γ -Glutamylcysteine (GGC) is a dipeptide and substrate for synthesis of the antioxidant glutathione (GSH), whose health promoting properties include reducing risks of oxidative stress-related injuries and diseases. The objective of this study was to investigate the efficacy of GGC on GSH synthesis and oxidative stress in human endothelial cells.

Main methods: We assessed oxidative stress, GSH, GSH synthetase (GSS) expression, and transcription factor DNA binding levels in human umbilical vein endothelial cells (HUVEC).

Key findings: We found significantly higher levels of PPAR γ DNA binding and lower levels of GSH, GSS protein, NF- κ B p65 DNA binding, thiobarbituric acid reactive substances (TBARS), and 8-epi-PGF $_{2\alpha}$ in a concentration-dependent manner, compared with the control. GSH and GSS protein levels showed a negative correlation with PPAR γ DNA binding levels and positive correlation trends with NF- κ B p65 DNA binding, TBARS, and 8-epi-PGF $_{2\alpha}$ levels. A putative binding site for NF- κ B was found at 4 227 bases upstream from the transcription start site of GSS gene, but none for PPARs. These findings suggest the involvement of NF- κ B in regulation of GSS expression. Subsequent GSH synthesis might be affected by the suppression of GSS expression in tested conditions.

Significance: Besides its substrate role in GSH synthesis, GGC may play a role in protection against oxidative stress by serving as an antioxidant and modulating the expression of protein(s) related to antioxidant defense. Thus, we speculate that GGC may serve as a novel intra- and intercellular therapeutic dipeptide for oxidative stress-related injuries and diseases.

© 2011 Elsevier Inc. All rights reserved.

Introduction

Hemorrhagic shock is a condition of reduced perfusion of vital organs with subsequent inadequate provision of oxygen and nutrients required for normal tissue and cellular function (Krausz 2006; Dutton 2007), and can be considered as global hypoxia or reoxygenation injury (Li and Jackson 2002). Exposure of hypoxic tissues to oxygen during reperfusion can lead to organ/tissue damage (Granger and Korthuis 1995), likely a consequence of excess of reactive oxygen species (ROS) generation. Modulation of oxidative stress by radical scavengers or antioxidants, such as glutathione (GSH), protects against organ/tissue damage.

GSH is a tripeptide composed of three amino acids, glutamate, cysteine, and glycine. GSH is the most abundant thiol-containing antioxidant in a cellular system, present at mM concentration (Glantzounis et al. 2006; Franco et al. 2007). GSH is synthesized in the cytosol of all mammalian cells (Huang et al. 2000; Wu et al. 2004). Increasing the levels of GSH is a topic of interest for preventative and therapeutic modulation of ROS in diabetes, cancer, AIDS, neurodegenerative and

liver diseases, ischemia reperfusion-induced injury and aging (Liu and Choi 2000; Glantzounis et al. 2006; Franco et al. 2007). However, delivery of GSH to tissues is limited by plasma membranes and the blood brain barrier (Zeevalk et al. 2008).

γ -Glutamylcysteine (GGC) is a dipeptide and precursor of GSH. Unlike GSH, supplemental GGC can be taken up into cells/tissues and directly used as a substrate for GSH synthesis (Dringen et al. 1997), suggesting its therapeutic potential. Two enzymes are involved in GSH biosynthesis: GGC synthetase (GCS) and GSH synthetase (GSS). GCS plays a role in the formation of GGC from glutamate and cysteine, while GSS requires GGC and glycine as substrates for the GSH synthesis (Franco et al. 2007).

In order to develop a better understanding about the relationship between GGC and GSH synthesis, we investigated the efficacy of GGC at graded concentrations on GSH synthesis, oxidative stress and redox-sensitive transcription factor DNA binding in human umbilical vein endothelial cells (HUVEC).

Materials and methods

Chemicals and reagents

GGC was purchased from Bachem (Torrance, CA, USA). EGM Complete Medium (#CC-3024), HEPES Buffered Saline, and Subculture

* Corresponding author at: 1664 North Virginia Street, Reno, Nevada 89557, USA. Tel.: +1 775 784 6447; fax: +1 775 784 6449.

E-mail address: omaye@unr.edu (S.T. Omaye).

Report Documentation Page				Form Approved OMB No. 0704-0188	
Public reporting burden for the collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to a penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.					
1. REPORT DATE 01 JAN 2012		2. REPORT TYPE N/A		3. DATES COVERED -	
4. TITLE AND SUBTITLE Î³-Glutamylcysteine inhibits oxidative stress in human endothelial cells.				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Nakamura Y. K., Dubick M. A., Omaye Stanley T.,				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) United States Army Institute of Surgical Research, JBSA Fort Sam Houston, TX				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION/AVAILABILITY STATEMENT Approved for public release, distribution unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT					
15. SUBJECT TERMS					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 6	19a. NAME OF RESPONSIBLE PERSON
a. REPORT unclassified	b. ABSTRACT unclassified	c. THIS PAGE unclassified			

Reagents were purchased from Lonza (Walkersville, MD, USA). Power SYBR® Green Cells-to-CT™ Kit, Synth-a-Freeze, and PCR primers were purchased from Invitrogen (Carlsbad, CA, USA). Nuclear Extraction Kit, PPAR γ and NF- κ B (human p50/p65 combo) Transcription Factor Assay Kits, Antioxidant Assay Kit, Glutathione Assay Kit, and 8-Isoprostane EIA Kit were purchased from Cayman Chemical (Ann Arbor, MI, USA). Primary antibody for human GSS was purchased from Abcam (Cambridge, MA, USA). Gelatin, o-phenylenediamine dihydrochloride tablets, and ExtrAvidin Peroxidase Staining Kit (EXTRA3) were purchased from Sigma-Aldrich (St. Louise, MO, USA).

Cell culture

Human umbilical vein endothelial cells (HUVEC, #CC-2517) cryogenically preserved were purchased from Lonza. After thawing, cells were grown in the EGM Complete Medium, containing fetal bovine albumin (2% final concentration) and all necessary growth factors, cytokines, and other supplements for cell growth/survival. Cells were subcultured by trypsin on 75 cm² gelatin-coated flasks and maintained at 37 °C in a humidified atmosphere of 5% CO₂ until becoming confluent. For RNA isolation and quantitative real-time PCR (qRT-PCR) analysis, cells were subcultured on a 96-well gelatin-coated plate.

Cell treatments and viability

HUVEC were grown on 75 cm² gelatin-coated flasks or 96-well gelatin-coated plate, and approximately 95% confluent cell (~10⁷ cells) were treated with GGC concentrations of 0, 50, 100, and 1000 μ mol/L for 24 h at 37 °C in a humidified atmosphere with 5% CO₂ (two flasks per treatment for nuclear fraction collection; two 96-wells per treatment for mRNA isolation; five flasks per treatment for cellular fraction collection). Physiological intracellular and extracellular (plasma) concentrations of GSH are: 0.5–10 mmol/L and 0.1–20 μ mol/L, respectively (Wu et al. 2004; Giustarini et al. 2008), while GGC levels in whole blood, plasma, and erythrocytes are: 25 $\pm$ 8 μ mol/L, 4.0 $\pm$ 0.3 μ mol/L, and 66 $\pm$ 24 μ mol/L, respectively (Hagenfeldt et al. 1978). Additionally, physiological concentrations of other di- and tripeptides range between 0.1 nM and 50 μ M in humans (Rubio-Aliaga et al. 2003; Wu et al. 2004). After GGC treatments for 24 h, viability of HUVEC was assessed microscopically. No cytotoxic effects of GGC were observed in each treatment. The sixth to ninth passages of tightly confluent mono-layered cells were collected after GGC treatments and used for subsequent analyses.

Cytoplasmic fraction preparation

Cytoplasmic fractions of HUVEC were collected for GSH and total antioxidant assays and GSS protein immunoassay. After treatments with GGC for 24 h, cells were rinsed, scraped, and suspended into ice-cold PBS (pH 7.4, 10 mmol/L of phosphate buffered saline, 138 mmol/L of NaCl, 2.7 mmol/L of KCl). Cells were collected from five 75 cm² flasks per each GGC treatment and pooled. Cells were homogenized for 15 s at the maximum speed (Tissue Tearor, Model 985–370, Biospec Products, Inc., Bartlesville, OK, USA), keeping cells cold in an ice-bath. Aliquots of the cell homogenate were kept at –70 °C until the performance of thiobarbituric acid reactive substances (TBARS) assay. The remaining cell homogenate was centrifuged for 15 min at 4 °C and 10000 $\times$ g. Supernatant (cytoplasmic fractions) was stored at –70 °C until the performance of other assays. All assays were performed within one month after the sample collection (except GSS protein assay within two months).

Extracellular fraction collection

Extracellular fractions of HUVEC were collected for an 8-epi-PGF_{2 α} immunoassay. The medium of confluent cell culture was used for the

immunoassay just before harvesting confluent cells. Samples of the medium were collected and pooled. The samples were stored at –70 °C within one month until the performance of 8-epi-PGF_{2 α} immunoassay.

Nuclear fraction preparation

Nuclear fractions of HUVEC were isolated with a commercial nuclear extraction kit (Cayman Chemical). After treatments with GGC for 24 h, cells were rinsed, scraped, suspended into ice-cold PBS containing phosphatase inhibitors, and centrifuged for 5 min at 4 °C and 300 $\times$ g. Cells were collected from two flasks per each GGC treatment and pooled. Then, cells were suspended and lysed with hypotonic buffer and 10% (w/v) Nonidet P-40. After spinning, the cell pellet was re-lysed and centrifuged for 10 min at 4 °C and 14000 $\times$ g. Supernatant was collected and stored at –70 °C until the performance of transcription factor assays. The assays were performed within three days after the sample collection.

Peroxisome proliferator-activated receptor- γ (PPAR γ) and nuclear factor- κ B (NF- κ B) p65 transcription factor assays

Both PPAR γ and NF- κ B p65 DNA binding activities in the nuclear fractions of HUVEC were assessed with PPAR γ and NF- κ B (human p50/p65 combo) transcription factor assays, respectively (Cayman Chemical). Either human PPAR γ bound to PPRE (5'-AGGTCAAAGGTCA-3') or human NF- κ B bound to a specific sequence (5'-GGGACTTTCC-3') immobilized within the bottoms of 96 wells was assessed at 450 nm spectrophotometrically with the enzyme-linked immunoassays. All sample tests were replicated (n = 4).

8-epi PGF_{2 α} enzyme immunoassay

8-epi PGF_{2 α} is commonly used as a biomarker of oxidative stress along with TBARS (Vincent et al. 2007). Extracellular levels of 8-epi-PGF_{2 α} (free 8-epi-PGF_{2 α} released into the EGM medium of cell culture) were measured at 405 nm spectrophotometrically with a commercial immunoassay (Cayman Chemical). All sample tests were replicated (n = 4).

Thiobarbituric acid reactive substance (TBARS) assay

TBARS is another biomarker of oxidative stress. Lipid peroxidation as the thiobarbituric acid/malondialdehyde (MDA) complex in the cell homogenate of HUVEC was assessed at 535 nm spectrophotometrically. A solution of 0.375% (w/v) thiobarbituric acid, 15% trichloroacetic acid, and 0.25 N hydrochloric acid was added to the cell homogenate, and the mixture was heated for 15 min at 100 °C (Burge and Aust 1978). After centrifugation for 10 min at 1000 $\times$ g, the supernatant was collected for spectrophotometrical reading. All sample tests were replicated (n = 4).

Glutathione (GSH) assay

Intracellular GSH levels of HUVEC were determined by the end point method with a commercial GSH assay (Cayman Chemical), and measured at 405 nm spectrophotometrically. All sample tests were replicated (n = 4).

Total antioxidant assay

Intracellular antioxidant levels of HUVEC were examined with a commercial antioxidant assay (Cayman Chemical). Total antioxidant capacity in the samples was measured at 405 nm spectrophotometrically. All sample tests were replicated (n = 4).

GSH synthetase (GSS) protein immunoassay

GSS protein levels of HUVEC were detected at 450 nm spectrophotometrically with immunoassay reagents (EXTRA3, SIGMAFAST OPD; Sigma-Aldrich) and rabbit primary antibodies to human GSS (polyclonal; Abcam). All sample tests were replicated ($n=4$).

RNA isolation, cDNA synthesis, and quantitative real-time PCR (qRT-PCR)

GSS mRNA levels of HUVEC were assessed by qRT-PCR method. Total RNA was extracted from HUVEC cultured on a 96-well plate (two wells per each GGC treatment) with Power SYBR® Green Cells-to-CT™ Kit (Invitrogen), and was used as a template for cDNA synthesis with oligo dT primers. The reverse transcription reaction was performed for 60 min at 37 °C and inactivated for 5 min at 95 °C. The cDNA was stored for one and half months at −20 °C until the performance of qRT-PCR. The primer sets used to amplify the GSS cDNA were: F-5′-GCAGGCTGATGGTATGGAAT-3′ and R-5′-TACGCCTTTCTAGGCTCCA-3′. Forty cycles of qRT-PCR reactions was performed for 15 s at 95 °C and for 1 min at 60 °C. Relative expression was calculated from cycle threshold values ($2^{-\Delta\Delta C_t}$ method), using 18S rRNA expression as an internal control for each sample. All sample tests were replicated ($n=4$).

Transcription factor binding site search

Transcription factor binding sites were identified with an online transcription factor search tool provided by Computational Biology Research Center, Japan. The following were analyzed to search putative transcription factor binding sites: the promoter region up to 6650 bases upstream from the transcription start site of human GSS gene and the promoter region up to 8600 bases upstream from the transcription start site of human GCS-HS gene (GCS catalytic unit).

Statistical analysis

Statistical analyses (ANOVA, Student's *t*-test, and Pearson's correlations) were performed with SPSS-PASW18. Differences with $p<0.05$ were considered to be statistically significant. All results were expressed as mean $\pm$ standard deviation.

Results

Transcription factor DNA binding

Compared to the control (GGC 0 $\mu\text{mol/L}$) through Student's *t*-test, we found significantly higher PPAR γ DNA binding levels (1.37 to 1.72 fold, $p<0.005$) and significantly lower NF- κ B p65 DNA binding levels (0.62 to 0.72 fold, $p<0.005$) at all GGC concentrations tested (Figs. 1 and 2).

Oxidative stress biomarkers

We found significantly lower 8-epi-PGF $_{2\alpha}$ levels (0.76 fold, $p<0.01$) at the higher GGC concentrations (100, 1000 $\mu\text{mol/L}$), compared to the control through Student's *t*-test (Fig. 3). Similarly, TBARS levels were markedly lower than the control (0.27 to 0.29 fold, $p<0.005$) at the two higher GGC concentrations (Fig. 4).

Antioxidant levels

No significant change in total antioxidant capacity was found at all GGC concentrations tested, compared to the control through Student's *t*-test (data not shown). In contrast, we found significantly lower GSH levels (0.66 to 0.41 fold, $p<0.01$) at all GGC concentrations tested, compared to the control (Fig. 5). GSH levels showed a negative

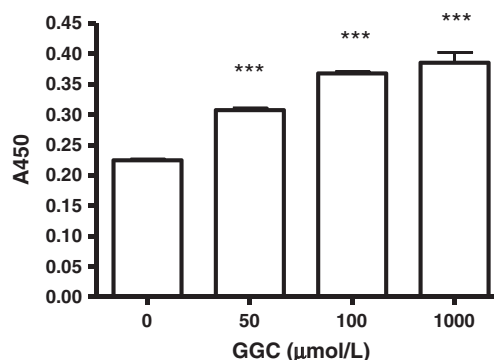


Fig. 1. Peroxisome proliferator-activated receptor γ (PPAR γ) DNA binding levels in human umbilical vein endothelial cells (HUVEC) after treatment with graded concentrations of 0, 50, 100, or 1000 $\mu\text{mol/L}$ of γ -glutamylcysteine (GGC). All treatments resulted in a significant increase in PPAR γ DNA binding levels, compared to the control treated with 0 $\mu\text{mol/L}$ of GGC through Student's *t*-test. Values are means $\pm$ SD. *** $p<0.005$. All test samples were replicated ($n=4$).

correlation with PPAR γ DNA binding levels ($p<0.05$) and positive correlation trends with NF- κ B p65 DNA binding ($p=0.059$), 8-epi-PGF $_{2\alpha}$, ($p=0.080$), and TBARS ($p=0.129$) levels through Pearson's correlations.

GSH synthetase (GSS) expression

We found statistically lower GSS protein levels (0.93 to 0.85 fold, $p<0.005$), compared with the control through Student's *t*-test (Fig. 6) after GGC incubation, though no significant change in GSS mRNA levels was observed at any GGC concentrations tested (data not shown). GSS protein levels showed a positive correlation with GSH levels ($p<0.01$), negative correlation with PPAR γ DNA binding levels ($p<0.05$), and positive correlation trends with NF- κ B p65 DNA binding ($p=0.076$), 8-epi-PGF $_{2\alpha}$ ($p=0.065$), and TBARS levels ($p=0.093$) through Pearson's correlations.

Transcription factor binding site search

One putative binding site for human NF- κ B was found at −4 227 bases in the GSS promoter region, whereas no binding site for human PPARs was identified, consistent with Lee et al.'s study (Lee et al. 2005). Similarly, there was one putative binding site for NF- κ B at −2 537-base in the GCS-HS promoter region, but none for PPARs, consistent with previous studies (Iwanaga et al. 1998; Kurozumi and Kojima 2005; Yang et al. 2005).

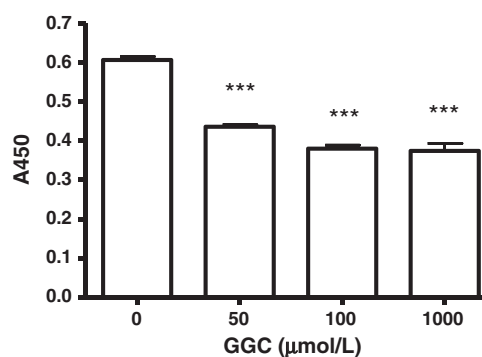


Fig. 2. Nuclear factor- κ B (NF- κ B) p65 DNA binding levels in human umbilical vein endothelial cells (HUVEC) after treatment with graded concentrations of 0, 50, 100, or 1000 $\mu\text{mol/L}$ of γ -glutamylcysteine (GGC). All treatments resulted in a significant decrease in NF- κ B p65 DNA binding levels, compared to the control treated with 0 $\mu\text{mol/L}$ of GGC through Student's *t*-test. Values are means $\pm$ SD. *** $p<0.005$. All test samples were replicated ($n=4$).

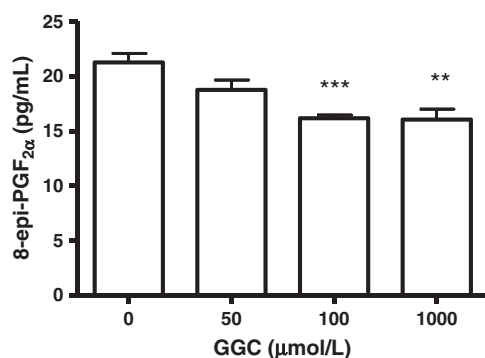


Fig. 3. Extracellular levels of 8-epi-PGF_{2α} after treatment with graded concentrations of 0, 50, 100, or 1000 μmol/L of γ-glutamylcysteine (GGC). There was a significant decrease in 8-epi-PGF_{2α} levels at higher concentrations of GGC, compared to the control treated with 0 μmol/L of GGC through Student's *t*-test. Values are means ± SD. ** *p* < 0.01 and *** *p* < 0.005. All test samples were replicated (*n* = 4).

Discussion

In our current study, GGC appears to lower oxidative stress levels (8-epi-PGF_{2α} and TBARS), despite in the presence of lower GSH levels. Our findings indicate antioxidant effects of GGC, possibly due to a -SH group in its structure. Although we found that in vitro GGC enrichment to oils (0, 0.05, 0.1, 1, 5 and 10 mmol/L) failed to inhibit lipid peroxidation (as TBARS, unpublished data not shown), a substitute role of GGC for the antioxidant GSH has been suggested in the absence of GSH in yeast and human subjects (Grant et al. 1997; Ristoff et al. 2002). If GGC can serve as a substitute of GSH or an antioxidant, rather than as a substrate for GSH synthesis, under our tested conditions or low oxidative conditions, GGC itself can lower oxidative stress levels even in the presence of lower GSH levels. However, it remains to be determined: 1) what factors influence the preference/tendency of GGC to become a substitute for GSH or substrate for GSH synthesis; 2) whether exogenous/excess GGC is shunted away from GSH synthesis; or 3) whether excess of GGC affects the stability or turnover of existing GSH.

Although its change is physiologically small, GGC also appears to, at least statistically, suppress GSS translation and/or stimulate GSH degradation/turnover, suggesting GGC-mediated inhibition on GSH synthesis. In fact, other investigators have reported mechanisms by which GSH synthesis is regulated by either endogenous or exogenous compounds. GCS, an enzyme in GSH synthesis *de novo*, is rate-limiting and feedback-inhibited by GSH and GGC in mammalian cells (Komlosch et al. 2001; Ristoff et al. 2002), but not yeast (Grant

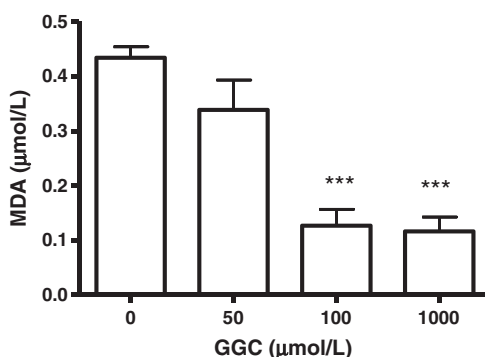


Fig. 4. Thiobarbituric acid reactive substance (TBARS) levels in cell homogenate of human umbilical vein endothelial cells (HUVEC) after treatment with graded concentrations of 0, 50, 100, or 1000 μmol/L of γ-glutamylcysteine (GGC). There was a significant decrease in TBARS levels (as MDA equivalent) at higher concentrations of GGC, compared to the control treated with 0 μmol/L of GGC through Student's *t*-test. Values are means ± SD. *** *p* < 0.005. All test samples were replicated (*n* = 4).

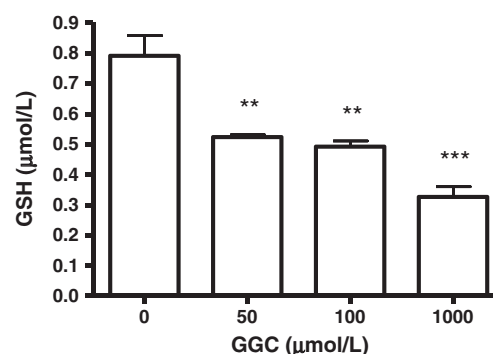


Fig. 5. Glutathione (GSH) levels in human umbilical vein endothelial cells (HUVEC) after treatment with graded concentrations of 0, 50, 100, or 1000 μmol/L of γ-glutamylcysteine (GGC). All treatments resulted in a significant decrease in GSH levels, compared to the control treated with 0 μmol/L of GGC through Student's *t*-test. Values are means ± SD. ** *p* < 0.01 and *** *p* < 0.005. All test samples were replicated (*n* = 4).

et al. 1997), and its age-related reduced activity results in decreased GSH levels in rat brain (Zhu et al. 2006). Similar to GCS-HS (Arab et al., 2006a, 2006b), GSS is an inducible enzyme (Nefedova et al. 2007). Inducers of GCS, such as treatments with sulfoximine and partial hepatectomy, also induce GSS expression in rat and human hepatocytes, and induction of both GSS expression and GSH synthesis occurs simultaneously (Huang et al. 2000; Huang et al. 2001). In addition, exogenous substrates for GSH synthesis are more effective when GSH are depleted after viral infection, but not before the infection or under normal conditions (Takagi et al. 2010). Moreover, induction of both GSS gene expression and GSH levels was observed with increased TBARS levels in ApoE^{−/−} mice fed folate and vitamin E deficient diets (Tchantchou et al. 2004). Correspondingly, we found that lower levels of both GSS protein and GSH occurred with lower levels of NF-κB p65 DNA binding and oxidative stress levels. Therefore, in the presence of prolonged and/or extensive oxidative stress causing GSH and/or GGC depletion, GGC may, in turn, tend to stimulate GSH synthesis, serving as a substrate. Thus, GSS may be a determinant of GSH synthesis capacity, similar to GCS (Huang et al. 2000; Komlosch et al. 2001; Ristoff et al. 2002).

Besides a possible substitute role of GGC, GGC may be involved in regulation of gene and protein expression, serving as a bioactive dipeptide. Amino acids (e.g., glutamate, glutamine, arginine) and/or small peptides are involved in gene and protein expression, and are associated with PPARγ, NF-κB, and antioxidant defense (Xu et al. 2005; Sato et al. 2006; Erdmann et al. 2008; Ringseis et al. 2009; White et al. 2009; Wu 2009; Brasse-Lagnel et al. 2010; Coeffier and Dechelotte 2010). Small (up to tripeptides) and large (up to 51

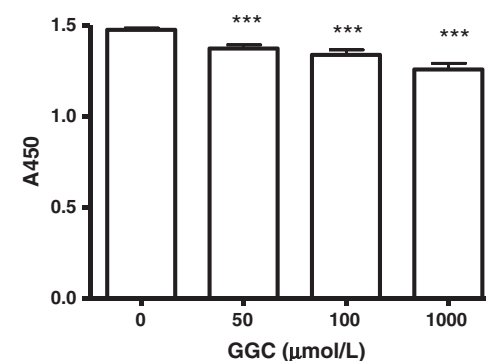


Fig. 6. Glutathione synthetase (GSS) protein levels in human umbilical vein endothelial cells (HUVEC) after treatment with graded concentrations of 0, 50, 100, or 1000 μmol/L of γ-glutamylcysteine (GGC). All treatments resulted in a significant decrease in GSH levels, compared to the control treated with 0 μmol/L of GGC through Student's *t*-test. Values are means ± SD. *** *p* < 0.005. All test samples were replicated (*n* = 4).

amino acids) peptides, possibly including GGC, can be taken up intact through plasma membranes via Na⁺-coupled peptide transporter 1 (PEPT1) and transporter 2 (PEPT2) in various tissues, such as intestine, brain, eye, kidney, lung, mammary gland, and prostate (Rubio-Aliaga et al. 2003; Zhou et al., in press; Chothe et al., 2011), and produce biological effects at the tissue levels (Roberts et al. 1999). Also, these peptide transporters are pharmacologically important to deliver drugs with cell-penetrating peptides, generally containing positively charged (e.g., arginine) and hydrophobic (e.g., lysine) groups (Rubio-Aliaga et al. 2003). Therefore, we currently investigate a role of GGC as a cell-penetrating peptide by assessing its cellular uptake using an animal model. Administration of dipeptides influence atherosclerotic development in apo E-deficient mouse models (Matsui et al. 2010) and immune responses and mRNA expression of GCS-HS in both humans and mice with compromised immune functions (Murakami et al. 2009; Takagi et al. 2010). Oligopeptides inhibit TNF α -induced activation of NF- κ B in human aortic endothelial cells (Ringseis et al. 2009) and ROS formation by inducing the expression of antioxidant enzymes, superoxide dismutase (SOD) and heme oxygenase, in mice (White et al. 2009). Thus, it is likely that GGC can also play a role in regulation of gene and/or protein expression as a bioactive dipeptide.

We found one putative binding site for NF- κ B at 2 537 bases upstream from the transcription start site of GCS-HS gene, but no binding sites for PPARs, suggesting NF- κ B-mediated GCS-HS gene expression. Also, a putative binding site for NF- κ B was found at -4 227 bases in the GSS promoter, but none for PPARs. These findings are consistent with previous studies which documented the existence of NF- κ B binding site in both GSS and GCS-HC promoters (Iwanaga et al. 1998; Kurozumi and Kojima 2005; Lee et al. 2005; Yang et al. 2005). Overexpression with either NF- κ B p50 or p65 increases promoter activities of GSS and GCS-modifier subunit (GCS-LC) (Yang et al. 2005). Deletion or mutagenesis of NF- κ B binding site in the GCS-HC promoter down-regulates GSH induction by nitroprusside (Kurozumi and Kojima 2005). In addition, the SH group of cysteine 62 of the NF- κ B p50 subunit is an important determinant of DNA recognition by the NF- κ B p50 subunit, and the DNA binding of NF- κ B p50 subunit is stimulated by reducing agents (Matthews et al. 1993), including perhaps GGC which contains a -SH group. The NF- κ B p50 homodimer binds DNA at NF- κ B p50/p65 heterodimer recognition sites (Muller et al. 1995), and the homodimer can act as either an activator or suppressor in gene regulation resulting in anti-inflammatory effects (Cao et al. 2006). Hence, there may be an involvement of either the NF- κ B p50/p65 heterodimer (de)activation or NF- κ B p50 homodimer formation in regulation of GSS gene expression. Our findings may support the idea, oxidative stress up-regulates, rather than down-regulates, expression of proteins involved in GSH synthesis (Iwanaga et al. 1998; Liu and Choi 2000) and antioxidant enzymes (Catani et al. 2004), perhaps through NF- κ B p50/p65 pathway, despite no significant change in GSS mRNA levels observed. It is noteworthy that we found significantly higher levels of PPAR γ DNA binding levels with lower levels of oxidative stress in a concentration-dependent manner in our current study. Our observation suggests that induction of other antioxidant enzymes, such as Cu/Zn SOD and catalase, through PPAR γ pathway (Nakamura and Omaye 2009; Okuno et al., 2010) partially contributes to lowering oxidative stress levels. Thus, redox-sensitive transcription factors, PPAR γ and NF- κ B, may be involved in gene expression of various proteins related to antioxidant defense. As a consequence, these transcription factors may modulate oxidative stress in a coordinated fashion. The exploration of endogenous and exogenous antioxidant defense network would be among future studies.

Riboswitches are mechanisms by which amino acids and/or metabolites regulate translation. Riboswitches are cis-acting RNA elements and monitor a physiological signal (Smith et al. 2010). Many riboswitches have been reported to exist in prokaryotes and plants, where amino acids (e.g., lysine, glycine), coenzymes (e.g. thiamine

pyrophosphate, flavin mononucleotide), and purines (e.g., guanine, adenine) serve as ligands of riboswitches to regulate translation for mainly amino acid synthesis (Blount and Breaker 2006). Recently, the first human riboswitch has been discovered in the mRNA 3' untranslated region (UTR) of human vascular endothelial growth factor-A (VEGF). This stress-responsive RNA switch senses two disparate stress stimuli, and up- or down-regulates VEGF translation (Ray et al. 2009). The investigators anticipate more human riboswitches to be discovered. In our study, we found no significant change of GSS mRNA levels, despite lower levels of GSS protein, GSH, and NF- κ B p65 DNA binding, suggesting post-transcriptional (or translational) inhibition. The notion of riboswitch-dependent mechanisms may provide partial explanation for our findings.

Conclusion

GGC plays a role in GSH synthesis as a substrate and in protection from oxidative stress by serving as a substitute for antioxidant GSH and modulating expression of proteins related to antioxidant defense as an inducer or suppressor. Further investigations would be warranted to elucidate GGC-mediated mechanisms to regulate the antioxidant defense network. However, GGC may serve as a novel intra- and intercellular therapeutic agent for oxidative stress-related injuries and diseases under optimized conditions, due to its permeability through cell membranes. Based on the current results, further studies to investigate GGC in an animal model of injury or disease are warranted.

Conflict of interest statement

None.

Acknowledgments

This work was supported by federal funds from U.S. Army (USAMRMC09070005).

We thank Dr. Stephen de St. Jeor for providing the access to his cell culture facility.

References

- Arab K, Rossary A, Flourie F, Tournier Y, Steghens JP. Docosahexaenoic acid enhances the antioxidant response of human fibroblasts by upregulating gamma-glutamyl-cysteine ligase and glutathione reductase. *Br J Nutr* 2006a;95(1):18–26.
- Arab K, Rossary A, Souler L, Steghens J. Conjugated linoleic acid, unlike other fatty acids, strongly induces glutathione synthesis without any lipoperoxidation. *Br J Nutr* 2006b;96:811–9.
- Blount K, Breaker RR. Riboswitches as antibacterial drug targets. *Nat Biotechnol* 2006;24(12):1558–64.
- Brasse-Lagnel C, Lavoinne AM, Husson AS. Amino acid regulation of mammalian gene expression in the intestine. *Biochimie* 2010;92(7):729–35.
- Burge JA, Aust SD. Microsomal lipid peroxidation. *Methods in Enzymology* Colowick SP and Kaplan NO, 52. New York, USA: Academic Press, Inc.; 1978. p. 302–10.
- Cao S, Zhang X, Edwards JP, Mosser DM. NF- κ B1 (p50) homodimers differentially regulate pro- and anti-inflammatory cytokines in macrophages. *J Biol Chem* 2006;281(36):26041–50.
- Catani MV, Savini I, Duranti G, Caporossi D, Ceci R, Sabatini S, Avigliano L. Nuclear factor κ B and activating protein 1 are involved in differentiation-related resistance to oxidative stress in skeletal muscle cells. *Free Radic Biol Med* 2004;37(7):1024–36.
- Chothe P, Singh N, Ganapathy V. Evidence for two different broad-specific oligopeptide transporters in the intestinal cell line Caco-2 and the colonic cell line CCD841. *Am J Physiol Cell Physiol* 2011;300(6):C1260–9.
- Coeffier M, Dechelotte P. Combine infusion of glutamine and arginine: does it make sense? *Curr Opin Clin Nutr Metab Care* 2010;13:70–4.
- Dringen R, Kranich O, Loschmann PA, Hamprecht B. Use of dipeptides for the synthesis of glutathione by astroglia-rich primary cultures. *J Neurochem* 1997;69(2):868–74.
- Dutton RP. Current concepts in hemorrhagic shock. *Anesthesiol Clin* 2007;25(1):23–34.
- Erdmann K, Cheung BWY, Schroder H. The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease. *J Nutr Biochem* 2008;19:643–54.

- Franco R, Schoneveld OJ, Pappa A, Panayiotidis MI. The central role of glutathione in the pathophysiology of human diseases. *Arch Physiol Biochem* 2007;113(4–5):234–58.
- Giustarini D, Milzani A, Dalle-Donne I, Rossi R. Red blood cells as a physiological source of glutathione for extracellular fluids. *Blood Cells Mol Dis* 2008;40:174–9.
- Glantzounis GK, Yang W, Koti RS, Mikhailidis DP, Seifalian AM, Davidson BR. The role of thiols in liver ischemia-reperfusion injury. *Curr Pharm Des* 2006;12:2891–901.
- Granger DN, Korthuis RJ. Physiologic mechanisms of postschismic tissue injury. *Annu Rev Physiol* 1995;57:311–32.
- Grant CM, MacIver FH, Dawes IW. Glutathione synthetase is dispensable for growth under both normal and oxidative stress conditions in the yeast *Saccharomyces cerevisiae* due to an accumulation of the dipeptide γ -glutamylcysteine. *Mol Biol Cell* 1997;8:1699–707.
- Hagenfeldt L, Arvidsson A, Larsson A. Glutathione and γ -glutamylcysteine in whole blood, plasma and erythrocytes. *Clin Chim Acta* 1978;85:167–73.
- Huang ZA, Yang H, Chen C, Zeng Z, Lu SC. Inducers of gamma-glutamylcysteine synthetase and their effects on glutathione synthetase expression. *Biochem Biophys Acta* 2000;1493(1–2):48–55.
- Huang ZZ, Chen C, Zeng Z, Yang H, Oh J, Chen L, Lu SC. Mechanism and significance of increased glutathione level in human hepatocellular carcinoma and liver regeneration. *FASEB J* 2001;15(1):19–21.
- Iwanaga M, Mori K, Iida T, Urata Y, Matsuo T, Yasunaga A, Shibata S, Kondo T. Nuclear factor kappa B dependent induction of gamma glutamylcysteine synthetase by ionizing radiation in T98G human glioblastoma cells. *Free Radic Biol Med* 1998;24(7–8):1256–68.
- Komlos A, Volohonsky G, Porat N, Tuby C, Bluvshstein E, Steinberg P, Oesch F, Stark AA. γ -Glutamyl transpeptidase and glutathione biosynthesis in non-tumorigenic and tumorigenic rat liver oval cell lines. *Carcinogenesis* 2001;22(12):2009–16.
- Krausz MM. Initial resuscitation of hemorrhagic shock. *World J Emerg Surg* 2006;1:14.
- Kurozumi R, Kojima S. Increase of intracellular glutathione by low-level NO mediated by transcription factor NF-kappaB in RAW 264.7 cells. *Biochem Biophys Acta* 2005;1744(1):58–67.
- Lee TD, Yang H, Whang J, Lu SC. Cloning and characterization of the human glutathione synthetase 5'-flanking region. *Biochem J* 2005;390:521–8.
- Li C, Jackson RM. Reactive species mechanisms of cellular hypoxia-reoxygenation injury. *Am J Physiol Cell Physiol* 2002;282(2):C227–41.
- Liu RM, Choi J. Age-associated decline in γ -glutamylcysteine synthetase gene expression in rats. *Free Radic Biol Med* 2000;28(4):566–74.
- Matsui T, Sato M, Tanaka M, Yamada Y, Watanabe S, Fujimoto Y, Imaizumi K, Matsumoto K. Vasodilating dipeptide Trp-His can prevent atherosclerosis in apoE-deficient mice. *Br J Nutr* 2010;103:309–13.
- Matthews JR, Kaszubska W, Turcatti G, Wells TNC, Hay RT. Role of cycteine62 in DNA recognition by the p50 subunit of NF-kappaB. *Nucleic Acids Res* 1993;21(8):1727–34.
- Muller CW, Rey FA, Sodeoka M, Verdine GL, Harrison SC. Structure of the NF-kappaB p50 homodimer bound to DNA. *Nature* 1995;373(6512):311–7.
- Murakami S, Kurihara S, Koikawa N, Nakamura A, Aoki K, Yosigi H, Sawaki K, Ohtani M. Effects of oral supplementation with cystine and theanine on the immune function of athletes in endurance exercise: randomized, double-blind, placebo-controlled trial. *Biosci Biotechnol Biochem* 2009;73(4):817–21.
- Nakamura YK, Omaye ST. Vitamin E-modulated gene expression associated with ROS generation. *J Funct Foods* 2009;1:241–52.
- Nefedova Y, Fishman M, Sherman S, Wang X, Beg AA, Gabrilovich DI. Mechanism of all-trans retinoic acid effect on tumor-associated myeloid-derived suppressor cells. *Cancer Res* 2007;67(22):11021–8.
- Okuno Y, Matsuda M, Miyata Y, Fukuhara A, Komoro R, Shimabukuro M, Shimomura I. Human catalase gene is regulated by peroxisome proliferator-activated receptor-gamma through a response element distinct from that of mouse. *Endocr J* 2010;57(4):303–9.
- Ray PS, Jia J, Yao P, Majumder M, Hatzoglou M, Fox PL. A stress-responsive RNA switch regulates VEGFA expression. *Nature* 2009;457:915–20.
- Ringseis R, Gotze V, Eder K. Tripeptides from dietary proteins inhibit TNFalpha-induced monocyte adhesion to human aortic endothelial cells. *Regul Pept* 2009;154(1–3):91–6.
- Ristoff E, Hebert C, Njalsson R, Norgren S, Rooyackers O, Larsson A. Glutathione synthetase deficiency: Is γ -glutamylcysteine accumulation a way to cope with oxidative stress in cells with insufficient levels of glutathione? *J Inherit Metab Disord* 2002;25:577–84.
- Roberts PR, Burney JD, Black KW, Zaloga GP. Effect of chain length on absorption of biologically active peptides from the gastrointestinal tract. *Digestion* 1999;60:332–7.
- Rubio-Aliaga I, Frey I, Boll M, Groneberg DA, Eichinger HM, Balling R, Daniel H. Targeted disruption of the peptide transporter *Pept2* gene in mice defines its physiological role in the kidney. *Mol Cell Biol* 2003;23(9):3247–52.
- Sato N, Moore FA, Kone BC, Zou L, Smith MA, Childs MA, Moore-Olufemi S, Scheultz SG, Kozar RA. Differential induction of PPAR- γ by luminal glutamine and iNOS by luminal arginine in the rodent postschismic small bowel. *Am J Physiol Gastrointest Liver Physiol* 2006;290:G616–23.
- Smith AM, Fuchs RT, Grundy FJ, Henkin TM. Riboswitch RNAs: regulation of gene expression by direct monitoring of a physiological signal. *RNA Biol* 2010;7(1):104–10.
- Takagi Y, Kurihara S, Higashi N, Morikawa S, Kase T, Maeda A, Arisaka H, Shibahara S, Akiyama Y. Combined administration of L-cystine and L-theanine enhances immune functions and protects against influenza virus infection in aged mice. *Immunology* 2010;72(2):157–65.
- Tchantchou F, Graves M, Ashline D, Morin A, Pimenta A, Ortiz D, Rogers E, Shea TB. Increased transcription and activity of glutathione synthetase in response to deficiencies in folate, vitamin E, and apolipoprotein E. *J Neurosci Res* 2004;75:508–15.
- Vincent HK, Innes KE, Vincent KR. Oxidative stress and potential interventions to reduce oxidative stress in overweight and obesity. *Diabetes Obes Metab* 2007;9:813–39.
- White CR, Datta G, Mochon P, Zhang Z, Kelly O, Curcio C, Parks D, Palgunachari M, Handattu S, Gupta H, Garber DW, Anantharamaiah GM. Vasculoprotective effects of apolipoprotein mimetic peptides: an evolving paradigm in HDL therapy (Vascular disease prevention, In Press). *Vasc Dis Prev* 2009;6:122–30.
- Wu G. Amino acids: metabolism, functions, and nutrition. *Amino Acids* 2009;37:1–17.
- Wu G, Fang Y, Yang S, Lupton JR, Turner ND. Glutathione metabolism and its implications for health. *J Nutr* 2004;134:489–92.
- Xu L, Sun J, Lu R, Ji Q, Xu J. Effect of glutamate on inflammatory responses of intestine and brain after focal cerebral ischemia. *World J Gastroenterol* 2005;11(5):733–6.
- Yang H, Magilnick N, Ou X, Lu SC. Tumour necrosis factor alpha induces coordinated activation of rat GSH synthetic enzymes via nuclear factor kappaB and activator protein-1. *Biochem J* 2005;391(Pt 2):399–408.
- Zeevalk GD, Razmpour R, Bernard LP. Glutathione and Parkinson's disease: is this the elephant in the room? *Biomed Pharmacother* 2008;62:230–49.
- Zhou J, Liu W, Pong RC, Hao G, Sun X, Hsieh JT. Analysis of oligo-arginine cell-permeable peptides uptake by prostate cells. *Amino Acids* in press, doi:10.1007/s00726-010-0817-7.
- Zhu Y, Carvey PM, Ling Z. Age-related changes in glutathione and glutathione-related enzymes in rat brain. *Brain Res* 2006;1090(1):35–44.