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PRINCIPAL INVESTIGATOR: Stanley T. Omaye, Ph.D.

CONTRACTING ORGANIZATION: Nevada System of Higher Education
Reno, NV 89557-001

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14. ABSTRACT Experiments were performed to study Gamma-glutamylcysteine (GGC) a precursor dipeptide for glutathione (GSH) for its ability to inhibit oxidative stress in human umbilical vein endothelial cells (HUVEC). HUVEC were used as a model to assess cellular biochemical changes that occur in hemorrhagic shock. We found that GGC protects against oxidative stress by alteration of gene expression of antioxidant defense independent of increased induction of endogenous GSH synthesis. In mixture studies looking at the effects of GGC with conjugated linoleic acid in HUVEC, we found that the mixture protected against oxidative stress similar to treatment with GGC alone. Therefore, GGC is likely a unique compound that exerts protection from oxidative stress by novel by modulation of gene expression of antioxidant defense enzymes. We have also develop a sensitive high performance liquid chromatographic method (HPLC) using fluorimetric detection to measure cellular changes in GGC.					
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INTRODUCTION:

Hemorrhagic shock is a leading cause of death in military combat and civilian trauma (Bellamy, 1984; Lieu, et al., 2004). Better understanding of the associated cellular biochemical changes that occur in ischemia-reperfusion (IR) injury can lead to efficacious therapies (Thomas et al., 2008). Therefore, we studied the feasibility of using gamma-glutamylcysteine (GGC) a dipeptide precursor for glutathione as a potential compound in modulating the oxidative stress associated with IR injury.

BODY:

Specific goal – Determine GGC doses to inhibit cellular oxidative stress and inhibit cellular death.

1. GGC inhibition of oxidative stress in human endothelial cells.

The objective of this study was to investigate the efficacy of GGC on GSH synthesis and oxidative stress in human endothelial cells, as a model for cellular oxidative stress. We found that GGC plays a role in GSH synthesis as a substrate for the antioxidant GSH and in modulating expression of proteins related to antioxidant defense as an inducer or suppressor.

2. Co administration of GGC and conjugated linoleic acid (CLA) in human endothelial cells.

The objective of this study was compare effects of co-administration of GGC and CLA with GGC alone on oxidative stress. We confirmed that GGC can substitute as an antioxidant for GSH without increasing GSH levels. Co-administration of CLA with GGC had differential effects depending on the dose of CLA. We believe that due to its ease of permeability through cell membranes, GGC could be used as an intra and intercellular therapeutic agent in oxidative stress-related injuries and diseases.

KEY RESEARCH ACCOMPLISHMENTS:

Two studies using human endothelial cells have been completed, peer reviewed and published. The studies indicate that GGC has efficacy in oxidative stress and suggest the potential usefulness of this compound in injuries and diseases associated with oxidative stress.

A sensitive method using high performance liquid chromatography (HPLC) and fluorimetric detection has been developed.

REPORTABLE OUTCOMES:

Published Abstracts

Nakamura, Y. K., Dubick, M. A., and **Omaye, S. T.** Gamma-Glutamylcysteine (GGC) inhibition of oxidative stress in human endothelial cells. Emerging Topics Section, Society of Toxicology Annual Meeting, 2011, Washington, D.C.

Nakamura, Y. K., Dubick, M. A., and **Omaye, S. T.** Effects of co-administration of gamma-glutamylcysteine (GGC) and conjugated linoleic acid (CLA) on oxidative stress in human endothelial cells. American Chemical Society Annual meeting, Denver, Colorado, 2011.

Published Peer-Reviewed Manuscripts

Nakamura, Y.K., Dubick, M.A., and Omaye, S. T. Gamma-glutamylcysteine inhibits oxidative stress in human endothelial cells. *Life Sciences* 90: 116-121, 2012.

Nakamura, Y.K., Dubick, M.A., and Omaye, S. T. Modulation of oxidative stress by γ -glutamylcysteine (GGC) and conjugated linoleic acid (CLA) isomer mixture in human umbilical vein endothelial cells. *Food and Chemical Toxicol* 60: 1854-1859, 2012.

CONCLUSION:

Although further studies are warranted to develop a better understanding about the efficacy of GGC supplementation under various conditions, GGC has potential as a therapeutic compound in modulation of oxidative stress. Additional studies are also needed to better understand the mechanisms of action and eventual application of GGC in oxidative stress associated with ischemia reperfusion and hemorrhagic shock

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Liu, L-M, Hu, D-Y, Chen, H-S, and Hu, P-H. 2004. The effect of different volumes of fluid resuscitation on traumatic-hemorrhagic shock at high altitude in the unacclimated rat. *Shock*. 21: 93-96.

Thomas, w., Krah, J. F., Kauvar, D. S. and Baer, D. G. 2008. The combined influence of hemorrhage and tourniquet application on the recovery of muscle function in rat. *J Ortho Trauma* 22: 47-51.

APPENDICES:

1. Abstract for published manuscript in Life Sciences
2. Abstract for published manuscript in Food and Chemical Toxicology



Modulation of oxidative stress by γ -glutamylcysteine (GGC) and conjugated linoleic acid (CLA) isomer mixture in human umbilical vein endothelial cells

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

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

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

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ABSTRACT

Individually, γ -glutamylcysteine (GGC), a dipeptide and precursor of glutathione (GSH), and conjugated linoleic acid (CLA), a trans-fatty acid, exhibit antioxidant properties. The objective of this study was to compare effects of co-administration of GGC and CLA to treatment with GGC alone on oxidative stress and GSH synthesis in human endothelial cells. Changes in levels of 8-epi-PGF2 α , thiobarbituric acid reactive substances (TBARS), GSH, total antioxidants, GSH synthetase (GSS) expression, and transcription factor DNA binding were assessed in human umbilical vein endothelial cells (HUVEC) treated with GGC alone (100 μ mol/L) or combined with CLA isomer mixture (10, 50, 100 μ mol/L) for 24 h. Significantly higher levels of TBARS, 8-epi-PGF2 α , GSH, and GSS protein were found in cells treated with GGC and 10 μ mol/L CLA, compared to cells treated with GGC alone, indicative of prooxidant effects of CLA. Approximately 40% cell death was microscopically observed in cells incubated with GGC and 100 μ mol/L CLA. Despite lower levels of GSH, treatment with GGC and 50 μ mol/L CLA appeared to be protective from oxidative stress similar to treatment with GGC alone, as indicated by lower levels of TBARS, compared to control cells not treated with GGC and CLA.

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1. Introduction

Oxidative stress is associated with various clinical conditions (e.g., ischemia–reperfusion injury) and chronic diseases (Granger and Korthuis, 1995; Li and Jackson, 2002; Willcox et al., 2004). Glutathione (GSH) is the prevalent thiol-containing tripeptide antioxidant in mammalian cellular systems, intracellularly present at millimolar concentrations (Glantzounis et al., 2006; Franco et al., 2007). Increasing GSH levels could be beneficial for modulating oxidative stress-related injuries, diseases, and aging (Liu and Choi, 2000; Wu et al., 2004; Zeevalk et al., 2008). γ -Glutamylcysteine (GGC) is a dipeptide and precursor of GSH. GGC is synthesized by catalytic activity of GGC synthetase (GCS) from glutamate and cysteine. GSH is subsequently produced by the activity of GSH synthetase (GSS) from GGC and glycine (Franco et al., 2007). In healthy humans, intracellular (erythrocytes) and extracellular (plasma) GGC levels are approximately 66 and 4 μ mol/L, respectively (Hagenfeldt et al., 1978). Unlike GSH, GGC uptake is not limited by plasma membranes or the blood brain barrier, and supplemental GGC can be directly used as a substrate for GSH synthesis (Dringen et al., 1997). Peptides with up to 51 amino acids, perhaps including GGC, can be taken up intact through plasma membranes

via Na⁺-coupled peptide transporter 1 (PEPT1) and transporter 2 (PEPT2) in various tissues (Rubio-Aliaga et al., 2003; Zhou et al., 2012; Chothe et al., 2011). In our recent GGC study (Nakamura et al., 2012), GGC appears to protect against oxidative stress by serving as a substitute for antioxidant GSH due to a SH group in its structure and modulating GSH synthesis.

Conjugated linoleic acid (CLA) has been reported to exhibit health promoting properties, such as anti-obesity, anti-carcinogenic, anti-inflammatory, and anti-atherogenic effects (Belury, 2002; Nakamura and Omaye, 2008; Kennedy et al., 2010; Gebauer et al., 2011). Previous studies have indicated that co-administration of nutraceuticals such as CLA with pharmaceuticals can augment the effects of the individual compounds. For instance, co-administration of CLA with a drug such as rosiglitazone or addition of resveratrol to the trans-10, cis-12 CLA isomer attenuates adverse effects associated with each compound (Liu et al., 2007; Kennedy et al., 2009; Halade et al., 2010). In addition, CLA can modulate oxidative stress by up-regulating GGC synthetase catalytic unit (GCS-HC) and subsequent GSH synthesis (Arab et al., 2006). Both GGC and CLA exhibit antioxidant properties. The heterogeneous nature of diets provided an environment for various interactions and relationships between endogenous/exogenous dietary substances. Our current interest is to establish a better understanding of such interactions and the subsequent effects on the antioxidant capacity of mixtures, including concentration dependent effects. These were thought to be timely studies because of the interest in the effects

* Corresponding author. Address: 1664 North Virginia Street, Reno, NV 89557, USA. Tel.: +1 775 784 6447; fax: +1 775 784 1375.

E-mail addresses: omaye@cabnr.unr.edu, omaye@unr.edu (S.T. Omaye).

in co-administration of multiple pharmaceuticals. In addition, it is crucial to evaluate the concept that each chemical has an optimal concentration range for beneficial effects with possible detrimental effects beyond such range. The objectives of this study were to investigate a synergistic antioxidant role of CLA as an adjuvant and the effects of mixtures of compounds/co-administration seeking an optimal concentration range for beneficial effects by comparing co-administration of GGC and CLA to treatment with GGC alone on oxidative stress and GSH synthesis in human endothelial cells. Since CLA-induced adverse effects, such as increases in insulin resistance and inflammation, have been observed mainly by use of single purified CLA isomer (in particular the *trans*-10, *cis*-12-CLA isomer, but not the *cis*-9, *trans*-11-CLA isomer) (Halade et al., 2010; Kennedy et al., 2010; Martinez et al., 2010), a mixture of CLA isomers was chosen in this study. We assessed changes in levels of 8-epi-PGF_{2α}, thiobarbituric acid reactive substances (TBARS), GSH, total antioxidants, GSS expression, and PPARγ and NF-κB DNA binding in human umbilical vein endothelial cells (HUVEC) treated with GGC alone (100 μmol/L: constant) or GGC together with CLA (the *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA isomer mixture; 50% each) at graded concentrations.

2. Materials and methods

2.1. Chemicals and reagents

GGC was purchased from Bachem (Torrance, CA, USA). EGM Complete Medium (#CC-3024), HEPES Buffered Saline, and Subculture 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). CLA isomers (the *cis*-9, *trans*-11- and *trans*-10, *cis*-12-CLA isomer mixture), Nuclear Extraction Kit, PPARγ and NF-κB (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-phenylenediaminedihydrochloride tablets (SIGMAFAST OPD), and Extravidin Peroxidase Staining Kit (EXTRA3) were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Cell culture

Human umbilical vein endothelial cells (HUVEC) were chosen in this study, since these cells are commonly used for investigations of molecule transport. HUVEC (#CC-2517) cryogenically preserved were purchased from Lonza. 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.

2.3. Cell treatments and viability

HUVEC were grown on 75 cm² gelatin-coated flasks or 96-well gelatin-coated plate, and approximately ≥95% confluent cells (~10⁷ cells) were treated with GGC alone (100 μmol/L), GGC (100 μmol/L: constant) and CLA (the *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA isomer mixture; 50% each; 0, 10, 50, 100 μmol/L), or not treated with GGC and CLA (control) for 24 h at 37 °C in a humidified atmosphere with 5% CO₂ (two flasks per each treatment for nuclear fraction collection; two 96-wells per each treatment for mRNA isolation; five flasks per each treatment for cellular fraction collection). The single concentration of GGC chosen (100 μmol/L) for each treatment group was the minimum concentration to significantly reduce levels of oxidative stress shown in our previous GGC study (Nakamura et al., 2012). After treatments for 24 h, cell viability was assessed microscopically. The sixth to ninth passages of tightly confluent mono-layered cells were collected after each treatment and used for subsequent analyses.

2.4. Cytoplasmic fraction preparation

After 24-h incubation with GGC alone or together with CLA, 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 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 for assay of thiobarbituric acid reactive substances (TBARS). The remaining cell homogenate was centrifuged for 15 min at 4 °C and 10,000g. Supernatant (cytoplasmic fractions) was stored at –70 °C for assays of GSH, GSS protein, and total antioxidants. All assays were performed within one month after the sample collection, except GSS protein immunoassay which was done within 2 months.

2.5. Extracellular fraction collection

Extracellular fractions of HUVEC were collected for the 8-epi-PGF_{2α} immunoassay. The medium of confluent cell culture was collected just before harvesting confluent cells. Samples were stored at –70 °C until the 8-epi-PGF_{2α} immunoassay was performed within one month.

2.6. Nuclear fraction preparation

Nuclear fractions of HUVEC were isolated with a commercial nuclear extraction kit (Cayman Chemical). After 24-h incubation with GGC alone or together with CLA, cells were rinsed, scraped, suspended into ice-cold PBS containing phosphatase inhibitors, and centrifuged for 5 min at 4 °C and 300g. Cells were collected from two flasks per each treatment and pooled. Then, cells were suspended and lysed with a 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 14,000g. Supernatant was collected and stored at –70 °C until transcription factor assays were performed. The assays were done within three days after the sample collection.

2.7. Peroxisome proliferator-activated receptor-γ (PPARγ) and nuclear factor-κB (NF-κB) p65 transcription factor assays

Because redox sensitive transcription factors, PPARγ and NF-κB, may play a role in regulating gene expression involved in antioxidant defense (Nakamura and Omaye, 2010), PPARγ and NF-κB p65 DNA binding activities in the nuclear fractions of HUVEC were assessed with PPARγ and NF-κB (human p50/p65 combo) transcription factor assays, respectively (Cayman Chemical). Either human PPARγ bound to PPRE (5'-AGGTCAAAGGTCA-3') or human NF-κB bound to a specific sequence (5'-GGGACTTTC-3') immobilized within the bottoms of 96 wells was assessed individually at 450 nm with the enzyme-linked immunoassays. All sample tests were replicated (*n* = 4).

2.8. 8-epi PGF_{2α} enzyme immunoassay

8-epi PGF_{2α} is commonly used as a biomarker of oxidative stress along with TBARS (Vincent et al., 2007). Extracellular levels of 8-epi-PGF_{2α} (free 8-epi-PGF_{2α} 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).

2.9. Thiobarbituric acid reactive substance (TBARS) assay

Lipid peroxidation as the complex of thiobarbituric acid and malondialdehyde in the cell homogenate of HUVEC was assessed at 535 nm spectrophotometrically. A mixture of thiobarbituric acid, trichloroacetic acid, and hydrochloric acid was added to the cell homogenate, and the mixture was heated for 15 min at 100 °C (Burge and Aust, 1978). The supernatant was collected for reading spectrophotometrically after centrifugation for 10 min at 1000g. All sample tests were replicated (*n* = 4).

2.10. Glutathione (GSH) assay

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

2.11. Total antioxidant assay

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

2.12. GSH synthetase (GSS) protein immunoassay

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

2.13. 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 treatment) with a Power SYBR® Green Cells-to-CT™ Kit (Invitrogen), and was used as a template for cDNA synthesis with oligodT primers. Reverse transcription reactions were performed for 60 min at 37 °C and inactivated for 5 min at 95 °C. The cDNA was stored for 6 weeks at –20 °C until the qRT-PCR method was performed. 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 were performed for 15 s at 95 °C and for 1 min at 60 °C. Relative expression was calculated from cycle threshold values ($2^{-\Delta\Delta Ct}$ method), using 18S rRNA expression as an internal control for each sample. All sample tests were replicated ($n = 4$).

2.14. 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.

3. Results

3.1. Cell viability

Cytotoxicity, approximately 40% cell death, was microscopically observed in cells treated with GGC and 100 $\mu\text{mol/L}$ CLA after 24 h-incubation. No change in cell viability was detected microscopically in cells with other treatments after the incubation. Consequently, the highest dose of CLA used in experiments reported here was 50 $\mu\text{mol/L}$.

3.2. Transcription factor DNA binding

Compared to control cells not treated with GGC and CLA, we found significantly higher PPAR γ DNA binding levels in cells treated with GGC alone (1.48-fold, $p < 0.005$) or together with 10 $\mu\text{mol/L}$ CLA (1.63-fold, $p < 0.005$) (Fig. 1). Significantly lower levels of PPAR γ DNA binding were observed in cells treated with GGC and 50 $\mu\text{mol/L}$ CLA (0.79-fold, $p < 0.05$), compared to cells treated with GGC alone (Fig. 1). The p -values of PPAR γ DNA binding levels were <0.0001 through one-way ANOVA (Fig. 1).

In contrast to PPAR γ DNA binding levels, significantly lower levels of NF- κ B p65 DNA binding were found in cells treated with GGC alone (0.64-fold, $p < 0.005$) or together with 10 and 50 $\mu\text{mol/L}$ CLA (0.69-fold, $p < 0.005$ and 0.66-fold, $p < 0.01$, respectively) in

comparison to controls (Fig. 2). In addition, statistically higher levels of NF- κ B p65 DNA binding were observed in cells treated with GGC and 10 $\mu\text{mol/L}$ CLA (1.08-fold, $p < 0.05$) than those treated with GGC alone, though this small increase is unlikely to be significant physiologically (Fig. 2). NF- κ B p65 DNA binding levels showed a statistical significance ($p < 0.0001$) through one-way ANOVA (Fig. 2).

3.3. Oxidative stress biomarkers

Compared to controls, we observed significantly lower levels of 8-epi-PGF $_{2\alpha}$ in cells treated with GGC alone (0.76-fold, $p < 0.01$), whereas cells treated with GGC and 10 $\mu\text{mol/L}$ CLA had 12% higher 8-epi-PGF $_{2\alpha}$ levels than controls ($p < 0.01$) (Fig. 3). Compared to cells treated with GGC alone, higher levels of 8-epi-PGF $_{2\alpha}$ were found in cells treated GGC and either dose of CLA (1.47-fold, $p < 0.005$ and 1.39-fold, $p < 0.01$, respectively) (Fig. 3). 8-epi-PGF $_{2\alpha}$ levels were significant with the p -values of <0.0001 through one-way ANOVA (Fig. 3).

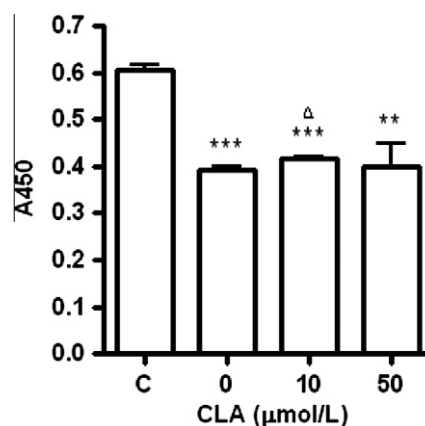


Fig. 2. Nuclear factor- κ B (NF- κ B) p65 DNA binding levels in human umbilical vein endothelial cells (HUVEC) after 24-h incubation with γ -glutamylcysteine (GGC) alone (100 $\mu\text{mol/L}$) or combined with conjugated linoleic acid (CLA; 10, 50, or 100 $\mu\text{mol/L}$). Values are means $\pm$ SD. ** $p < 0.01$ and *** $p < 0.005$, compared to control not treated with GGC and CLA through Student's *t*-test. $\Delta p < 0.05$, compared to treatment with GGC alone (100 $\mu\text{mol/L}$). The p -values of one-way ANOVA is <0.0001 . All test samples were replicated ($n = 4$).

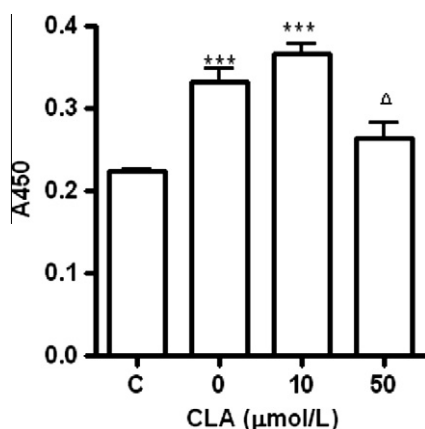


Fig. 1. Peroxisome proliferator-activated receptor γ (PPAR γ) DNA binding levels in human umbilical vein endothelial cells (HUVEC) after 24-h incubation with γ -glutamylcysteine (GGC) alone (100 $\mu\text{mol/L}$) or combined with conjugated linoleic acid (CLA; 10, 50, or 100 $\mu\text{mol/L}$). Values are means $\pm$ SD. *** $p < 0.005$, compared to control not treated with GGC and CLA through Student's *t*-test. $\Delta p < 0.05$, compared to treatment with GGC alone (100 $\mu\text{mol/L}$). The p -values of one-way ANOVA is <0.0001 . All test samples were replicated ($n = 4$).

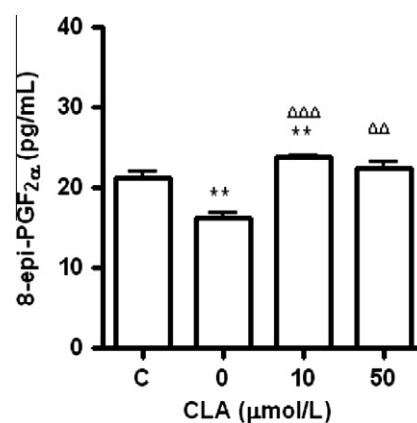


Fig. 3. Extracellular levels of 8-epi-PGF $_{2\alpha}$ after 24-h incubation with γ -glutamylcysteine (GGC) alone (100 $\mu\text{mol/L}$) or combined with conjugated linoleic acid (CLA; 10, 50, or 100 $\mu\text{mol/L}$). Values are means $\pm$ SD. ** $p < 0.01$, compared to control not treated with GGC and CLA through Student's *t*-test. $\Delta\Delta\Delta p < 0.01$ and $\Delta\Delta p < 0.005$, compared to treatment with GGC alone (100 $\mu\text{mol/L}$). The p -values of one-way ANOVA is <0.0001 . All test samples were replicated ($n = 4$).

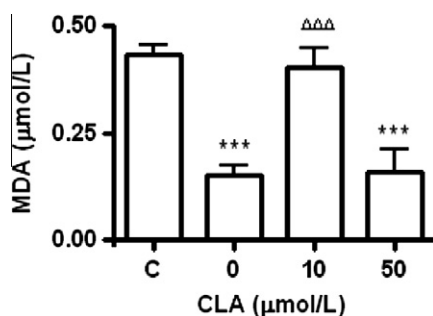


Fig. 4. Thiobarbituric acid reactive substance (TBARS) levels in cell homogenate of human umbilical vein endothelial cells (HUVEC) after 24-h incubation with γ -glutamylcysteine (GGC) alone (100 μ mol/L) or combined with conjugated linoleic acid (CLA; 10, 50, or 100 μ mol/L). Values are means $\pm$ SD. *** p < 0.005, compared to control not treated with GGC and CLA through Student's t -test. $\Delta\Delta\Delta p$ < 0.005, compared to treatment with GGC alone (100 μ mol/L). The p -values of one-way ANOVA is <0.0001. All test samples were replicated (n = 4).

Compared to controls, we found significantly lower levels of TBARS in cells treated with GGC alone (0.35-fold, p < 0.005) or GGC and 50 μ mol/L CLA (0.37-fold, p < 0.005) (Fig. 4). In contrast, TBARS concentrations were near control levels in cells treated with GGC and 10 μ mol/L CLA and were significantly higher (2.67-fold, p < 0.005), when compared to cells treated with GGC alone (Fig. 4). The p -values of TBARS levels were <0.0001 through one-way ANOVA (Fig. 4).

3.4. Antioxidant levels

No significant changes in total antioxidant levels were found in cells with all treatments, compared to either controls or cells treated with GGC alone (Fig. 5). Treatment with GGC alone did not result in a statistically significant decrease in GSH levels, compared to controls (Fig. 6). In contrast, significantly higher levels of GSH were found in cells treated with GGC and 10 μ mol/L CLA, compared to either controls (1.44-fold, p < 0.01) or cells treated with GGC alone (1.87-fold, p < 0.01) (Fig. 6). However, treatment of cells with GGC and 50 μ mol/L CLA resulted in markedly lower GSH levels when compared to either controls (0.3-fold, p < 0.005) or cells treated with GGC alone (0.39-fold, p < 0.005) (Fig. 6). GSH levels exhibited a statistical significance (p < 0.0001) through one-way ANOVA (Fig. 6).

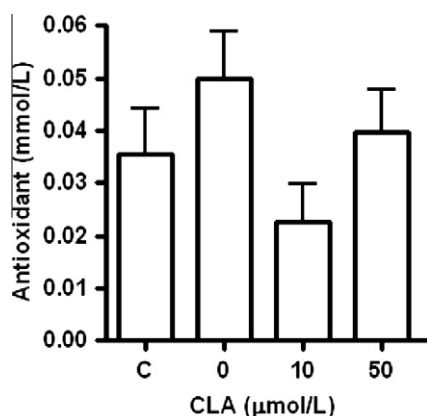


Fig. 5. Total antioxidant levels in human umbilical vein endothelial cells (HUVEC) after 24-h incubation with γ -glutamylcysteine (GGC) alone (100 μ mol/L) or combined with conjugated linoleic acid (CLA; 10, 50, or 100 μ mol/L). Values are means $\pm$ SD. No statistical significance was obtained through either Student's t -test or one-way ANOVA. All test samples were replicated (n = 4).

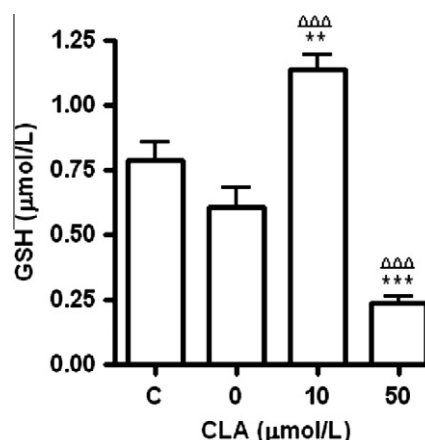


Fig. 6. Glutathione (GSH) levels in human umbilical vein endothelial cells (HUVEC) after 24-h incubation with γ -glutamylcysteine (GGC) alone (100 μ mol/L) or combined with conjugated linoleic acid (CLA; 10, 50, or 100 μ mol/L). Values are means $\pm$ SD. ** p < 0.01 and *** p < 0.005, compared to control not treated with GGC and CLA through Student's t -test. $\Delta\Delta\Delta p$ < 0.005, compared to treatment with GGC alone (100 μ mol/L). The p -values of one-way ANOVA is <0.0001. All test samples were replicated (n = 4).

3.5. GSH synthetase (GSS) expression

Although no significant changes in GSS mRNA levels were found in cells with all treatments (Fig. 7), those levels showed a positive correlation trend with PPAR γ DNA binding levels (r = 0.946, p = 0.054). GSS protein levels were 10% lower than controls in cells treated with GGC alone (Fig. 8), while GSS protein levels were 8% higher in cells treated with GGC and either dose of CLA (Fig. 8). These data translated into GSS protein levels being 20% higher in these CLA groups compared to those treated with GGC alone (p < 0.005) (Fig. 8). A positive correlation was found between GSS protein and 8-epi-PGF $_{2\alpha}$ levels (r = 0.972, p < 0.05). GSS protein levels had a statistical significance (p < 0.0001) through one-way ANOVA (Fig. 8).

4. Discussion

Higher levels of TBARS, 8-epi-PGF $_{2\alpha}$, GSH, and GSS protein were found in human umbilical vein endothelial cells (HUVEC) treated with 100 μ mol/L GGC and 10 μ mol/L CLA, compared to treatment with GGC alone, suggesting prooxidant effects of CLA at the low

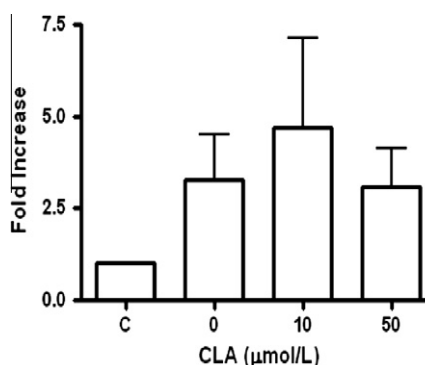


Fig. 7. Glutathione synthetase (GSS) mRNA levels in human umbilical vein endothelial cells (HUVEC) after 24-h incubation with γ -glutamylcysteine (GGC) alone (100 μ mol/L) or combined with conjugated linoleic acid (CLA; 10, 50, or 100 μ mol/L). Values are means $\pm$ SD. No statistical significance was obtained through either Student's t -test or one-way ANOVA. All test samples were replicated (n = 4).

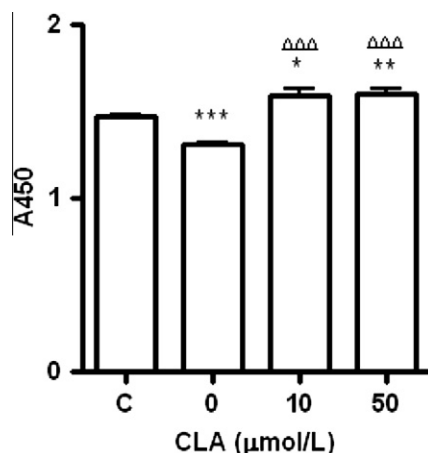


Fig. 8. Glutathione synthetase (GSS) protein levels in human umbilical vein endothelial cells (HUVEC) after 24-h incubation with γ -glutamylcysteine (GGC) alone (100 μ mol/L) or combined with conjugated linoleic acid (CLA; 10, 50, or 100 μ mol/L). Values are means $\pm$ SD. * p < 0.05, ** p < 0.01 and *** p < 0.005, compared to control not treated with GGC and CLA through Student's t -test. $\Delta\Delta\Delta p$ < 0.005, compared to treatment with GGC alone (100 μ mol/L). The p -values of one-way ANOVA is <0.0001. All test samples were replicated (n = 4).

dose similar to our previous CLA study. Higher levels of TBARS were observed only in HUVEC treated with low dose of each CLA isomer (5 μ mol/L), compared to controls not treated with CLA isomers (Nakamura and Omaye, 2009). Compared to linoleic acid, CLA is more susceptible to oxidation, likely due to their conjugated bonds (van den Berg et al., 1995; Campbell et al., 2003), thus, CLA may be oxidized and serve as a prooxidant at the low dose of 10 μ mol/L (i.e., increased exposure of each CLA molecule to oxygen). In our previous GGC study (Nakamura et al., 2012), we found a possible binding site for NF- κ B, but none for PPAR γ , in the promoter region of GSS gene and lower levels of GSS protein, NF- κ B p65 DNA binding, GSH, and oxidative stress, compared to control cells treated without GGC. In the current study, GSH synthesis mediated by oxidative stress involves the pro-inflammatory NF- κ B p50/p65 pathway. GSS expression and GSH levels may be induced through the pro-inflammatory NF- κ B p50/p65 pathway when oxidative stress (or prooxidant activity of CLA) surpasses the antioxidant capacity of GGC co-administrated and other antioxidant defense. GCS and GSS are involved in GSH synthesis and are inducible (Huang et al., 2000), and exogenous substrates for GSS synthesis (i.e., GGC) are more effective when GSH levels are depleted (Takagi et al., 2010). Thus, GGC may serve as a substitute for GSH likely because of the $-SH$ group in its structure (Grant et al., 1997; Ristoff et al., 2002) in normal or low to mild oxidative stress situations (i.e., 50 μ mol/L CLA, see details below) and as a substrate for GSH synthesis under extensive oxidative stress (i.e., 10 μ mol/L CLA). However, it is unsure whether NF- κ B p65 DNA binding levels were high enough to produce physiological affects despite its small but statistically significant increase in comparison to the treatment with GGC alone. Hence, there is the possibility of other pro-inflammatory mechanisms may be involved in up-regulation of GSS protein and GSH levels at the low dose of CLA. Unexpectedly, cytotoxicity of approximately 40% cell death was observed in cells treated with GGC and 100 μ mol/L CLA after 24 h-incubation. Cytotoxicity of CLA, in particular the trans-10, cis-12 CLA isomer, to cancer cells has previously been reported only at low concentrations (5 to 10 μ mol/L), but not at high concentrations of the CLA isomer (100 μ mol/L total) (Yamasaki et al., 2005). Further investigations are warranted to determine the mechanism of CLA-induced cytotoxicity in interactions between GGC and CLA.

Despite lower levels of GSH, treatment with GGC and 50 μ mol/L CLA appears to be protective from oxidative stress similar to treatment with GGC alone, which is indicated by lower levels of TBARS, when compared to control cells not treated with GGC and CLA. GSH levels were even lower in cells treated with GGC and 50 μ mol/L CLA than in cells treated with GGC alone, while levels of 8-epi-PGF $_{2\alpha}$ and GSS protein were higher than the treatment with GGC alone and positively correlated. These changes in 8-epi-PGF $_{2\alpha}$ and GSS protein seem not to be related to GSH, TBARS, and NF- κ B p65 or PPAR γ DNA binding levels. The increase in 8-epi-PGF $_{2\alpha}$ levels was near control concentrations along with low TBARS levels, suggesting the increase was not due to higher free radical or ROS generation. This inconsistency was not seen in our GGC study, indicating it is related to CLA-specific induction. Because CLA (in particular the trans-10, cis-12-CLA isomer) increases free 8-epi-PGF $_{2\alpha}$ levels through competition between CLA and 8-epi-PGF $_{2\alpha}$ for peroxisomal β -oxidation and modulation of its enzyme system activities, the increase in 8-epi-PGF $_{2\alpha}$ levels along with CLA supplementation does not result from increased lipid peroxidation, as suggested by Iannone et al. (Iannone et al., 2009).

Low levels of GSH without increasing oxidative stress observed at the 50 μ mol/L dose CLA together with GGC suggest CLA-mediated suppression of GSH synthesis through post-translational modification of GSS (e.g., inhibition of its enzymatic activity) or CLA-induced stability of existing GSS protein and/or GSH degradation. Exogenous GGC may serve as a substitute for GSH under our conditions or in absence of extensive oxidative stress. CLA may also have synergistic antioxidant effects on GGC due to lower levels of GSH compared to GGC treatment. In addition, CLA-induced antioxidative changes observed with the treatment seem to be modulated in a PPAR γ independent and NF- κ Bp50/p65 dependent manner. In fact, CLA has been reported to down-regulate NF- κ B p50/p65 activation and the expression of its target gene COX-2 as a ROS generator (Iwakiri et al., 2002; Cheng et al., 2004; Ringseis et al., 2006; Park et al., 2010). Although other investigators have reported that the trans-10, cis-12 CLA isomer induces prooxidative and inflammatory effects through NF- κ B p50/p65 activation (Kennedy et al., 2009; Martinez et al., 2010), this does not appear to be a factor at the 50 μ mol/L dose used under the conditions of the current study. In contrast, our data at the 10 μ mol/L dose of CLA with GGC is consistent with previous reports of prooxidant effects of CLA as mentioned above. Thus, CLA seems to induce 8-epi-PGF $_{2\alpha}$ and existing GSS stability and GSH degradation at the intermediate dose of 50 μ mol/L CLA, rather than inducing GSS expression. In our study, both CLA and GGC exhibit differential effects, and the effects depend on doses of CLA or oxidative stress.

5. Conclusions

The results of the present study confirm previous reports that GGC can substitute as an antioxidant for GSH without increasing GSH levels. The efficacy of GGC supplementation in lowering oxidative stress is consistent with our previous findings. Co-administration of CLA with GGC had differential effects depending on dose of CLA in our experimental system. A dose of 100 μ mol/L was cytotoxic, whereas a dose of 10 μ mol/L seemed to have prooxidant activity without inducing cytotoxicity. In contrast, an intermediate dose of 50 μ mol/L CLA with GGC seemed to have antioxidant activity despite a reduction in GSH levels greater than that seen with GGC alone. GGC may play a role not only in GSH synthesis as a substrate but also in protection from oxidative stress as a substitute/antioxidant mediated through a $-SH$ group and as a modulator of GSH synthesis. Due to its ease of permeability through cell membranes, GGC could be used as an intra- and intercellular

therapeutic agent in oxidative stress-related injuries and diseases. Further studies are warranted to develop a better understanding about the efficacy of GGC supplementation under various conditions, for example, in the presence of prolonged/extensive oxidative stress, in either cell culture or animal models. In addition, the value of CLA as an adjunct to GGC to reduce oxidative stress seems to be limited by its narrow range of efficacy. Additional studies with CLA are warranted to better understand its mechanisms of action and which isomers are most effective.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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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

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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.

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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).

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. Louis, 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'-GGGACTTTC-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

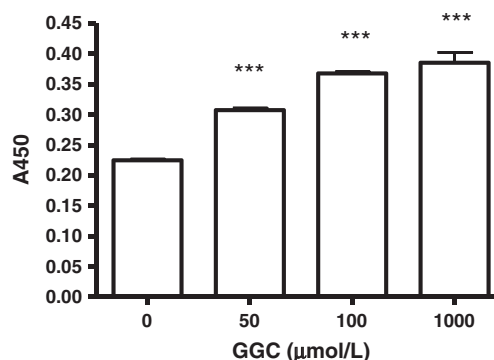


Fig. 1. Peroxisome proliferator-activated receptor γ (PPAR γ) DNA binding levels in human umbilical vein endothelial cells (HUVEC) after treatment with graded concentrations of 0, 50, 100, or 1000 $\mu\text{mol/L}$ of γ -glutamylcysteine (GGC). All treatments resulted in a significant increase in PPAR 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).

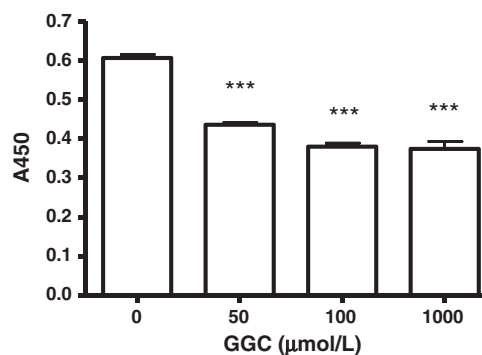


Fig. 2. Nuclear factor- κ B (NF- κ B) p65 DNA binding levels in human umbilical vein endothelial cells (HUVEC) after treatment with graded concentrations of 0, 50, 100, or 1000 $\mu\text{mol/L}$ of γ -glutamylcysteine (GGC). All treatments resulted in a significant decrease in NF- κ B p65 DNA binding 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 ($n=4$).

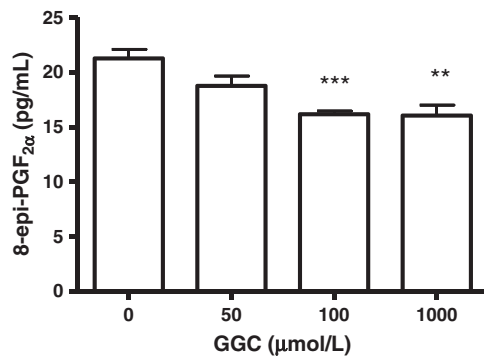


Fig. 3. Extracellular levels of 8-epi-PGF_{2α} after treatment with graded concentrations of 0, 50, 100, or 1000 μmol/L of γ-glutamylcysteine (GGC). There was a significant decrease in 8-epi-PGF_{2α} levels at higher concentrations of GGC, compared to the control treated with 0 μmol/L of GGC through 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

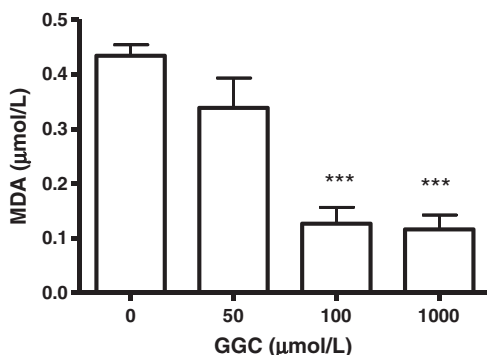


Fig. 4. Thiobarbituric acid reactive substance (TBARS) levels in cell homogenate of human umbilical vein endothelial cells (HUVEC) after treatment with graded concentrations of 0, 50, 100, or 1000 μmol/L of γ-glutamylcysteine (GGC). There was a significant decrease in TBARS levels (as MDA equivalent) 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).

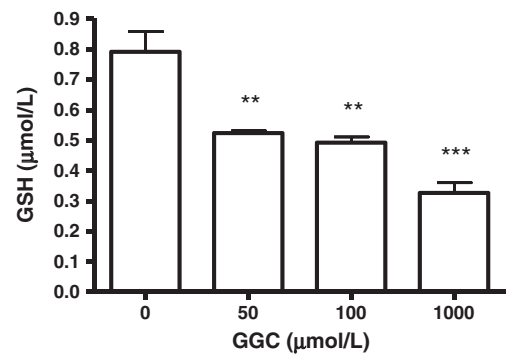


Fig. 5. Glutathione (GSH) levels in human umbilical vein endothelial cells (HUVEC) after treatment with graded concentrations of 0, 50, 100, or 1000 μmol/L of γ-glutamylcysteine (GGC). All treatments resulted in a significant decrease in GSH levels, compared to the control treated with 0 μmol/L of GGC 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

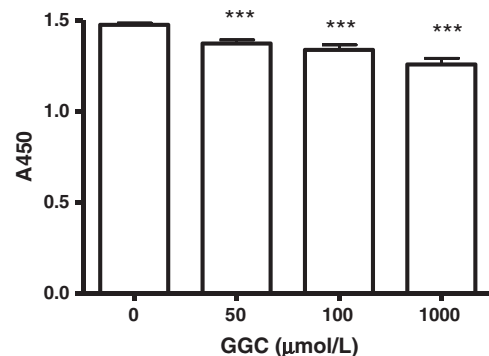


Fig. 6. Glutathione synthetase (GSS) protein levels in human umbilical vein endothelial cells (HUVEC) after treatment with graded concentrations of 0, 50, 100, or 1000 μmol/L of γ-glutamylcysteine (GGC). All treatments resulted in a significant decrease in GSH levels, compared to the control treated 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

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