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내독소유발 세포손상에 대한 환원형 글루타치온 투여의 효과 및 작용 기전

Effect and Mechanism of Reduced Glutathione Treatment on Lipopolysaccharide-induced Cellular Injury

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Effect and Mechanism of Reduced Glutathione Treatment on Lipopolysaccharide-induced Cellular Injury

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Abstract

Effect and Mechanism of Reduced Glutathione Treatment on Lipopolysaccharide-induced Cellular Injury

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Reactive oxygen species (ROS) are chemically reactive materials including radical and non-radical oxygen species formed by the partial reduction of oxygen. Oxidative stress caused by excess ROS is one of many mechanisms by which multi-organ failure occurs in sepsis. Reduced glutathione (GSH) is a tripeptide formed by glutamic acid, cysteine, and glycine, which acts as an antioxidant via the

glutathione redox cycle in eliminating hydrogen peroxide (H_2O_2). This study aimed to prove that GSH has protective effects against oxidative stress in the lipopolysaccharide (LPS)-exposed cell. We induced oxidative stress in human lung microvascular endothelial cells (HMVEC-L) by incubating them with various concentrations of LPS. In 10 μg/mL LPS-exposed cells, cell viability and the dose related effects of GSH were assessed 24 hours after treatment with 0, 0.4, and 0.8 mM of GSH, respectively. After a twelve hour incubation period, GSH level, glutathione disulfide (GSSG) level, the GSH/GSSG ratio, and H_2O_2 level were measured. For measuring the effects of GSH on intracellular protective mechanisms against oxidative stress, we measured expression levels of phosphorylated inhibitor κB-α (p-Iκ B-α), cytoplasmic phosphorylated Akt (p-Akt), and nuclear factor erythroid 2‐related factor 2 (Nrf2) using western blots. As the concentration of GSH increased, the intra cellular GSH level and the GSH/GSSG ratio increased along with a decreasing level of H_2O_2 and proinflammatory cytokines. Cell viability increased as GSH concentration increased and this result was associated with an increase of cytoplasmic p-Akt expression and Nrf2 expression. In conclusion, GSH treatment attenuated LPS-induced cell injury by up-regulating Nrf2 signaling in the Akt survival pathway.

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Keywords: Glutathione, glutathione redox cycle, oxidative stress reactive oxygen species, Akt survival pathway, Nrf2

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I. Introduction

Reactive oxygen species (ROS) are chemically reactive materials including radical and non-radical oxygen species formed by the partial reduction of oxygen (1, 2). Commonly, ROS are generated as natural products of mitochondrial oxidative metabolism and have important roles in cell signaling and homeostasis such as apoptosis, cytokine release, and defense to infections (3, 4). When ROS overwhelm the ability to detoxify the reactive intermediates or to repair the resulting damage, oxidative stress occurs (5).

Sepsis, a syndrome of physiologic, pathologic, and biochemical abnormalities induced by infection, features tissue hypo-perfusion, hypo-oxygenation and multi-organ failure, eventually (6, 7). Oxidative stress in sepsis by excessive production of ROS or by dysregulated antioxidant defense systems is one of the many mechanisms of multi-organ failure in sepsis (8, 9). Reducing the oxidative stress by using antioxidants can be an effective strategy in treatment of sepsis (10). Various antioxidants such as niacin (11), N-acetylcysteine (NAC) (12), vitamin-C (13), and reduced glutathione (14, 15) have been studied as potential medicines in sepsis.

Reduced glutathione (GSH) is a tripeptide formed by glutamic acid, cysteine, and glycine, which acts as an antioxidant via the glutathione redox cycle in eliminating hydrogen peroxide (H_2O_2) (16). The glutathione redox cycle consists of two enzyme reactions in which glutathione peroxidase eliminates H_2O_2 via converting GSH to

glutathione disulfide (GSSG) and glutathione reductase subsequently reconverts GSSG to GSH using reduced nicotinamide adenine dinucleotide phosphate (NADPH). In respect of GSH as a potent protector against oxidative stress, many studies have been conducted in various fields such as oncology (17), Parkinson's diseases (18), and inflammatory bowel diseases (19). Recently, the study by Kwon et al. showed that the combination therapy of niacin and selenium attenuated lung injury and improved survival during sepsis with the synergistic activation of the glutathione redox cycle and up-regulation of nuclear factor erythroid 2-related factor 2 (Nrf2) and down-regulation of nuclear factor-κB (NF-κB) (20). Although GSH seems to be one of the most probable candidates for treatment of sepsis, few studies have tested the effectiveness of GSH in presenting a molecular level of defense mechanisms against oxidative stress in sepsis.

We performed this study to determine whether treatment with clinically relevant doses of GSH attenuates cell injury by oxidative stress through changes in the Akt survival pathway including Nrf2 signaling and proinflammatory cytokine expression.

II. Materials and Methods

2.1. Materials

Human lung microvascular endothelial cells (HMVEC-L) and EGM-2MV Bulletkit media were purchased from Lonza (Walkers-ville, MD). Fetal bovine serum was purchased from Gibco (Grand Island, NY). Reduced glutathione purchased from Well Pharm (Seoul, Korea). Lipopolysaccharide (LPS, from Escherichia coli, O26:B6) from Sigma-Aldrich (St Louis, MO).

2.2 Clinically Relevant Doses of reduced Glutathione

In humans, the intravenous infusion of 2 g/m2 of reduced glutathione (GSH) made the concentration of GSH in plasma increase from 17.5 ± 13.4 μM (mean ± SD) to 82.3 ± 326 μM (21). In another in vitro study, 1 mM of GSH was used as a control group (22). For this in vitro study, we selected 0.4 mM and 0.8 mM of GSH as intervention groups.

2.3 Cell Culture and Drug Treatment

HMVEC-L were cultured in EGM-22MV Bulletkit media containing 10% fetal bovine serum and incubated at 37°C in a humidified atmosphere with 5% CO_2. After the cells were grown to 90%

confluence, they were seeded on 96-well plates (2×10^4 per well) or six-well plates (2×10^6 per well) and used for subsequent experiments.

For the dose-response of LPS experiments, the cells were incubated with different concentrations of LPS (0, 5, 10, and 20 μg/mL) for 24 hours. The LPS concentration of 10 μg/mL was selected and used for the time-dependent experiments. Then, 10 μg/mL of LPS-exposed cells were treated with various concentrations of reduced glutathione (0, 0.4, and 0.8 mM). Twenty-four hours after LPS and drug treatment, cell viability was assessed using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole assay with a Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) (23). For the molecular variable measurements, an independent set of HMVEC-L was harvested at 12 hours after LPS and drug treatment. Six independent experiments were performed.

2.4 Antioxidant and Oxidative Stress Measurements

GSH and GSSG levels were measured using a glutathione assay kit (20). The H_2O_2 level in the culture media was determined using a hydrogen peroxide colorimetric detection kit (Enzo Life Science, Farmingdale, NY).

2.5 Nuclear and Cytoplasmic Extracts

The extraction of nuclear and cytoplasmic proteins was performed using an NE-PER nuclear and cytoplasmic extraction kit (Pierce

Biotechnology, Rockford, IL).

2.6 Real-Time Polymerase Chain Reaction

Real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed to detect and quantify the gene expressions of tumor necrosis factor (TNF)-α and interleukin (IL)-6 (11). Briefly, total RNA was extracted using an RNeasy total RNA extraction kit (Qiagen, Chatsworth, CA). Next, 1 μg of total RNA was used in a complementary DNA reaction. Quantitative real-time RT-PCR was performed using an ABI Prism 7000 Sequence Detection system (Applied Biosystems, Foster City, CA). Data were normalized with respect to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primers and probes were obtained from Applied Biosystems: GAPDH (Hs02758991_g1), TNF-α (Rn99999017_m1), and IL-6 (Rn99999011_m1). All of the experiments were performed in triplicate.

2.7 NF-κB p65 DNA - Binding Activity Measurement

NF-κB p65 DNA - binding activity was determined using a TransAM NF-κB p65 transcription factor assay kit (Active Motif, Carlsbad, CA).

2.8 Western Blotting

To determine the expressions of cytoplasmic phosphorylated inhibitor κB-α (p-IκB-α), cytoplasmic phosphorylated Akt (p-Akt), and nuclear factor erythroid 2-related factor 2 (Nrf2), we performed Western blotting as previously described using the following primary antibodies: rabbit anti-p-IκB-α (diluted 1:500; Cell Signaling, Beverly, CA), anti-p-Akt (diluted 1:1,000; Cell Signaling), and mouse anti-Nrf2 (diluted 1:500; Abnova, Taipei City, Taiwan) (11, 24). To ensure equivalent protein loading, the blots of cytoplasmic and nuclear extracts were normalized against β-actin and histone H3 using mouse monoclonal anti-β-actin (Cell Signaling) and anti-histone H3 antibodies (Pierce Biotechnology), respectively (25).

2.9 Statistical Analysis

We compared variables using via the Kruskal-Wallis test with the post hoc Mann-Whitney U test and a Bonferroni correction. Adjusted p values less than 0.05 were considered statistically significant, and the significance levels quoted are two-sided. The statistical analyses were performed using SPSS version 20.0 for Windows (SPSS, Chicago, IL).

III. Results

3.1 Toxicity of LPS on HMVEC-L

The exposure of HMVEC-L to 5, 10, and 20 μg/mL of LPS decreased cell viability by 16.9, 51.4, and 62.8%, respectively (Fig. 1A). After incubation for 24 hours with 10 μg/mL of LPS, cell viability decreased significantly (adjusted $p<0.05$) (Fig. 1B).

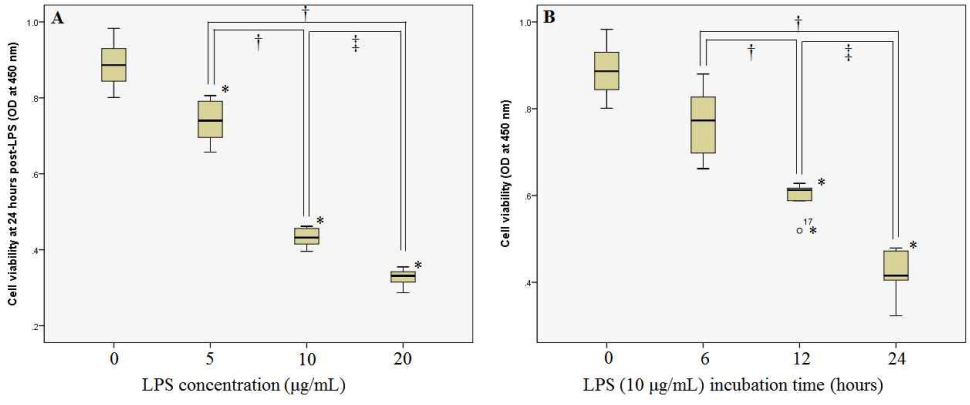

Figure 1. The toxicity of lipopolysaccharide (LPS) on human lung microvascular endothelial cells (HMVEC-L)

The dose-response LPS experiments (A) and time-dependent experiments (B). Data are presented as the median (quartiles, range) from six independent experiments. *Adjusted p value<0.05 vs. control group. † Adjusted p value<0.05 vs. 5 µg/mL LPS group. ‡ Adjusted p<0.05 vs. 10 µg/mL LPS group (A). *Adjusted p<0.05 vs. control group. † Adjusted p<0.05 vs. 6-hour incubation group. ‡ Adjusted p<0.05 vs. 12-hour incubation group (B).

3.2 Dose Related Effect of GSH on LPS-Exposed Cells

LPS exposure substantially decreased cell viability (adjusted $p<0.05$). High-dose GSH treatment (0.8 mM) improved cell viability compared with that of low-dose GSH group (0.4 mM) and untreated group (0 mM) (adjusted $p<0.05$).

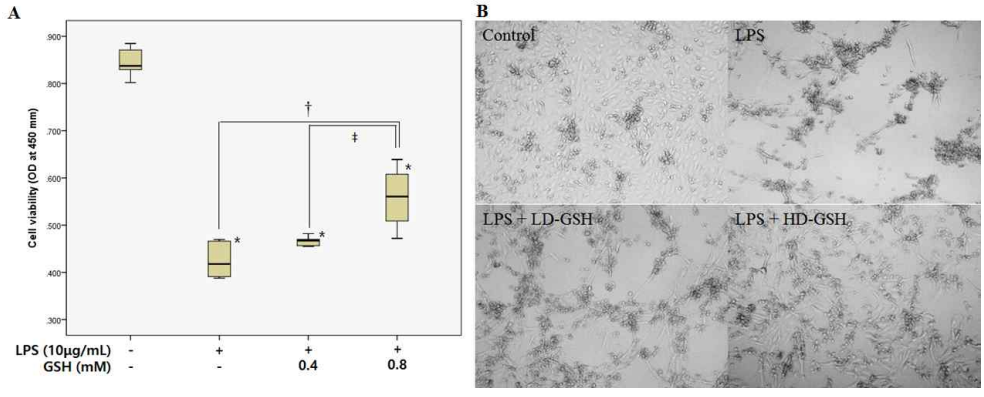

Figure 2. Dose-related effects of reduced glutathione (GSH) on cell viability of 10 μg/mL lipopolysaccharide (LPS)-exposed cells

Cell viability (A), the representative photomicrographs of Human lung microvascular endothelial cells (HMVEC-L) (B). Data are presented as the median (quartiles, range) from six independent experiments. *Adjusted $p<0.05$ vs. control group. † Adjusted $p<0.05$ vs. LPS group. ‡ Adjusted $p<0.05$ vs. low-dose GSH group.

3.3 Antioxidant and Oxidative Stress in LPS-Exposed Cells

When HMVEC-L were exposed to 10 μg/mL of LPS, intracellular GSH levels were decreased and the GSH levels were recovered with administration of GSH. In the high-dose group (0.8 mM of GSH), excess GSH levels, more than that of the control group, were measured (adjusted $p<0.05$) (Fig. 3A). Although LPS exposure increased GSSG levels (adjusted $p<0.05$), no significant difference was observed among the three LPS exposed groups (Fig. 3B). As the dose of GSH increased, the GSH/GSSG ratio was elevated significantly (adjusted $p<0.05$), but all ratios in the LPS-exposed groups were lower than that of the control group (adjusted $p<0.05$) (Fig. 3C). After oxidative stress was induced by LPS treatment, the H_2O_2 level significantly increased (adjusted $p<0.05$). The elevated H_2O_2 levels were decreased by GSH treatment in a dose dependent manner (Fig. 3D). However, the H_2O_2 level in the high-dose GSH group was still higher than that of the control group, even though excess GSH levels were measured in that group (adjusted $p<0.05$) (Fig. 3D).

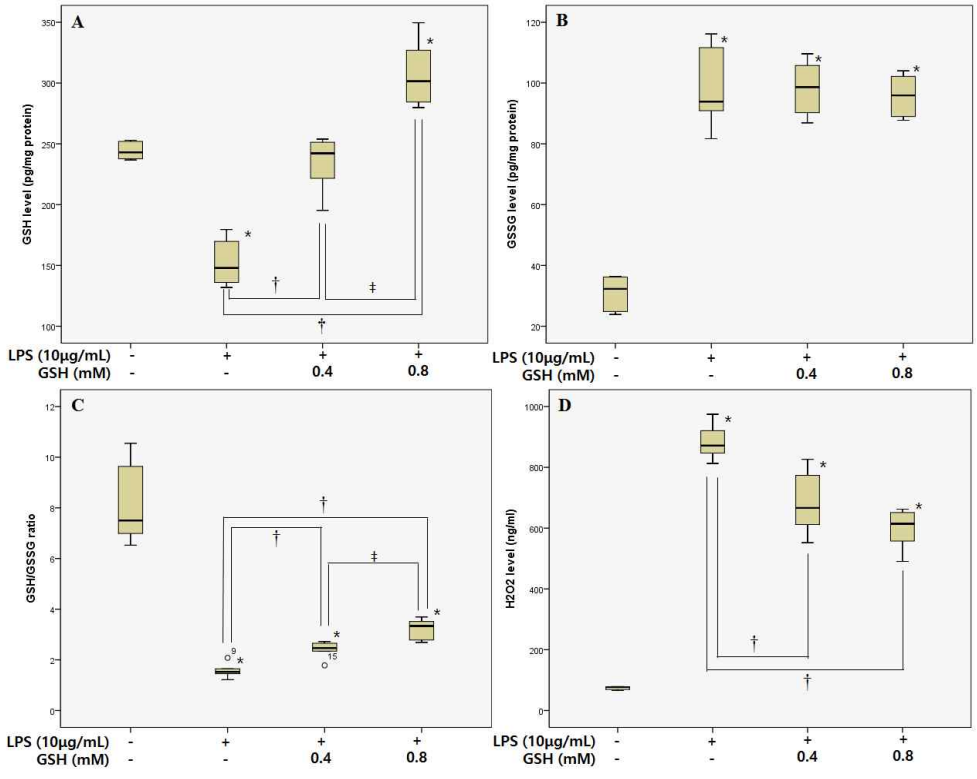

Figure 3. Dose-related effects of reduced glutathione (GSH) on oxidative stress in 10 μg/mL lipopolysaccharide (LPS)-exposed cells

Reduced glutathione (GSH) level (A), glutathione disulfide (GSSG) level (B), the GSH/GSSG ratio (C), hydrogen peroxide (H_2O_2) level (D). Data are presented as the median (quartiles, range) from six independent experiments. *Adjusted $p<0.05$ vs. control group. † Adjusted $p<0.05$ vs. LPS group. ‡ Adjusted $p<0.05$ vs. low-dose GSH (0.4mM) group.

3.4 Intracellular Proinflammatory Cytokine Levels

Tumor Necrosis Factor-alpha (TNF-α) mRNA expression levels were significantly higher in LPS-exposed groups (adjusted $p<0.05$) and decreased as the treatment concentration of GSH increased (Fig. 4A). There was no significant difference in TNF-α expression levels between the high-dose GSH group and the control group (adjusted $p>0.05$). The exposure of LPS increased interlukin-6 (IL-6) mRNA expression levels (adjusted $p<0.05$) and GSH treatment decreased Interlukin-6 (IL-6) mRNA levels in LPS-exposed groups (Fig. 4B). As well as TNF-α, there was no significant difference in IL-6 expression levels between the high-dose GSH group and the control group (adjusted $p>0.05$). After LPS exposure, high-dose GSH treatment significantly decreased levels of both TNF-α and IL-6 mRNA expression compared with those of control groups (adjusted $p<0.05$).

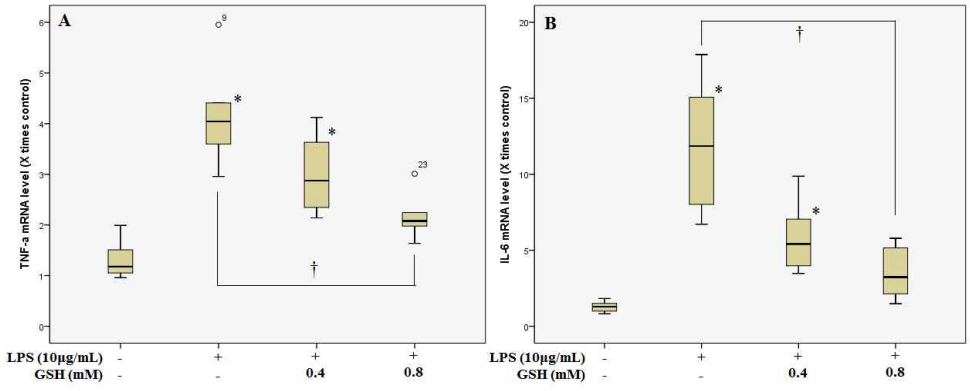

Figure 4. Dose related effects of reduce glutathione (GSH) on intracellular proinflammatory cytokine levels

Tumor Necrosis Factor-alpha (TNF-α) mRNA levels (A) and Interlukin-6 (IL-6) mRNA levels (B). Data are presented as the median (quartiles, range) from six independent experiments. *Adjusted $p<0.05$ vs. control group. † Adjusted $p<0.05$ vs. LPS group.

3.5 Intracellular Signaling in LPS-Exposed Cells

To determine expression levels of cytoplasmic phosphorylated inhibitor κB-α (p-IκB-α), cytoplasmic phosphorylated Akt (p-Akt), and nuclear factor erythroid 2‐related factor 2 (Nrf2), we performed Western blotting. Blots are representative of three wells per group (Fig. 5). The LPS exposure increased cytoplasmic p-IκB-α expression and as the dose of GSH increased, these expression levels decreased (Fig. 6A). In the high-dose GSH group, cytoplasmic p-Akt significantly increased compared with the untreated GSH group (adjusted $p<0.05$) (Fig. 6B). Nuclear Nrf2 expression increased as the GSH dose increased. The high-dose GSH group had a significantly higher Nrf2 expression level than any other group (adjusted $p<0.05$) (Fig. 6C). The LPS exposure resulted in a significant increase in NF-κB p65 DNA‐binding activity and GSH treatment reduced NF-κB p65 DNA ‐binding activity. High-dose GSH treatment reduced the NF-κB p65 DNA‐binding activity compared with that of the untreated GSH group (adjusted $p<0.05$) (Fig. 6D).

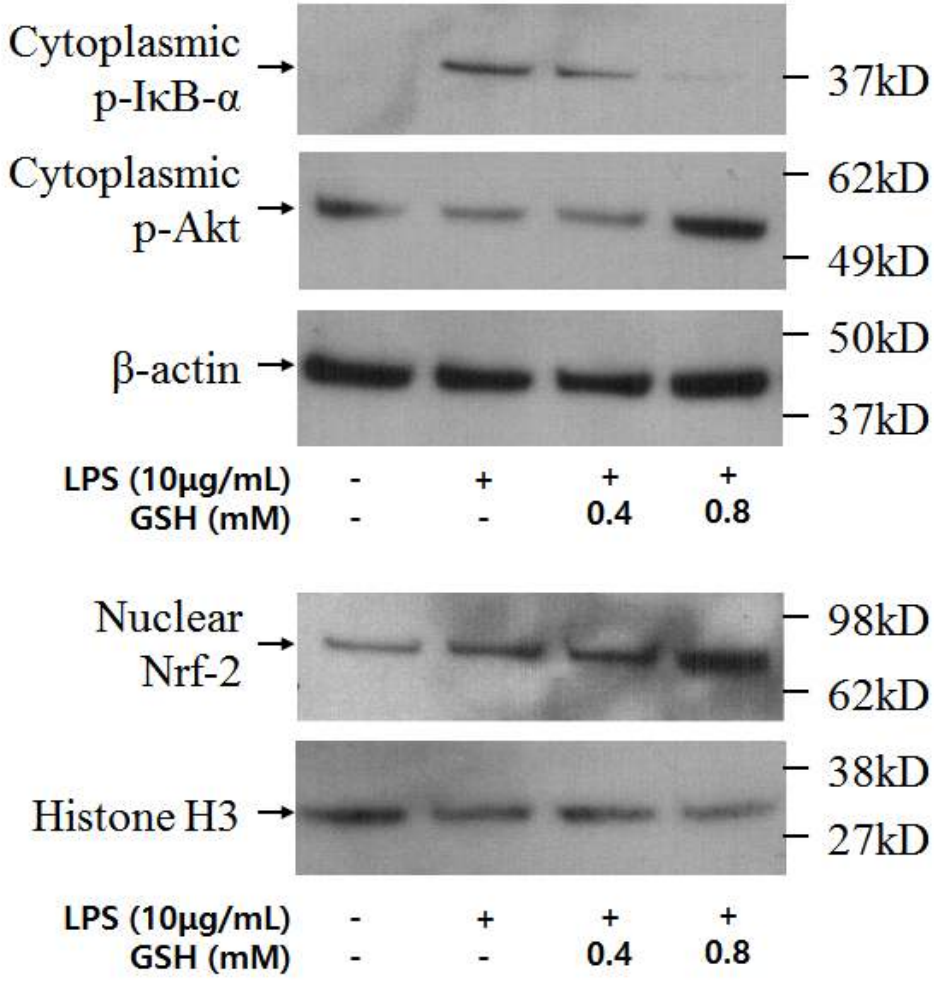

Figure 5. The representative blots of the intracellular signaling molecules in 10 μg/mL lipopolysaccharide (LPS)-exposed cells with reduce glutathione (GSH) treatment

The blots of cytoplasmic and nuclear extracts were normalized against β-actin and histone H3, respectively.

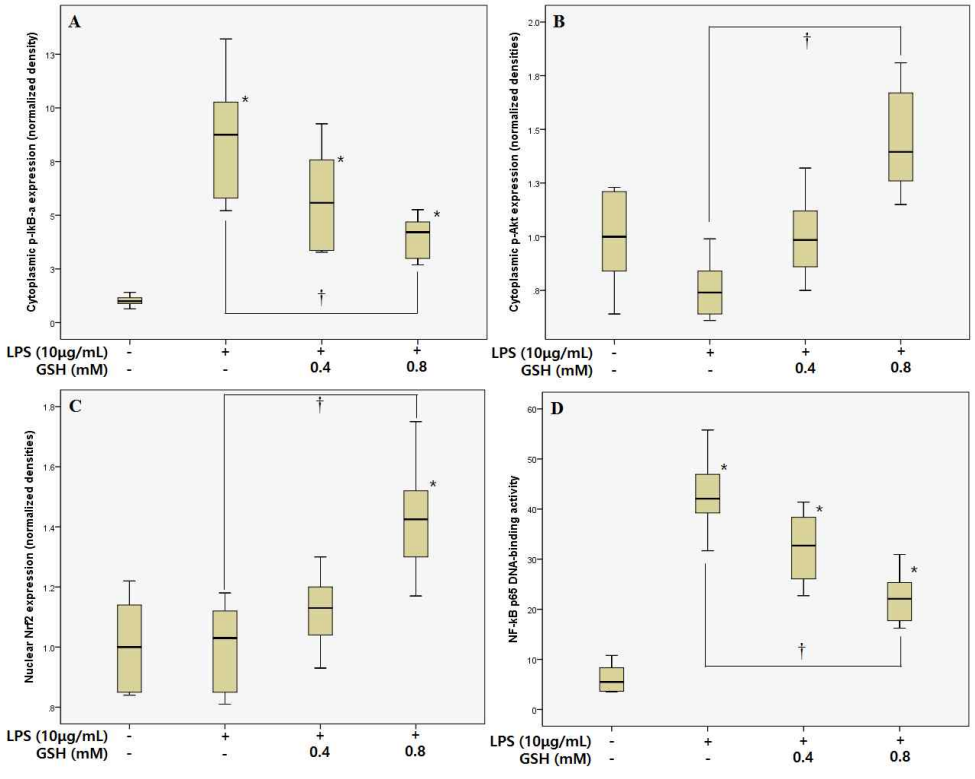

Figure 6. Dose-related effects of reduced glutathione (GSH) on the intracellular signaling in 10 μg/mL lipopolysaccharide (LPS)-exposed cells

Cytoplasmic phosphorylated inhibitor κB-α (p-IκB-α) (A), phosphorylated Akt (p-Akt) (B), nuclear factor erythroid 2-related factor 2 (Nrf2) expressions (C), and nuclear factor κB (NF-κB) p65 DNA - binding activity (D). *Adjusted $p<0.05$ vs. control group. † Adjusted $p<0.05$ vs. LPS group.

IV. Discussion

The present study showed that administration of reduced glutathione (GSH) eliminated excessive production of ROS and improved cell viability in a dose dependent manner. Although excess GSH was measured in the high-dose GSH treatment group, a considerable amount of H_2O_2 was still detected in that group and the GSSG levels of the three LPS-treated groups (0, 0.4 mM, and 0.8 mM of GSH, respectively) were substantially higher than that of the control group. This might be because the glutathione redox cycle was saturated and the excess GSH in the high-dose GSH group was not used to eliminate H_2O_2 effectively. Additionally, in oxidative stress conditions, decreased activities of enzymes in the glutathione redox cycle could result in a disturbance of effective H_2O_2 elimination. In previous studies, niacin and selenium increased the enzyme activity and improved survival rates in endotoxemia rat models and cardiac arrest rat models (11, 20, 26). It seems reasonable that using a cocktail therapy which includes niacin, selenium and GSH could be a new strategy for antioxidant therapy in sepsis. In the cocktail therapy, niacin and selenium activate glutathione redox cycles and the high-dose GSH acts as an excess substrate by which the enzyme reactions can be carried on in progress toward eliminating H_2O_2.

In respect of the GSH/GSSG ratio, a box plot of the cell viability with increasing doses of GSH (Fig. 2A) showed similarities with that of the GSH/GSSG ratio (Fig. 3C). This might not be GSH itself, but that the GSH/GSSG ratio mirrors the actual intracellular

conditions indicative of oxidative stress and cell viability. Recently, there have been studies showing that the GSH/GSSG ratio can be used as a prognostic factor for sepsis (27, 28). As a prognostic factor, the GSH/GSSG ratio would suggest that the effective reconversion of GSSG to GSH is important in eliminating the H_2O_2 produced by oxidative stress as well as being indicative of sufficient GSH administration.

Our study also showed that GSH attenuates cell death by proinflammatory cytokine release suppression and activation of the Akt survival pathway. Especially in the high-dose GSH-treated groups, a significant increase in cytoplasmic p-Akt and nuclear Nrf2 expression was observed. Likewise, in the high-dose GSH treatment group, there was a significant decrease in proinflammatory cytokines, TNF-α and IL-6. This means that high-dose GSH treatment reduces the excessive oxidative stress induced by LPS exposures and switches on protective mechanisms that include Akt and Nrf2 signaling (24). In the previous study, niacin and selenium combination therapy synergistically activates the glutathione redox cycle and reduces H_2O_2 (20). The H_2O_2 reduction inhibits NF-κB p65 DNA-binding activity via the up-regulation of Akt-Nrf2 survival pathway and via the suppression of cytoplasmic IκB-α phosphorylation. And the up-regulation of Nrf2 was associated with down-regulation of NF-κB pathway (11). Although our study results generally agreed with the previous studies, we could not elicit a new signaling pathway involved in cell responses to oxidative stress in regard to GSH concentration and intracellular redox balance.

This study has several limitations. First, this in vitro study

did not show the survival benefits of GSH in sepsis animal models. The concentration of LPS used this study was 10 μg/mL which might be higher than that of in vivo settings. Further studies using endotoxemic rat models are under consideration. Second, we could not measure activities of glutathione peroxidase and glutathione reductase and a concentration of NADPH. Further studies measuring those activities with the administration of niacin and selenium are needed. Finally, a novel pathway in detecting oxidative stress, activating survival signaling and reducing oxidative stress should be studied.

In conclusion, reduced glutathione treatment attenuated cell injury in LPS-exposed cells. Its therapeutic effects were associated with the activation of the Akt-Nrf2 survival pathway.

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요약(국문초록)

활성산소물질(reactive oxygen species)의 과량 발생에 의한 산화스트레는 패혈증에서 세포의 손상 및 사멸을 일으키고 결과적으로 다발성 장기 손상을 초래하게 되어 패혈증 환자의 사망률을 높이는 원인 중 하나이다. 환원형 글루타치온 (reduced glutathione, GSH)은 자연적으로 존재하는 트리 펩타이드(glutamyl - cysteinyl -glycine)로 체내에서 글루타치온 산화 환원 싸이클(glutathione redox cycle)을 통하여 과산화수소를 제거하는 대표적인 항산화물질이다. 본 연구는 사람 폐미세혈관 내피세포에 내독소(lipopolysaccharide)를 처리하여 인위적으로 세포에 산화스트레스를 만든 후 글루타치온의 항산화효과로 인한 세포 보호효과를 측정할 목적으로 시행되었다. 10 μg/mL의 내독소를 처리한 세포에 각각 0, 0.4, 0.8mM 의 글루타치온을 처리 후 12시간 뒤 환원형 글루타치온(GSH), 산화형 글루타치온(GSSG), 환원형/산화형 글루타치온 비, 과산화수소농도 및 nuclear factor (NF)-κB p65 DNA -binding activity 측정하였다. 또한 세포 내 NF-κB 분자작용신호에 관련된 세포질 내 phosphorylated inhibitor κB-α (p-IκB-α), phosphorylated Akt (p-Akt), 및 핵내 nuclear factor erythroid 2-related factor 2 (Nrf2) 발현 정도를 측정하였다. 처리한 글루타치온의 농도가 높아짐에 따라 배양된 세포의 글루타치온 농도 및 환원형/산화형 글루타치온 비가 상승하였고, 세포 생존률은 상승하였다. 이와 더불어 Akt 생존분자작용신호에 관계된 세포질 내 p-Akt 및 핵 내 Nrf2 발현이 촉진되며 염증관련 사이토카인이 줄어드는 것을 관찰 할 수 있었다. 결론적으로 환원형 글루타치온은 내독소 투여로 비롯된 세포의 산화손상을 지연시키며 이러한 항산화효과의 분자수준 작용기전은 Akt-Nrf2 경로의 활성화와 관련되어 있었다.

주요어: 글루타치온, 글루타치온 산화환원 싸이클, 활성산소물질 산화스트레스, Akt survival pathway, Nrf2

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