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Temporal changes in glutathione biosynthesis during the lipopolysaccharide-induced inflammatory response of THP-1 macrophages

Hongqiao Zhang[1], **Honglei Liu**[2], **Lulu Zhou**[1], **Jenay Yuen**[1], and **Henry Jay Forman**[1]

[1]Andrus Gerontology Center of the Leonard Davis School of Gerontology, University of Southern California, 3715 McClintock Ave, Los Angeles, CA 90089-0191, USA

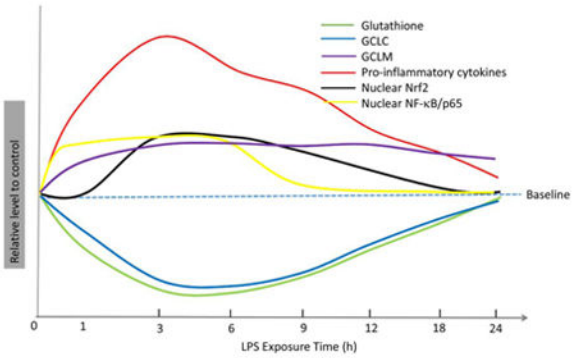
[2]Children's Hospital Los Angeles, 4650 Sunset Blvd, Los Angeles, CA 90027

Abstract

How macrophages maintain redox homeostasis in the inflammatory process, in which a large amount of oxidants are produced, remains elusive. In this study, we investigated the temporal changes in the intracellular glutathione (GSH), the master antioxidant, and the expression of glutamate cysteine ligase (GCL), the rate-limiting enzyme for GSH biosynthesis, in the inflammatory response of human macrophages (THP1 cells) to lipopolysaccharide. Intracellular GSH concentration was decreased significantly in the early phase (~6 h) of LPS exposure, and then gradually went back to the basal level in the late phase (9~24 h). The expression level of the catalytic subunit of GCL (GCLC) followed a similar pattern of change as GSH: its mRNA and protein levels were reduced in the early phase and then back to basal level in the late phase. In contrast, the expression of the modifier subunit of GCL (GCLM) was significantly increased in the phase of LPS exposure. Activation Nrf2, the transcription factor involved in the induction of both GCLC and GCLM, occurred at as early as 3 h after LPS exposure; whereas the activation of NF-κB occurred at as early as 30 min. Inhibition of NF-κB signaling with SN50 prevented the decrease of GCLC and inhibited Nrf2 activation in response to LPS. These data demonstrate time-dependent changes in the expression of GCL and Nrf2 signaling during the inflammatory response, and that the regulation of GCLC and GCLM might be through different pathways in this process.

Graphical abstract

Corresponding author: Hongqiao Zhang, PhD, Research Assistant Professor, Andrus Gerontology Center of the Leonard Davis School of Gerontology, University of Southern California, 3715 McClintock Ave GER306B, Los Angeles, CA 90089-0191, USA, hongqiaz@usc.edu, Phone: (626) 272-7968.



Keywords

inflammation; macrophage; glutathione; GCLC; lipopolysaccharide

Introduction

Macrophages play a critical role in the initiation, maintenance, and resolution of inflammation [1]. These cells patrol the tissues and become activated in response to invading pathogens, particles [2], lipopolysaccharide [3], and other stimuli. The activation of macrophages is critical for inflammatory processes including phagocytosis and immunomodulation.

In response to inflammatory stimuli, macrophages and other inflammatory cells produce a large amount of reactive oxidants at the inflammation site [4], including hypochlorous acid (HOCl), superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), nitric oxide ($NO^{\bullet}$), and peroxynitrite ($ONOO^-$). These oxidants may play multiple roles in the inflammatory response such as facilitating the elimination of invading microorganisms, acting as second messenger that mediates the signaling pathways involved in gene expression, cell proliferation, and apoptosis [5], and/or modulating immune cell functions [6, 7]. On the other hand these oxidants carry the potential to cause oxidative damage to the macrophages and other cells at the inflammation site [8]. Therefore, the intracellular redox status of macrophages and others needs to be tightly regulated to accomplish the oxidant-dependent functions while simultaneously preventing extensive destruction of inflammatory cells themselves by oxidants.

Glutathione (GSH) is the most abundant nucleophile in the cells and plays a predominant role in regulating the intracellular redox homeostasis and protecting against oxidative injury, by serving as the substrate of many of the enzymes that remove oxidants and other electrophiles, and to restore reduced cysteine moieties in proteins [9]. Under inflammatory conditions, both the oxidation of GSH (to GSSG, the oxidized form) and consumption of GSH (as a result of GSSG export or formation of GSH-conjugates) are significantly increased [10-14], suggesting that GSH production does not keep up with its consumption during the inflammatory process. The *de novo* GSH biosynthesis is mainly regulated through glutamate cysteine ligase (GCL), the rate-limiting enzyme in the biosynthesis reaction. GCL activity is determined by several factors including the absolute protein levels of both its

catalytic subunit (GCLC) and modifier subunit (GCLM) [15, 16], the ratio of GCLC/GCLM [17], and the feedback inhibition of GCL by GSH itself [18]. Studies from past decades have demonstrated that the expression of both GCLC and GCLM subunits could be induced in response to oxidative stress, through nuclear factor (erythroid-derived 2)-like 2 (Nrf2) signaling [16, 19-22]. Nrf2 is a transcription factor that is constantly and rapidly degraded in the cytosol through the Keap1-mediated ubiquitination-proteasome system under non-stimulated condition. Upon exposure of oxidants/electrophiles, specific cysteine residues on Keap1 are oxidized or alkylated, and modified Keap1 loses its ability to assist in Nrf2 degradation. Nrf2 is then phosphorylated and translocated into the nucleus, where it binds to the electrophile response element (EpRE) in the promoters of GCLC and GCLM, and enhances their transcription [15, 23]. The Nrf2 signaling plays a critical role in maintaining cellular redox homeostasis and protecting against oxidative injuries during oxidative stress [24].

It has been reported that both GSH concentration and the expression level of GCL were decreased in the inflammatory response of inflammatory cells including macrophages [12, 13, 25]. This however, is inconsistent with the well-established finding that GCL expression is induced (increased) in response to oxidative stress. Thus, it might have been expected that GSH would increase during the inflammatory response. But, previous studies only determined the alteration of GSH concentration and GCL expression at specific time point after exposure to inflammatory stimuli. So, whether these changed over time during the inflammatory response remained unknown. To address these questions we examined the changes in intracellular GSH level and GCL expression, the change of NF-κB and Nrf2 activation, and the potential role of NF-κB signaling in alteration of Nrf2 and GCL expression in human macrophages as a function of time during the response to LPS. Our data demonstrated that the intracellular level of GSH and GCL expression varied with time of incubation with LPS in inflammatory macrophages, and that the regulation on GCLC and GCLM in the inflammatory response might be through different signaling pathways. Our data also suggest that NF-κB pathway was involved in both the decrease of GCLC and activation of Nrf2 in LPS-stimulated inflammatory response.

Methods and Materials

Chemicals and reagents

Unless otherwise noted, chemicals were from Sigma (St. Louis, MO, USA). Antibodies to Nrf2, NF-κB p65, and lamin B1, and β-tubulin were from Cell Signaling Technology, Inc. (Danvers, MA, USA). TRIzol reagent, NE-PER Nuclear and Cytoplasmic Extraction Reagents, Reverse Transcription kit, SYBR Green PCR master mixture, and RPMI 1640 cell culture medium were from Thermal Fisher Scientific Inc. (Thermal Fisher, Rockford, IL, USA). NF-κB SN50 inhibitor was from EMD Millipore (Billerica, MA, USA).

Cell culture and treatment

THP-1 cells (Human acute monocytic leukemia cell line, from American Type Culture Collection) were cultured in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum (FBS), 100 μg/ml penicillin and 100 μg/ml streptomycin, and 0.05 mM β-

mercaptoethanol at 37° C in a humidified 5% CO_2 incubator. Prior to experiments, THP1 cells at a density of 3×10^5 cells/cm2 were differentiated into macrophages in medium containing 7.5 ng/ml phorbol 12-myristate-13-acetate (PMA) for 2 days, and then the medium was replaced with normal medium one day before further treatment.

GSH assay

GSH content was measured as described before [26]. Briefly, cells were lysed in 0.1% Triton X-100/0.1 M sodium phosphate buffer and then deproteinized with 5-sulfosalicylic acid. Cell lysate was added to a reaction mixture containing 10 μM diethylenetriaminepentaacetic acid (DETAPAC), 1.5 U/ml glutathione reductase, 0.2 mM NADPH, and 0.6 mM 5,5′-dithio-bis-[2-nitrobenzoic acid] (DTNB), and the reaction rate of DTNB reduction was monitored at 412 nm.

Western Analysis

Briefly, cell lysate was extracted with NE-PER and 20 μg protein was electrophoresed on a 4-20% Tris-glycine acrylamide gel (Thermal Fisher Scientific, Rockford, IL, USA) and transferred to polyvinylidene difluoride (PVDF) membrane. Membrane was blocked with 5% fat-free milk and then incubated with primary antibody overnight at 4oC in 5% BSA dissolved in Tris-buffered saline (TBS). After being washed with 1XTBS containing 0.05% Tween 20 (TTBS), the membrane was incubated with secondary antibody at room temperature for 2 h. After TTBS washing, the membrane was exposed to an enhanced chemiluminescence reagent mixture (ECL Plus; Thermal Fisher Scientific, Rockford, IL) for 5 min and then imaged and analyzed using Syngene PXi6 imaging system (Syngene, Cambridge, UK). The blots were analyzed with ImageJ.

mRNA assay

RNA extraction, reverse transcription, real time PCR assay, and calculation of relative mRNA levels, were performed as described before [27]. The primer sequences for real time PCR assay were as listed in Table 1.

Statistical analysis

Data were expressed as mean ± standard error. One-way ANOVA with post hoc analysis was used for significance testing. Wilcoxon rank-Sign test was used for statistical analysis of Western densitometry data. Statistical significance was accepted when $p < 0.05$.

Results

Pro-inflammatory cytokine induction by LPS in THP1 macrophages

We first determined the mRNA expression of pro-inflammatory cytokines in THP1-differentiated macrophages in response to LPS as a function of time, to establish the model of the inflammatory response. The mRNA level of pro-inflammatory cytokines (TNF-α and IL-1β) remained unchanged throughout the observation when cells were treated with vehicle control (PBS). Upon LPS exposure (10 ng/ml), the mRNA level of these cytokines was

increased significantly at 1 h, peaked at 3-6 h, and then gradually decreased after 12 h, but it remained higher than basal level even at 24 h after LPS exposure.

Temporal alteration of GSH level in response to LPS exposure

To examine the temporal change of GSH level, the total GSH content was measured in THP1 macrophages treated with 10 ng/ml LPS for a various period of time. As shown in Fig. 2, the intracellular total GSH concentration exhibited a time-dependent change after LPS exposure. It started to decrease at 1 h, down to the lowest at about 3-6 h (decreased by about 40%), and went back to basal level at 18 h after LPS exposure, suggesting that total GSH level was reduced in the early phase (1-9 h) of LPS exposure, but returned to basal level in the late phase (after 18 h). Without LPS, intracellular GSH concentration barely changed.

Time dependent changes in GCL expression following LPS exposure

As GCL is the rate-limiting enzyme for the *de novo* synthesis of GSH and its expression was reportedly decreased by LPS [13, 25], we hypothesized that the temporal change of GSH content in the inflammatory response observed above was due to alteration of GCL expression. To test it, the mRNA and protein levels of both GCLC and GCLM, the two subunits of GCL, were determined in THP1 macrophages exposed to 10 ng/ml LPS for various time periods. As shown in Fig. 3A, the mRNAs of both GCLC and GCLM remained unchanged in the observation period in THP1 macrophages without stimulation. Following LPS exposure, the mRNA level of GCLC began to decrease at 1 h, went down to the lowest level at around 3 h, and then gradually went back to the basal level at 18 h. On the other hand, the GCLM mRNA level was increased at 3 h after LPS exposure and remained higher than basal (or control) level until the end of observation (Fig. 3A). The change of GCLC protein level was similar to its mRNA; GCLC protein decreased to as low as 40% of the start time (0 h) in the early phase of exposure (3-9 h) and then returned to start level (0 h) at 24 h. GCLM protein level was increased in a similar pattern as that of its mRNA in the observation period (24 h) (Fig. 3B). These data indicate that GCLC expression was decreased in the early phase of LPS exposure and back to basal level in the late phase, while GCLM expression was increased in this whole process.

Nrf2 activation during LPS-stimulated inflammatory response

The expression of both GCLC and GCLM genes is regulated through Nrf2 signaling at transcription level [16, 19-22]. We hypothesized that Nrf2 signaling would also be involved in the alterations of GCLC and GCLM mRNAs in LPS-triggered inflammatory response. To test this, the nuclear translocation of Nrf2 (nuclear Nrf2 protein, a marker of Nrf2 activation) was measured at various time points under conditions of with and without LPS exposure. We compared the timing of Nrf2 translocation into nucleus with that of the p65 subunit of NF-κB. Without LPS exposure, nuclear protein levels of both Nrf2 and NF-κB/p65 remained constant during the observation. Nuclear p65 protein level, a marker of NF-κB signaling activation, was increased significantly at as early as 30 min after LPS stimulation, peaked at 3 h, and went back to the unstimulated level at 9 h. On the other hand, nuclear Nrf2 protein was increased at 3 h and remained higher than basal level until 6 h, and then went back to

basal level at 9 h (Fig. 4). These data suggest that the activation of Nrf2 signaling occurred later than that of NF-κB signaling.

NF-κB signaling inhibitor prevented LPS-caused decrease in GCLC mRNA

Given the coincidence of the decrease in GCLC expression and the induction of pro-inflammatory cytokines at the early phase of the inflammatory response, we hypothesized that NF-κB signaling pathway was involved in the decrease in GCLC expression. THP1 macrophages were pretreated with SN50, a specific NF-κB peptide inhibitor [28], before LPS stimulation (10 ng/ml), and then its effect on GCL expression was determined. As expected, SN50 (50 μg/ml) significantly reduced the induction of both TNF-α and IL-1β by LPS (Fig. 5A), indicating a significant decrease in NF-κB-mediated induction of pro-inflammatory cytokines/mediators. Meanwhile, SN50 pretreatment prevented the decrease of GCLC mRNA (Fig. 5B) and protein (Fig.5C) by LPS at 3 h, although it had no effect on LPS-induced GCLM induction (Fig. 5A), suggesting that NF-κB signaling was involved in the decrease in GCLC expression in the early phase of LPS-triggered inflammation but not in the induction of GCLM by LPS.

NF-κB inhibitor blocked Nrf2 activation by LPS

To explore the potential role of early activation of NF-κB signaling (occurs in 30 min after LPS exposure) in the activation of Nrf2 signaling (at 3 h of LPS exposure), we investigated the effect of NF-κB inhibitor SN-50 on Nrf2 activation by LPS. SN50 pretreatment prevented the increase of nuclear NF-κB p65 protein (marker of NF-κB activation) caused by LPS, meanwhile it abrogated LPS-mediated increase of nuclear Nrf2 protein (Fig. 6), suggesting that activation of NF-κB signaling is involved in the following activation of Nrf2 in the process of inflammatory response of THP1 macrophages to LPS.

Discussion

Glutathione, the most abundant non-protein thiol in cells, plays an essential role in the inflammatory and/or immune response. In this study, we demonstrated that the intracellular GSH level exhibited a temporal change during the inflammatory response of THP1 macrophages to LPS. It was decreased in the early phase (3-9 h) and back to basal level in the late phase (after 18). The temporal change in GSH level was highly associated with that of GCLC, the catalytic subunit of GCL, while the expression of GCLM was increased in the observational period of LPS exposure. We also found that the early activation of NF-κB signaling was involved in both the decrease in GCLC expression and the activation of Nrf2 signaling.

Consistent with previous reports, total GSH in THP1 macrophages was decreased significantly in the early phase of LPS exposure (Fig. 2). It was postulated that the decrease was due to the deficiency of precursor amino acids for GSH synthesis such as cysteine [29, 30] or the increased consumption of GSH during inflammation [10-14]. Accumulating evidence indicate that the decrease in GSH level in LPS-triggered inflammatory response might be largely attributed to the decrease in GCL expression [13, 25]. The current finding that the decrease in the intracellular GSH occurred coincidentally with the decrease in

GCLC expression (protein and mRNA) level in the early phase of the inflammatory response (Fig. 3) provides further evidence for this. On the other hand, GCLM expression (mRNA and protein) was increased over the period of LPS exposure, demonstrated that it was the decline in GCLC rather than GCLM that was responsible for the decrease in GSH level in the inflammatory response to LPS. It is noteworthy that other mechanisms, such as increased GSH consumption inside of the cells and/or increased exportation of GSSG to the outside of cells, also contribute to decreased GSH concentration in LPS-challenged macrophages.

The gradual recovery of the intracellular GSH concentration from its lowest level (3-6 h) to the basal level (18 h) after LPS exposure demonstrated that macrophages had a system to increase intracellular GSH level during the inflammatory response. Although several mechanisms including decreased consumption, decreased feedback inhibition of GCL activity by GSH, and increased GSH recycle (thus providing precursor cysteine) [15] may contribute to increased GSH level during oxidative stress, up regulation of GCL expression (both GCLC and GCLM) has been proved to be the major one of this well coordinated adaptive mechanism; i.e., increasing the capacity for *de novo* GSH synthesis through increasing GCL expression [31]. In this study, the expression of GCLC gradually increased from its lowest level at 3 h (after LPS exposure) to the basal level at 18 h, and GCLM expression was induced and remained higher than control after 3 h of LPS exposure. The increase in GSH level from the lowest level was well associated with the increase in GCLC expression, indicating that GCLC plays a key role in regulating the GSH level in the inflammatory response.

The differential alteration of GCLC and GCLM expression in the inflammatory response to LPS suggests that they were regulated through different mechanisms. As aforementioned, both genes could be regulated at the transcription level through the Nrf2-EpRE signaling pathway. Since Nrf2 signaling was activated only after 3 h of LPS exposure, it was inferred that this pathway was involved in the increase of GCLC and GCLM expression after 3-6 h of LPS exposure at transcriptional level. On the other hand, GCLC gene expression could also be regulated at post-transcriptional level. The mRNA stability of GCLC was regulated through the binding of HuR to an AU rich motif (+2785AUUUA) in its 3′-untranslated region [32]. As HuR is also required for mRNA stability of many inflammatory cytokines [33], whose mRNAs were immediately increased in the early phase (1-3 h) of the inflammatory response, its binding to GCLC mRNA could be dramatically decreased due to the competition, leading to faster decay of GCLC mRNA. This hypothesis was at least partially supported by the finding that LPS-mediated GCLC decrease was prevented by NF-κB signaling inhibitor SN50, which inhibits translocation of NF-κB to the nucleus thereby simultaneously suppressed the induction of pro-inflammatory cytokines (Fig.5). Although Tomasi and collaborators [25] reported that a lowered sumolation of Nrf2 and its partners might be responsible for the decreased expression of both GCLC and GCLM in a murine macrophage cell line (Raw264.7) in response to LPS, the differential change of GCLC and GCLM mRNA as found here suggests that a mechanism other than Nrf2 signaling might be involved.

It is noteworthy that the interaction between NF-κB and Nrf2 signaling may also be involved in the down-regulation of GCLC in LPS challenged macrophages. For example, both NF-κB

and Nrf2 pathways require the transcriptional co-activator cAMP response element binding protein (CREB) binding protein (CBP)/p300 for the transcription activity, and CBP/p300 could become rate limiting for the gene transcription when both pathways are activated [34]. In addition, NF-κB/p65 could antagonize Nrf2 binding to EpRE through competing for co-activators and promoting a corepressor (histone deacetylase 3) [34]. Considering the overlapping time of the increased nuclear levels of both NF-κB and Nrf2 after LPS exposure (Fig. 4), antagonistic competition for transcription co-activators from NF-κB could impair the efficacy of Nrf2 signaling and decrease GCLC transcription.

Regarding the underlying mechanism involved in GCLM induction before Nrf2 activation (~3h) in the inflammatory response to LPS, limited information is available. Based on the evidence that Nrf2 was just activated at 3 h of LPS exposure, and that there was a synergic effect on the increase of GCLM mRNA between LPS and Nrf2 activator sulforaphane at this time point (data not shown), it is postulated that signaling pathway other than Nrf2 was involved. NF-κB signaling was reportedly involved in the regulation of GCLM [35]. However, NF-κB inhibitor SN50 had no effect on GCLM induction at 3h of LPS exposure even though it suppressed Nrf2 signaling (Fig.5 and Fig.6). This evidence however, cannot exclude the possibility that NF-κB subunit combinations other than the p65/p50 dimer, which was the target of SN50, is involved in GCLM regulation by LPS. In addition, GCLM expression was also up regulated through AP-1 activation [36], which was activated by LPS in THP1 cells [37]. Therefore, the involvement of AP-1 signaling in GCLM induction cannot be excluded. Nonetheless the underlying mechanism of how GCLM expression was induced in response to LPS exposure was beyond the scope of the current study.

Another novel finding in this study was that activation of NF-κB signaling pathway was prerequisite for later Nrf2 activation by LPS (Fig. 6). Combined with the evidence that NF-κB activation and cytokine induction occurred earlier than Nrf2 nuclear translocation, and that SN50 inhibited both cytokine induction and Nrf2 activation, it could be inferred that Nrf2 activation in response to LPS was through NF-κB-mediated mechanism. Consistent with this, we recently reported that a delayed activation of Nrf2 signaling by iron-coated silica nanoparticles required the activation of NF-κB signaling in THP1 macrophages [38]. It remains to be determined how NF-κB signaling causes Nrf2 activation in the response of THP1 cells to these inflammatory stimuli. One possible mechanism is that it is mediated through NF-κB-regulated gene products. The induction and release of NF-κB-regulated pro-inflammatory cytokines (including TNFα and IL-1β) [39-42] and/or mediators including cyclooxygenase-2 (COX-2) [43, 44] usually occurs in as soon as 1h in the inflammatory response [45]. These cytokines/inflammatory mediators could activate NADPH oxidases [46] to produce H_2O_2, and/or increase production of electrophiles [47, 48], and subsequently activate Nrf2 signaling. This hypothesis is supported by reports from Barrett *el al.*, that TNF-α induction by silica was required for the subsequent up regulation of chemokine in alveolar type II cells [49, 50] and Herseth *et al.* that the early induction of IL-1β and COX-2 genes was prerequisite for a later up regulation of IL-6 [51]. In addition, 15-deoxy-delta-12, 14-prostaglandin J2 (15d-PGJ2), a product of COX-2, is a well-established Nrf2 activator [52]. With the large number of NF-κB regulated genes [53], which of them was responsible for Nrf2 activation would be too speculative to suggest here. In summary, intracellular GSH and the expression of GCLC are temporally altered in the inflammatory response to LPS in

THP1 macrophages, and it opposes in parallel the temporal change in pro-inflammatory cytokine expression (Fig.7). The opposing temporal changes in pro-inflammatory cytokines and GSH, and the temporal activation of NF-κB and Nrf2 signaling, suggest this is a well-coordinated process. Nrf2-target gene products including GSH have been recognized as anti-inflammation effectors in many studies, and it is reasonable to expect an increased Nrf2 signaling as an adaptive response to inflammation-caused oxidative stress. However, the pathophysiologic significance underlying the decrease of GSH level in the early phase of inflammatory response when large amount of pro-inflammatory cytokines are produced, remains to be determined, especially considering the implication that GSH deficiency has in various inflammatory and infectious diseases [54]. Based on current findings and available evidence from others, a putative mechanism and signaling pathways involved in the decrease of GSH concentration in the early phase of inflammatory response of THP1 macrophages to LPS are summarized (Fig. 8). In response to LPS exposure, GSH consumption is increased due to excessive production of oxidants while its biosynthesis is decreased due to decreased GCL expression, this results in the decrease in GSH concentration. The decreased GCL expression is mainly attributed to a decline in GCLC expression, probably resulted from the combined effect of increased mRNA decay rate and decreased transcription, both processes are mediated through NF-κB signaling. The exact mechanisms involved of course, need further to be determined.

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Highlights

- GSH concentration altered kinetically in macrophages in response to LPS
- GSH decrease in the early phase of LPS exposure coincided with a decreased expression of GCLC
- NF-κB inhibition prevented LPS-caused decrease in GCLC but did not affect LPS-induced GCLM
- NF-κB inhibition abrogated LPS-mediated Nrf2 activation in macrophages

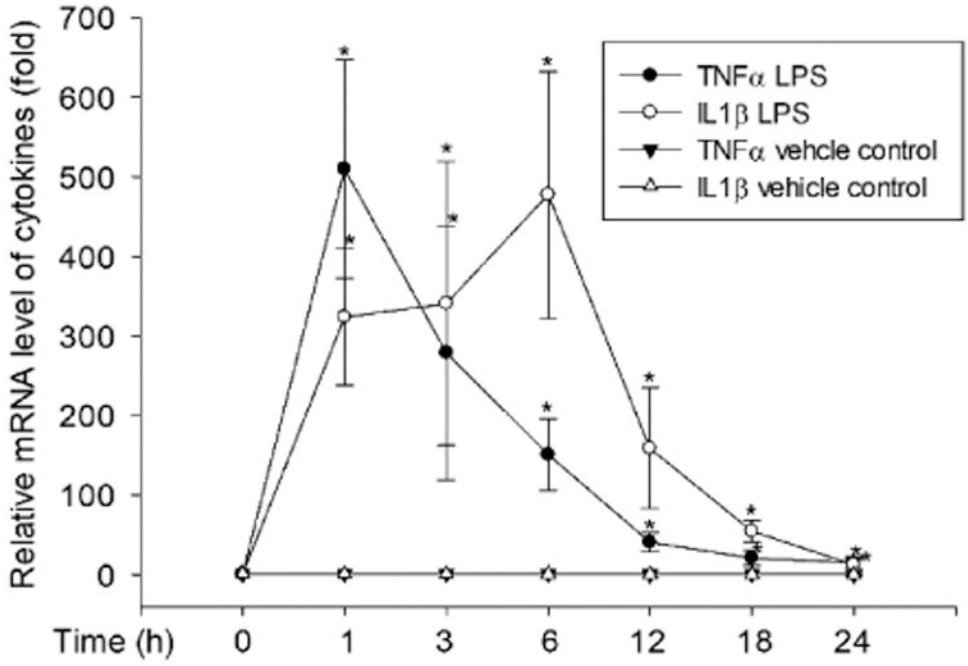


Figure 1.

Temporal induction of pro-inflammatory cytokines by LPS. Differentiated THP1 macrophages were stimulated with 10 ng/ml LPS for time as indicated and the mRNAs of pro-inflammatory (TNFα and IL-1β) were determined using real time PCR assay. The mRNA induction by LPS was shown as fold change compared to vehicle control at the same treatment time, and the mRNA level of vehicle control was shown as fold change compared to start time (0 h), N=4, * P<0.05.

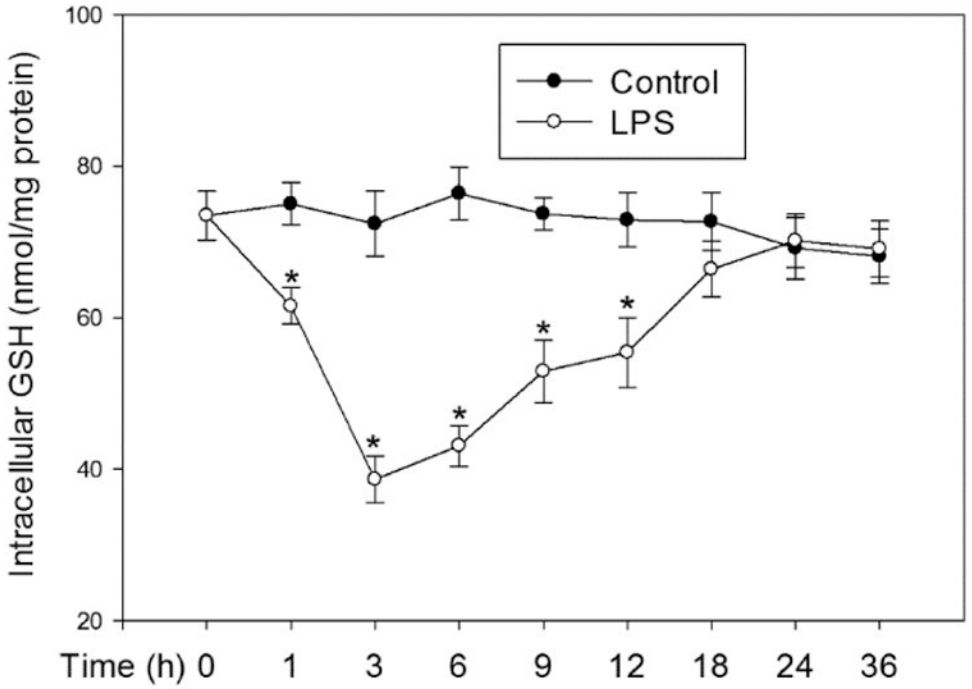


Figure 2.

Change of intracellular total GSH concentration in the inflammatory response to LPS. After being stimulated with 10 ng/ml LPS for indicated time, THP-1 macrophages were collected and the total GSH level was measured. N=3, *, $P<0.05$ compared to vehicle control at the same treatment time.

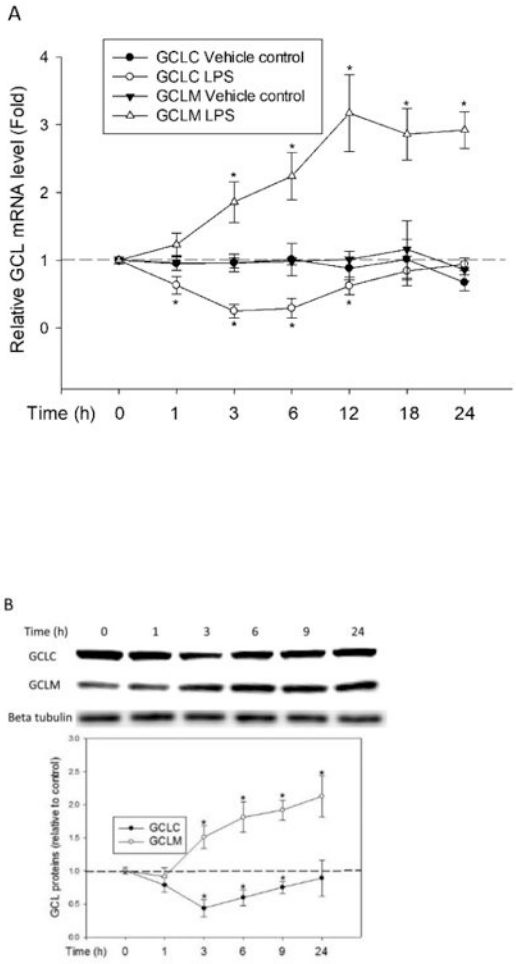


Figure 3.

Temporal change of GCL expression upon LPS stimulation. Differentiated THP-1 macrophages were exposed with 10 ng/ml LPS for indicated time, and the mRNA (A) and protein (B) level of both GCLC and GCLM were determined real time PCR and Western blotting assay, in respective. The mRNA induction by LPS was shown as fold change compared to vehicle control at the same treatment time, the relative mRNA level of vehicle control and the induction of proteins were shown as fold change compared to start time (0 h). N=3, *, P<0.05.

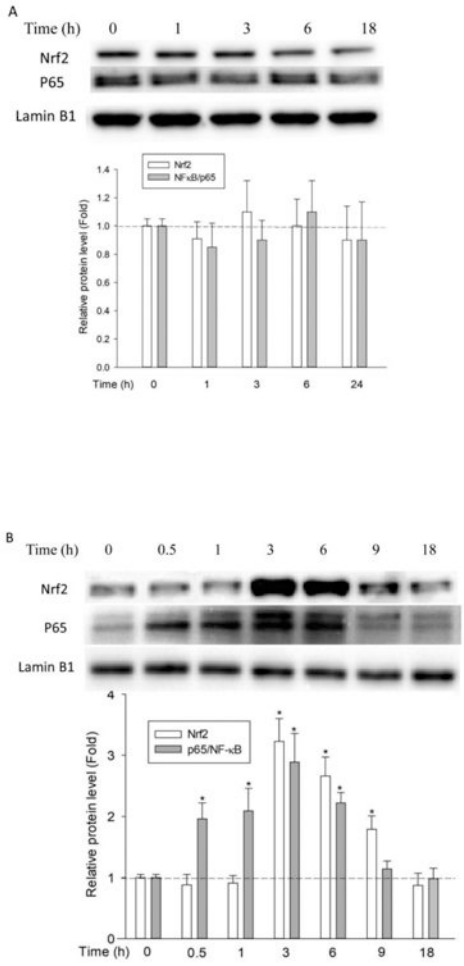
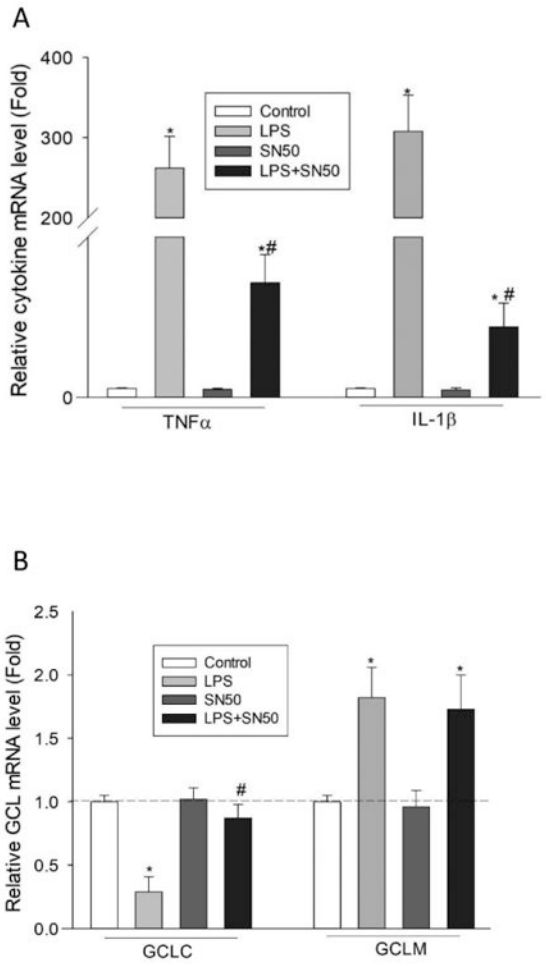


Figure 4.

Temporal change of protein levels of nuclear Nrf2 and NF-κB/p65. Differentiated THP-1 macrophages were treated with vehicle control (A) or 10 ng/ml LPS (B) for indicated time, and the nuclear protein levels of NF-κB p65 and Nrf2 were determined with Western blotting. N=3, *, P<0.05 compared to the protein level at start time (0 h).



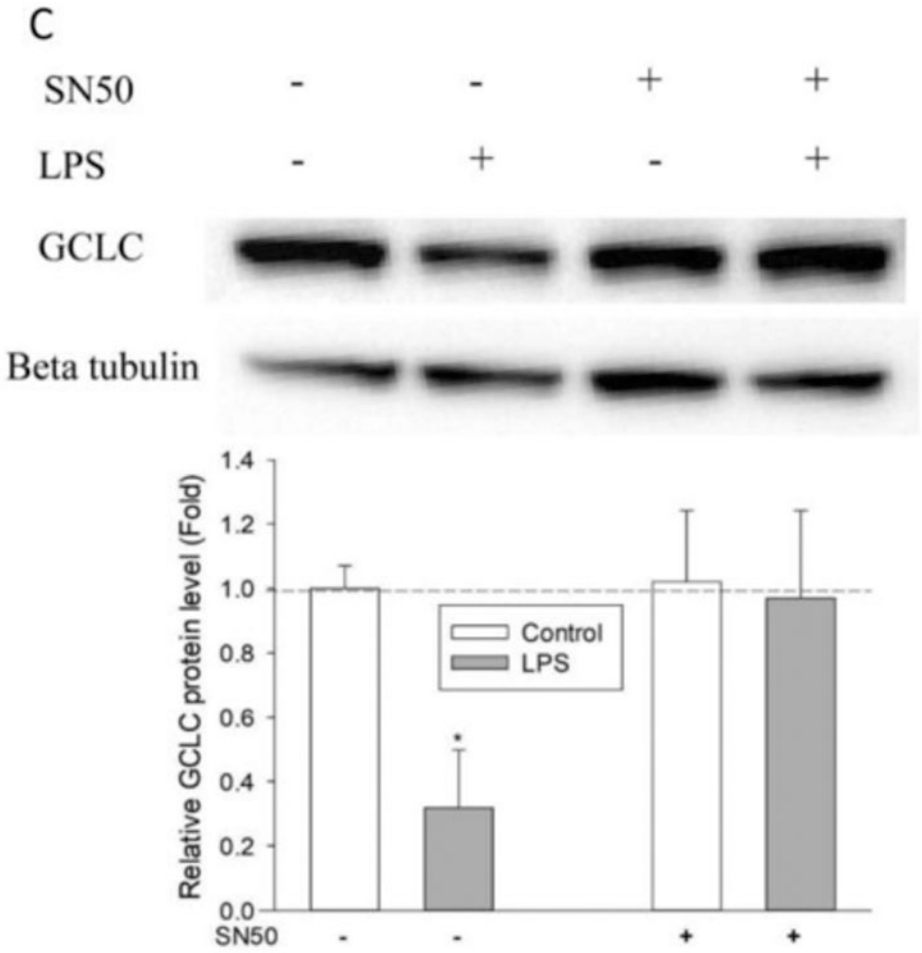


Figure 5.

NF-κB inhibitor prevented LPS-mediated decrease of GCLC expression. THP-1 macrophages were pretreated with 50 μg/ml SN-50 for 15 min and then exposed to 10 ng/ml LPS for 3h. The mRNA levels of pro-inflammatory cytokines (A), GCL mRNA (B), and GCLC protein level (C) were determined. N=3; *, P<0.05 compared to vehicle control; #, P<0.05 compared with LPS exposure alone.

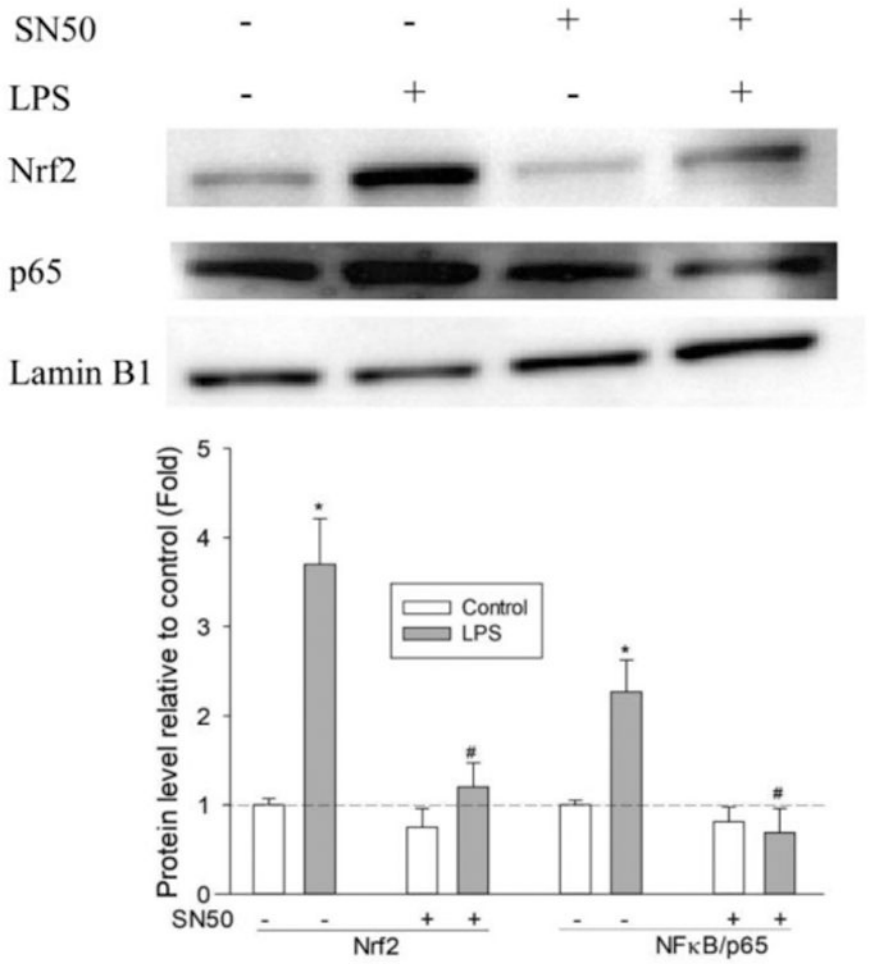


Figure 6.

NF-κB inhibitor inhibited LPS-caused Nrf2 activation. THP-1 macrophages were pretreated with 50 μg/ml SN-50 for 15 min and then exposed to 10 ng/ml LPS for 3h. The nuclear NF-κB p65 and Nrf2 protein levels were determined with Western blotting. N=3; *, P<0.05 compared to vehicle control; #, P<0.05 compared with LPS exposure alone.

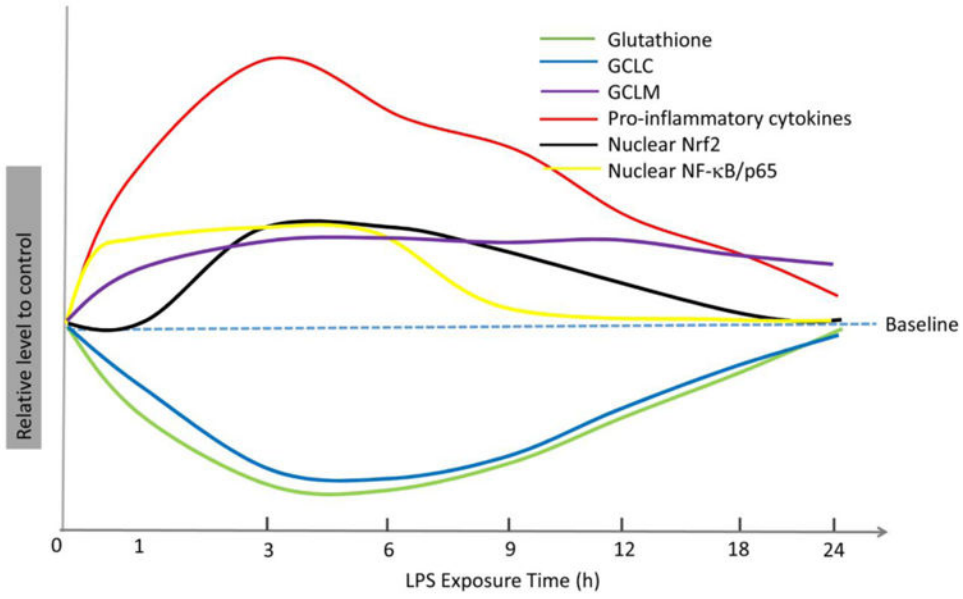


Figure 7.

Summary of the kinetic change of cytokine, total GSH, and GCL mRNAs during LPS-stimulated inflammatory response. X Axis shows the exposure time and Y Axis shows the change of pro-inflammatory cytokines (red), total GSH (green), GCLC (blue), GCLM (purple), Nrf2 (black), and NF-κB/p65.

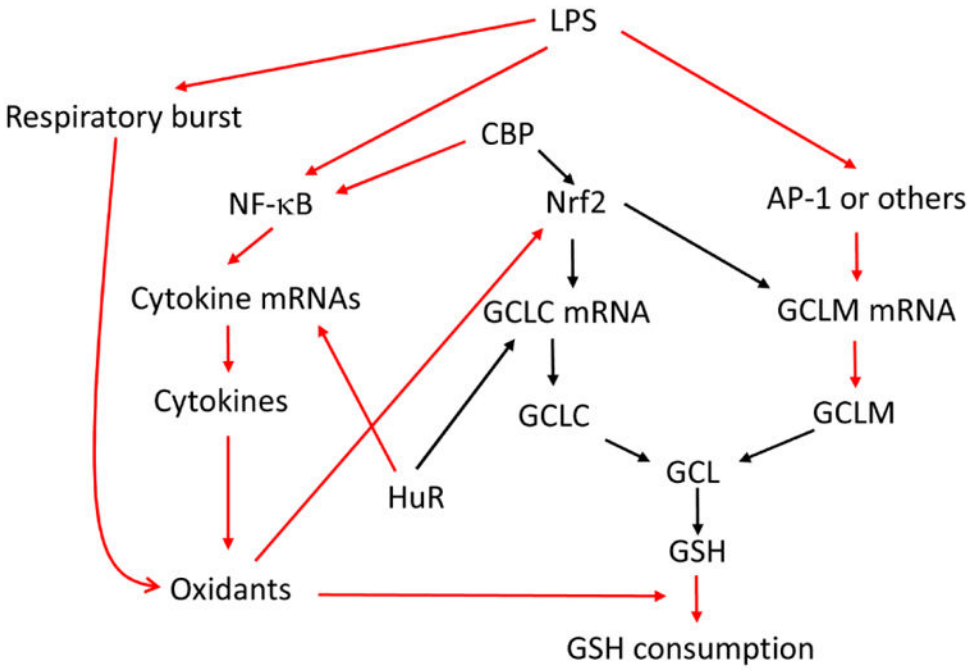


Figure 8.

Putative signaling pathways involved in GSH decrease in the inflammatory response of macrophages to LPS. Red arrow shows activation or increase and black arrow shows inactivation or decrease. In the early phase of LPS exposure, NF-κB is activated and pro-inflammatory cytokine mRNAs are induced immediately. The former antagonizes Nrf2 signaling by competition for co-activator CBP thus decreases GCLC gene transcription, while the latter competes with GCLC mRNA for HuR binding that is required for its mRNA stability, thus increases GCLC mRNA decay rate. Although GCLM can be induced through LPS-activation of AP-1 signaling, the overall expression of GCL activity is decreased. The decrease in GSH biosynthesis and increase in GSH consumption contribute to the decrease in GSH concentration.

Table 1

Primer sequences for mRNA assay

Genes	Primers
Actin	Forward 5′-CATGGAGTCCTGTGGCATC-3′; Reverse 5′-GGAGCAATGATCTTGATCTTC-3′
GCLC	Forward 5′-ATGGAGGTGCAATTAACAGAC-3′; Reverse 5′-ACTGCATTGCCACCTTTGCA-3′
GCLM	Forward 5′-GCTGTATCAGTGGGCACAG-3′; Reverse 5′-CGCTTGAATGTCAGGAATGC-3′
TNF-α	Forward 5′-CCCAGGGACCTCTCTCTAATCA-3′; Reverse 5′-AGCTGCCCCTCAGCTTGAG-3′
IL-1β	Forward 5′-CGACACATGGGATAACGAGGCTT-3′; Reverse 5′-TCTTTCAACACGCAGGACAGGTA-3′