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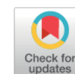
Original article

Down regulation of glutathione and glutamate cysteine ligase in the inflammatory response of macrophages



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ABSTRACT

Glutathione (GSH) plays critical roles in the inflammatory response by acting as the master substrate for antioxidant enzymes and an important anti-inflammatory agent. In the early phase of the inflammatory response of macrophages, GSH content is decreased due to the down regulation of the catalytic subunit of glutamate cysteine ligase (GCLC). In the current study we investigated the underlying mechanism for this phenomenon. In human THP1-differentiated macrophages, GCLC mRNA had a half-life of 4 h under basal conditions, and it was significantly reduced to less than 2 h upon exposure to lipopolysaccharide (LPS), suggesting an increased decay of GCLC mRNA in the inflammatory response. The half-life of GCLC protein was > 10 h under basal conditions, and upon LPS exposure the degradation rate of GCLC protein was significantly increased. The pan-caspase inhibitor Z-VAD-FMK but not the proteasome inhibitor MG132, prevented the down regulation of GCLC protein caused by LPS. Both caspase inhibitor Z-LEVD-FMK and siRNA of caspase-5 abrogated LPS-induced degradation of GCLC protein. In addition, supplement with γ-GC, the GCLC product, efficiently restored GSH content and suppressed the induction of NF-κB activity by LPS. In conclusion, these data suggest that GCLC down-regulation in the inflammatory response of macrophages is mediated through both increased mRNA decay and caspase-5-mediated GCLC protein degradation, and γ-GC is an efficient agent to restore GSH and regulate the inflammatory response.

1. Introduction

The innate immune/inflammatory response is a fundamental defense mechanism against invading pathogens and/or tissue damages. This complex process involves activation of multiple signaling pathways and production of various mediators including cytokines and oxidants, often referred to by the nonspecific misnomer, reactive oxygen species (ROS). Among these mediators, H_2O_2 plays a critical role by acting as a signaling messenger to enhance the respiratory burst and activate NF-κB signaling [1–4], and by facilitating the elimination of pathogens [5,6]. Excessive H_2O_2 and other reactive oxidants however, can exacerbate inflammation and cause oxidative stress [7–10], which is implicated in the pathogenesis of a variety of pathologies, including acute respiratory distress syndrome (ARDS) [11], idiopathic pulmonary fibrosis (IPF) [12,13], and others. Excessive H_2O_2 production can also distort redox signaling and disrupt cell function without causing irreversible oxidation of macromolecules [14]. Therefore, it is important to understand how redox homeostasis is regulated in the inflammatory response.

Glutathione (GSH) is the master antioxidant that maintains redox homeostasis and it is critically involved in the inflammatory response. In addition to functioning as an antioxidant, GSH is involved in modification of NF-κB and AP-1 signaling [15,16], leukotriene synthesis [17], and monocyte differentiation [18]. GSH depletion can augment [19,20], and its supplementation can suppress, the inflammatory response [20–25].

GSH content is temporally changed during the inflammatory response. In the early phase, its biosynthesis and content are decreased [26–29]; and in the late phase, the biosynthesis and content of GSH are increased [29,30]. This temporal alteration of GSH content is coincident with the inflammatory progress, with lower GSH level corresponding to the initiation phase when H_2O_2 production is increased (e.g., by NADPH oxidase production of superoxide which then dismutates to H_2O_2) and an oxidative milieu is needed (e.g., for activation

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of NF-κB and elimination of pathogens), and higher GSH content corresponding to the resolution phase when oxidative damage needs to be repaired and pro-inflammatory signals need to be silenced. Thus, temporal change of GSH biosynthesis and content are likely to be an important component of the inflammatory response.

GSH is a tripeptide composed of glutamate, cysteine and glycine, that is synthesized from two sequential reactions catalyzed by glutamate cysteine ligase (GCL) and glutathione synthetase. GCL, which catalyzes the formation of gamma-glutamylcysteine (γ-GC) from glutamate and cysteine, is the rate-limiting enzyme for *de novo* GSH biosynthesis. Previous studies showed that GSH depletion in the inflammation was largely due to the decreased expression of GCL [30–33]. GCL is an enzyme composed of catalytic (GCLC) and modifier (GCLM) subunit. In prior studies we showed that GSH depletion in the inflammatory response of macrophages was due to a temporal down-regulation of GCLC [30,34], but the underlying mechanism remained unclear.

In the current study we found that the down regulation of GCLC in the inflammatory response of macrophages was mediated at both post-transcriptional and post-translational levels, and that GCLC protein was degraded through caspase-5. In addition, we demonstrated that supplementation with γ-GC, the GCL product, efficiently restored GSH content and suppressed the inflammatory response.

2. Methods and materials

2.1. Chemicals and reagents

Unless otherwise noted, chemicals were from Sigma (St. Louis, MO, USA). Antibody of α-actin was from Cell Signaling Technology, Inc. (Danvers, MA, USA). The antibody and siRNA of caspase 5 were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Pan-caspase inhibitor Z-VAD-FMK and caspase-1 inhibitor Z-YVAD-FMK were from R&D Systems (Minneapolis, MN, USA), and caspase 4/5 inhibitor Z-LEVD-FMK was from Biovision, Inc. (Milpitas, CA, USA). THP1 Blue NF-κB reporter monocyte and QUANTI-Blue were from InvivoGen (San Diego, CA, USA). TRIzol reagent, Mammalian Protein Extraction Reagent (M-PER), Reverse Transcription kit, SYBR Green PCR master mixture, RNAiMAX, OPTI-MEM, and RPMI 1640 medium were from Thermal Fisher Scientific Inc. (Thermal Fisher, Rockford, IL, USA).

2.2. Cell culture and treatment

THP1 cells (Human acute monocytic leukemia cell line, from American Type Culture Collection) were cultured under condition as previously described [30]. Prior to experiments, THP1 cells at a density of 3×10^5 cells/cm^2 were differentiated into macrophages in medium containing 7.5 ng/ml of phorbol 12-myristate-13-acetate (PMA) for 2 days. The cell medium was replaced with normal medium one day before further treatment.

2.3. Transfection

THP1 macrophages were transfected with scrambled or caspase-5 siRNA by using RNAiMAX. In brief, siRNA and RNAiMAX were diluted in equal volume of OPTI-MEM, then mixed at a ratio of 1 μl (RNAiMAX): 25 nmol (siRNA). The mixture was added into cultured cells after being incubated for 5 min at RT, and 48 h later, the transfected cells were treated with LPS.

2.4. NF-κB reporter assay

THP1-Blue NF-κB reporter monocytes were maintained in RPMI1640 medium containing 10% FBS, 1% pen/strep, 100 μg/ml Normocin, and 10 μg/ml Blasticidin at 4×10^5-$1x10^6$ cells/ml with 5% CO_2 at 37 °C. To measure NF-κB activity, THP1-Blue monocytes were seeded in 96-well plates at 8×10^5 cells/ml. After treatment with LPS for 24 h, cells were centrifuged at 2,000g for 10 min and supernatants were assayed for SEAP activity with QUANTI-Blue (InvivoGen). In brief, supernatant was added to QUANTI-Blue solution in 96-well assay plates, incubated at 37 °C/1 h, followed by spectrophotometry at 652 nm.

2.5. Western blotting

Western blotting was performed as previously described [30]. Briefly, protein was extracted with M-PER and 15 μg protein was electrophoresed on a 4–20% Tris-glycine acrylamide gel and transferred to polyvinylidene difluoride (PVDF) membrane. Membrane was blocked with 5% fat-free milk and then incubated with primary antibody overnight at 4 °C in 5% milk 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 and ECL exposure, image was captured using a Syngene PXi6 imaging system (Syngene, Cambridge, UK). The blots were analyzed with ImageJ.

2.6. Quantitative PCR assay of mRNA

Quantitative PCR (qPCR) assay of mRNAs was performed as previously described [30]. For mRNA stability assay, 18S RNA was used as internal control; for other mRNA assays, actin mRNA was used as internal control. PCR primer sequences for GCLC and actin were as described before [30] and the primer sequences for 18S are 5'-GGCCCT GTAATTGGAATGAGTC-3' and reverse 5'-CCAAGATCCAACTACGAG CTT-3'.

2.7. Statistical analysis

Data were expressed as mean ± standard deviation. For significance analysis, one-way ANOVA with post hoc analysis was performed for time-dependent change, and student t-test with Welch's correction for others, by using Prism 7. Statistical significance was accepted when $p < 0.05$.

3. Results

3.1. GCLC mRNA decay was increased in the inflammatory response

We previously demonstrated that the expression of GCLC mRNA was decreased in the early phase of the inflammatory response of macrophages [30,34], but whether it happens at transcriptional or post-transcriptional level remained unclear. To examine this question, we determined the decay rate of GCLC mRNA in THP1-differentiated macrophages with/without inhibiting transcription. Treatment with actinomycin D (AD, 5 μg/ml), an inhibitor of RNA transcription, decreased GCLC mRNA level in a time-dependent manner, with a half-life of about 4 h. Upon LPS exposure, the decay of GCLC mRNA was about 2 fold faster than that caused by AD treatment (Fig. 1A). Co-treatment with AD and LPS did not enhance the decrease in GCLC mRNA caused by AD alone (Fig. 1B), suggesting that the down regulation of GCLC mRNA in the inflammatory response is due to increased mRNA decay.

3.2. The degradation of GCLC protein was enhanced in the inflammatory response

In the inflammatory response, GCLC protein was decreased with a similar time pattern as its mRNA [30,34]. To examine how the decline in GCLC mRNA contributes to the decrease in protein level, we first compared the relative decrease in GCLC mRNA and protein at different time upon transcription inhibition by AD. Compared to its mRNA (Fig. 1A), it took 4 more hrs for GCLC protein to reach the same

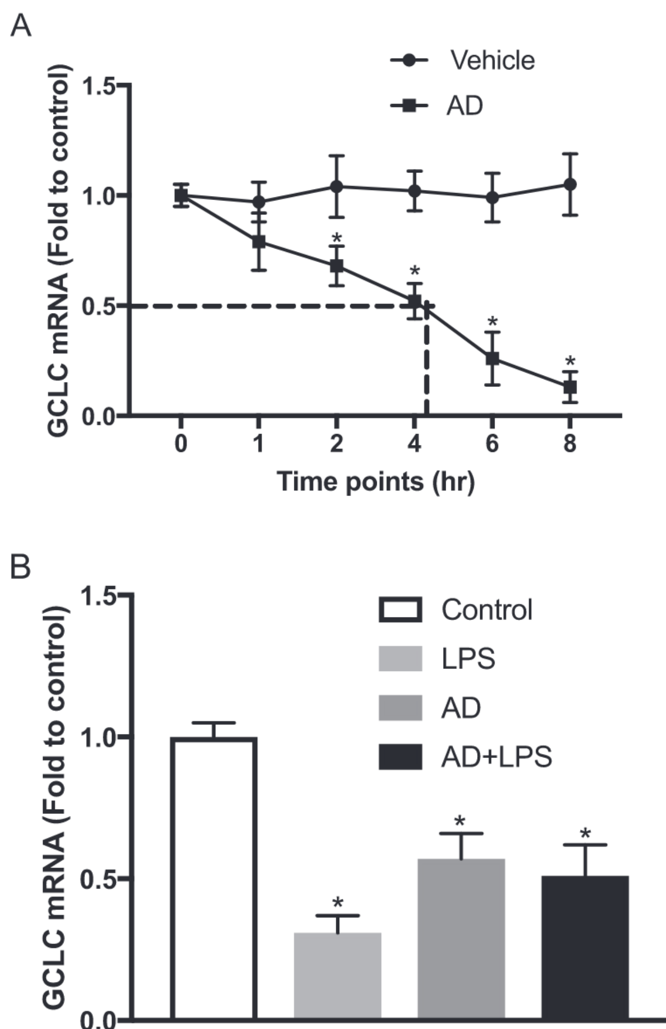

Fig. 1. LPS increased GCLC mRNA decay. (A) GCLC mRNA decay rate. THP1-differentiated macrophages were treated with/without 5 μg/ml actinomycin D (AD) for indicated time and GCLC mRNA was measured using qPCR. N = 4, *P < 0.05 compared with vehicle control at the same time point. (B) LPS increased the decay of GCLC mRNA. THP1-differentiated macrophages were treated with/without AD (5 μg/ml) and LPS (10 ng/ml) for 3 h and GCLC mRNA was measured using qPCR. *P < 0.05 compared with vehicle control.

magnitude of decrease (Fig. 2A). This suggests that the decline in mRNA level only partially contributed to the LPS-caused decrease in GCLC protein. We then determined the protein degradation rate of GCLC protein. Upon exposure to cycloheximide (10 μg/ml), an inhibitor of protein translation, GCLC protein was degraded gradually with a half-life of about 12 h (Fig. 2). GCLC protein was decreased by ~60% at 3 h after LPS exposure [30], therefore it was unlikely due to a decline in protein translation, but rather due to enhanced protein degradation.

3.3. GCLC protein was degraded by caspase-5 in the inflammatory response

To determine how GCLC protein was degraded in the inflammatory response, we first tested the involvement of proteasomes. Pre-treatment of macrophages with MG-132 (10 μg/ml), a proteasome inhibitor, did not change the expression of GCLC protein and its decrease upon LPS exposure (3 h) (Fig. 3A). Since it was reported that caspase mediated GCLC protein degradation in apoptosis [35], we then examined the involvement of caspases. Pretreatment of macrophages with pan-caspase inhibitor Z-VAD-FMK (10 μg/ml) completely prevented GCLC protein degradation caused by LPS exposure (Fig. 3A), suggesting that GCLC protein is degraded through caspases.

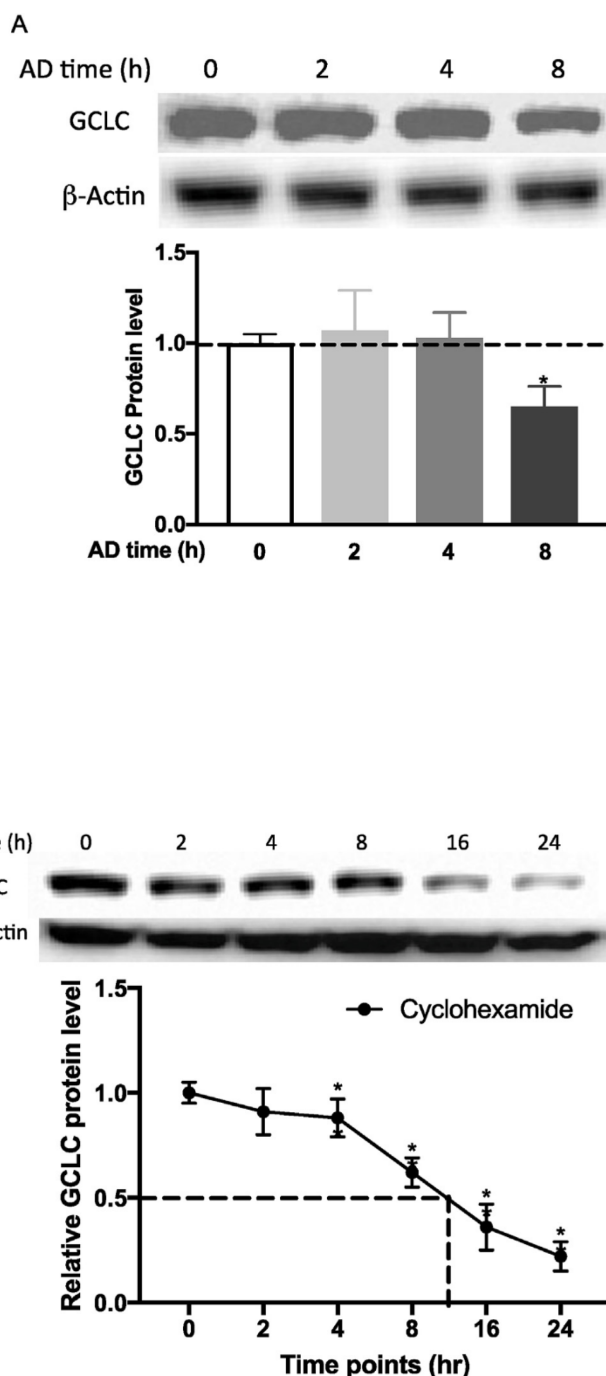

Fig. 2. Down regulation of GCLC protein in the inflammation was due to enhanced protein degradation rather than decreased mRNA level. (A) The decrease in GCLC protein upon transcription inhibition. THP1-differentiated macrophages were treated with/without 5 μg/ml actinomycin D (AD) for indicated time and GCLC protein was determined using Western blotting. N = 3, *P < 0.05 compared with starting time. (B) Time-dependent turnover of GCLC protein. THP1-differentiated macrophages were treated with cycloheximide (CH, 10 μg/ml)) for indicated time and GCLC protein was determined using Western blotting. N = 3, *P < 0.05 compared with starting time.

Caspases are a family of endoproteases consisting of 12 members in human, including 4 caspases (caspase-1, -4, -5, and -12) involved in the inflammation [36]. To identify the specific caspase that targets GCLC, we tested the effect of specific inhibitors of the inflammatory caspases on GCLC protein degradation. Among them, Z-LEVD-FMK (5, 10 μg/ml), an inhibitor for both caspase-4 and -5, prevented LPS-induced

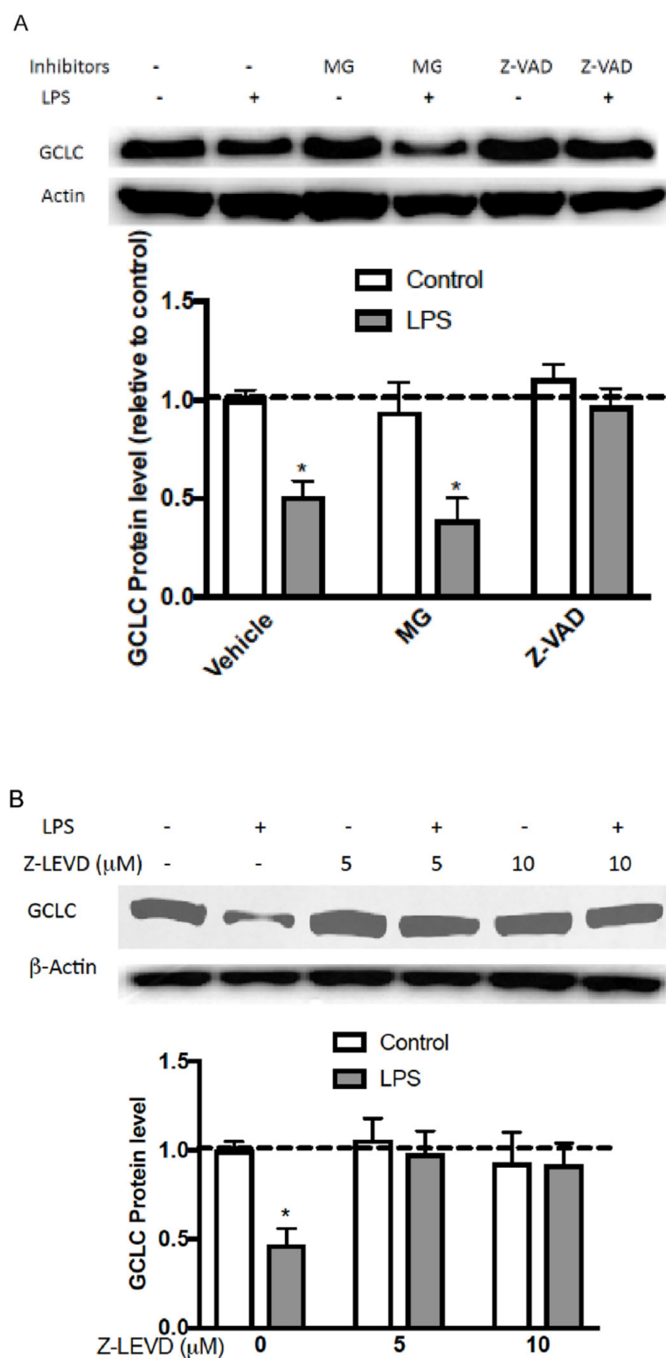

Fig. 3. GCLC protein was degraded by caspases in the inflammatory response of macrophages to LPS. (A) Pan-caspase inhibitor but not proteasome inhibitor prevented LPS-induced down regulation of GCLC protein. THP1 macrophages were pretreated with/without proteasome inhibitor (MG132, 10 μM) or pan-caspase inhibitor (Z-VAD-FMK, 10 μM) for 30 min and then treated with LPS (10 ng/ml) for 3 h. GCLC protein was measured using Western blotting. N = 3, *P < 0.05 compared with vehicle control, #P < 0.05 compared with LPS alone. (B) Caspase-4/5 inhibitor prevented LPS-induced down regulation of GCLC protein. THP1 macrophages were pretreated with caspase-4/5 inhibitor (Z-LEVD-FMK, 5 and 10 μM) for 30 min before being treated with LPS (10 ng/ml) for 3 h and GCLC protein was determined using Western blotting. N = 3, *P < 0.05 compared with vehicle control.

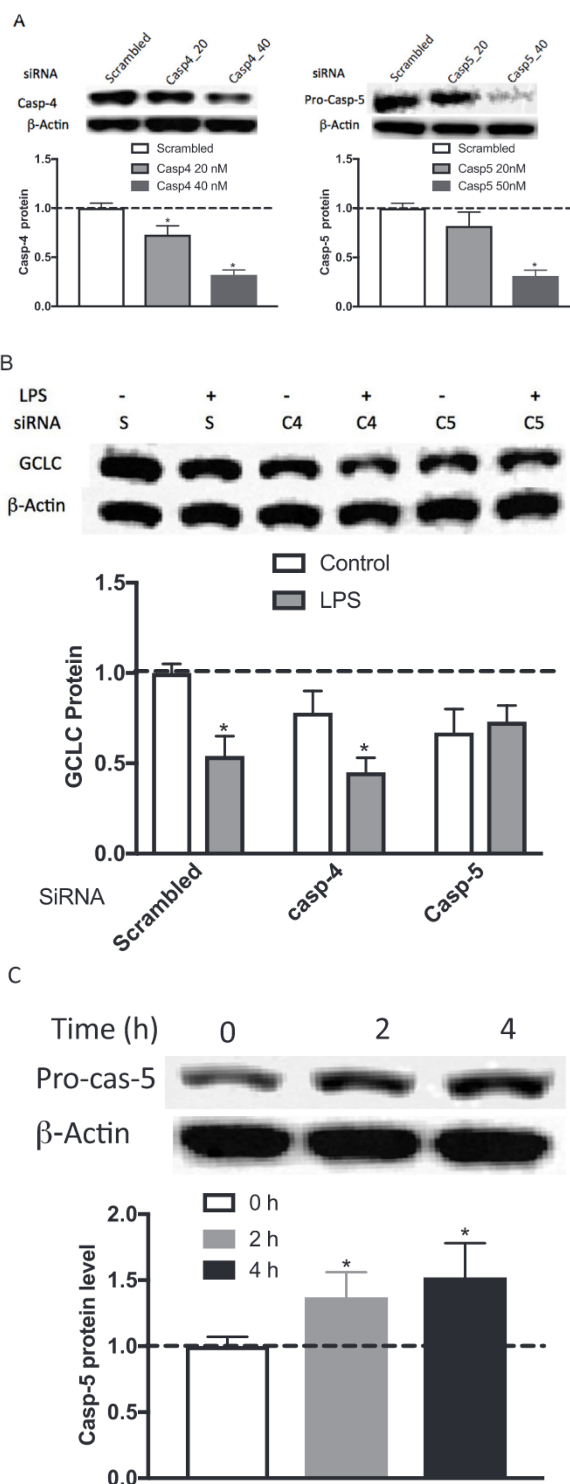

Fig. 4. Caspase 5 mediated GCLC degradation in the inflammatory response. (A) Silencing Caspase-4 and -5 with siRNA. THP1-differentiated macrophages were transfected with siRNA for 48 h and protein was determined using Western blotting. *, P < scrambled siRNA, N = 3. (B) Silencing caspase 5 prevented LPS-induced down regulation of GCLC protein. THP1 macrophages were transfected with scrambled or caspase-4 or -5 siRNA (40 nM) for 48 h and then treated with LPS (10 ng/ml) for 3 h. Protein was determined using Western blotting. N = 3, *P < 0.05 compared with vehicle of scrambled, #P < 0.05 compared with LPS of scrambled. (C) Induction of caspase-5 by LPS. Cells were treated with LPS for indicated time and protein was determined using Western blotting. Only pro-caspase-5 (43kD) was observed. N = 3, *P < 0.05 compared with start time (0 h).

GCLC protein degradation (Fig. 3B), and caspase-1 inhibitor (Z-YVAD-FMK, 10 μM) showed no effect (data not shown). To further determine the caspase involved, caspase-4 or -5 was silenced with siRNA (Fig. 4A). LPS-caused decrease in GCLC protein was prevented by caspase 5

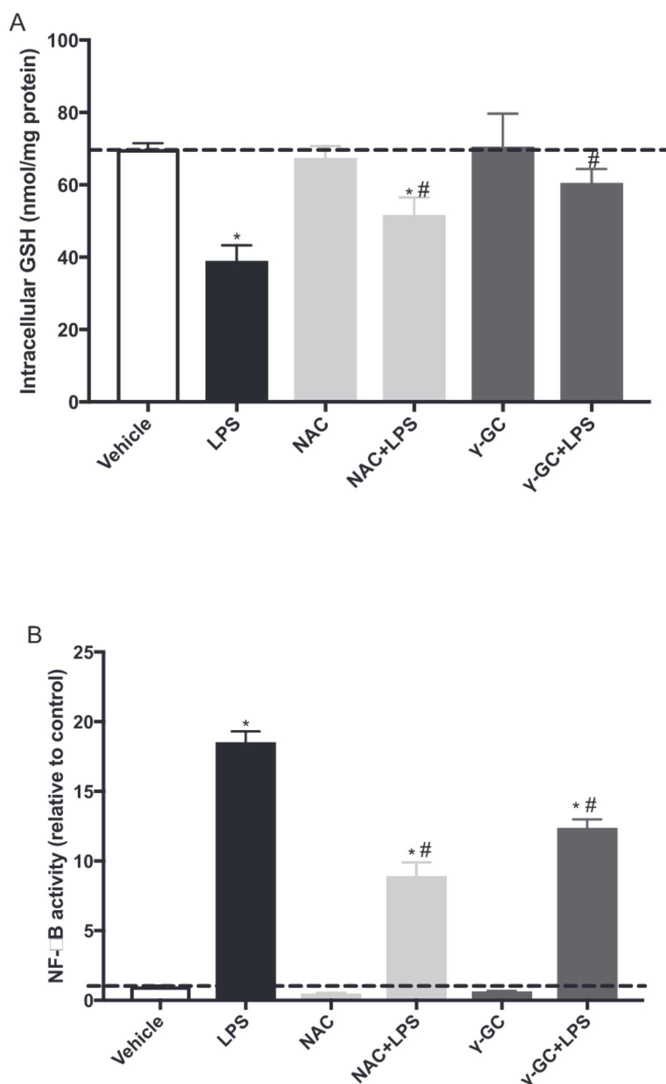

Fig. 5. Supplement of γ-GC restored GSH content and alleviated NF-κB activation by LPS. (A) γ-GC restored LPS-caused decline in GSH content. THP1-differentiated macrophages were pretreated with γ-GC (2 mM) or NAC (2 mM) for 1 h before being exposed to LPS (10 ng/ml) for another 3 h. GSH content was measured using protocol described in Method. N = 3, *P < 0.05 compared with vehicle control; #P < 0.05 compared with LPS. (B) γ-GC supplementation alleviated NF-κB activation by LPS. THP1 Blue NF-κB reporter cells were pretreated with γ-GC (2 mM) or NAC (2 mM) for 2 h before being exposed to LPS (10 ng/ml) for 24 h, and the NF-κB activity was measured. N = 4, *P < 0.05 compared with vehicle control; #P < 0.05 compared with LPS.

siRNA, but not by caspase-4 siRNA (Fig. 4B). Consistently LPS exposure increased the expression of pro-caspase-5 (43kD) as early as 2 h (Fig. 4C), however, the cleaved form of caspase-5 (p20) was not detected (data not shown). In brief, these data suggest that caspase-5 is responsible for the degradation of GCLC protein in the inflammatory response.

3.4. Gamma-GC restored GSH content and suppressed the activation of NF-κB signaling

Being the rate-limiting enzyme for GSH biosynthesis, the alteration of GCLC expression is directly related to the change of GSH content in the inflammatory response [30]. We hypothesize that γ-GC, the product of GCLC, can bypass the reaction catalyzed by GCLC and restore GSH content more efficiently. Pretreatment with γ-GC (2 mM) for 1 h before LPS exposure prevented the decrease in total GSH content (restored GSH content by 85%); N-acetyl cysteine (NAC, 2 mM), a precursor of cysteine, restored GSH content by 74% (Fig. 5A). Meanwhile, both γ-GC and NAC significantly decreased the basal NF-κB activity and inhibited LPS-caused activation of NF-κB reporter, although the inhibitory effect of NAC was more significant than that of γ-GC (Fig. 5B). Taken together, these data indicate that γ-GC is an efficient agent to restore GSH deficiency and suppress inflammation in the inflammatory response of macrophages.

4. Discussion

GCLC expression is down-regulated in the inflammatory response of macrophages and it contributes to GSH depletion and oxidative stress. This study explored the underlying mechanism for this phenomenon and demonstrated that GCLC down regulation in the inflammatory response of macrophages to LPS is due to both increased decay of mRNA and increased degradation of GCLC protein by caspase-5. We also found that supplementation of γ-GC, the GCL product, efficiently restored GSH content and alleviated the inflammatory response caused by LPS.

Prior studies suggest that changing mRNA stability is an important mechanism in regulating GCLC expression. Liu et al. first reported that GCLC mRNA stability was increased in response to oxidative stress [37], and later Song et al. further showed that oxidative stress stabilized GCLC mRNA through increasing the binding of HuR to GCLC mRNA [38]. The current study demonstrated that GCLC mRNA stability was decreased due to the increased mRNA decay in the inflammatory response of macrophages (Fig. 1). It is likely that certain products encoded by NF-κB target genes mediate the decay of GCLC mRNA, as either inhibiting transcription (Fig. 1B) or inhibiting NF-κB signaling prevented the decrease in GCLC mRNA [30,34].

Another significant finding reported here is that the decline in GCLC protein in the inflammatory response of macrophages is due to increased degradation by caspase-5 (Fig. 5). This confirms the report by Franklin et al. that GCLC protein was degraded by caspase cleavage [35]. In contrast to their finding that GCLC protein was cleaved by caspase-3, however, our data suggests that it was by caspase-5, as inhibiting/silencing caspase-5 but not others (caspase-1, and -4) prevented LPS-caused decrease in GCLC protein (Fig. 4). The difference in specific caspase involved could be due to the different model systems: Franklin et al. examined the GCLC degradation in apoptosis in which caspase-3 was activated [35], while the current study used a model of inflammatory response (THP1 macrophages response to LPS), in which no apoptosis and caspase-3 activation was observed [39,40]. Caspase-5 is induced by LPS [41] and plays critical roles in regulating the inflammatory response [36,42] including inflammasome activation [43]. Our data suggests that caspase-5 may also be involved in altering redox homeostasis in macrophages in during the inflammatory response. It is noteworthy that although we observed an induction of precursor form of caspase-5 (pro-caspase-5, 43 kD) as early as 2 h upon LPS stimulation (Fig. 4C), the cleaved form of caspase-5 (20kD), which is considered a marker of caspase-5 activation, was not detected. In addition, silencing caspase-5 by siRNA caused a decrease in the basal level of GCLC protein by 25%. The basal GCLC expression is regulated by basal level of electrophiles including H_2O_2 and we speculate that silencing caspase-5 would decrease the basal inflammasome activity and thus further H_2O_2 production. It is also remains to be determined how caspase-5 becomes inactivated in the later phase of the inflammatory response when GCLC protein is increased. Nonetheless, in our current model system, the data obtained using both a specific inhibitor and siRNA support the conclusion that GCLC degradation is by caspase-5 in the inflammatory response of macrophages.

Although both increased mRNA decay and protein degradation contributed to the down regulation of GCLC in the inflammatory response of macrophages, the latter contributed a larger portion. GCLC protein has a longer half-life (12 h) relative to its immediate decrease in the inflammatory response (Fig. 2B), and the effect of mRNA alteration

on protein level is not immediate (Fig. 2A), suggesting that LPS-caused decrease in GCLC protein is attributable more to the protein degradation than to the mRNA decay. The dual mechanisms that target both mRNA and protein of GCLC predominate in down regulating GSH content in the early phase of inflammatory response.

Several approaches have been used to restore GSH levels under pathophysiological conditions implicated with GSH deficiency, these including supplementation of N-acetyl cysteine (NAC) and GSH ester. Supplementation of γ-GC, the product of GCL, was more efficient than NAC in restoring GSH content in the inflammatory response (Fig. 5A). This is reasonable considering that GCL is the rate-limiting enzyme for GSH biosynthesis and the decrease in GCLC expression would lead to a declined production of γ-GC even with increase availability of cysteine derived from NAC, while supplementation of γ-GC can bypass the need for the reaction catalyzed by GCL. Consistent with reports that GSH supplementation could alleviate the inflammatory response [20–25], supplementation of γ-GC and NAC also suppressed the activation of NF-κB signaling caused by LPS (Fig. 5B). In term of inhibiting NF-κB activation, NAC was more potent than γ-GC. Nonetheless the results that γ-GC restored GSH content and suppressed NF-κB activity further confirm the notion that altering GSH and GCLC is an important mechanism in regulating the inflammatory response.

In summary, GCLC expression was down regulated through an enhanced mRNA decay and increased protein degradation by caspase-5, and the latter pathway played a larger role. In addition, we showed that γ-GC supplement was able to prevent GSH deficiency and alleviate the NF-κB activation in the inflammatory response of macrophages. These findings suggest that regulation of GSH and GCLC might be a well-coordinated component of the inflammatory response of macrophages and provide new clues and targets to manage inflammation. However, several puzzles remain to be answered: e.g., what product coded by NF-κB target gene mediates the decay of GCLC mRNA? Also, how are the signals that down regulate GCLC decline and permit the gradual increase in GCLC expression in the later phase of the inflammatory response controlled? Elucidation of these questions will further our understanding of the regulatory mechanism of GSH and GCLC and the regulation of the inflammatory response of macrophages.

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