Glutaredoxin-1 modulates the NF-κB signaling pathway to activate inducible nitric oxide synthase in experimental necrotizing enterocolitis

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Article

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Abstract

Glutaredoxin-1 (Grx1) is a cytosolic thioltransferase that catalyzes reduction of GSH-protein adducts and plays an important role in pathophysiological of Necrotizing enterocolitis (NEC). The Nuclear factor kappa B (NF-κB) pathway is inhibited by S-glutathionylation of inhibitory kappa B kinase beta (IKKβ), which can be restored by Grx1. Inducible nitric oxide synthase (iNOS) regulated by NF-κB is crucial in the progression of NEC. We aim to explore the role of Grx1 in experimental NEC. Wild-type (WT) and Grx1-knockout (Grx1−/−) mice were treated with a NEC-inducing regimen. The production of iNOS, NO, and inflammation injuries were assessed. NF-κB and involved signaling pathways were also explored. The severity of NEC was attenuated in Grx1−/− mice. Grx1 ablation promoted IKKβ glutathionylation, NF-κB inactivation, and decreased iNOS and NO production in NEC mice. Grx1 ablation protected NEC through iNOS and NO inhibition, which may be related to S-glutathionylation of IKKβ to inhibit NF-κB signaling. Grx1-related signaling pathways maybe provide a new therapeutic target in NEC.

Introduction

Necrotizing enterocolitis (NEC) is a common gastrointestinal emergency characterized by an increased inflammatory response and necrosis in premature, which is associated with high morbidity and mortality. [1–3]. Several risk factors, containing prematurity, formula feeding, hypoxia, intestinal ischemia, and bacterial infection have been linked to the etiology of NEC [4]. Current research suggests intestinal injuries, for instance, microvascular disruption and hypoxiation, are the initiating event that leads to bacterial translocation over the epithelial tissue function as a barrier and increases innate immune system reactions [5–7]. Certain luminal bacteria and their constituents promote the development of inflammatory factors, containing pro-inflammatory cytokines, nitric oxide (NO), and peroxynitrite (ONOO−) [8–11]. Although this scenario is widely accepted, the exact molecular mechanism involved in the pathogenesis of NEC remains undefined.

NO, caused by inducible nitric oxide synthase (iNOS), is important in intestinal barrier degradation because it induces enterocyte death and inhibits epithelial restoration processes. Peroxynitrite, a powerful oxidant produced by the interaction of NO with superoxide (O2•−), is thought to mediate cytotoxic effects [12]. The factors that regulate iNOS overexpression in the gut remain not well known, hampering efforts to create NO/iNOS-targeted therapeutics.

Nuclear factor kappa B (NF-κB) is a transcription factor that influences pro-survival, pro-inflammatory, and immunological regulatory sequences. NF-κB dysregulation has been associated with several persistent inflammatory disorders such as tumor, asthma, and sepsis. Inhibitory kappa B kinase beta (IKKβ) activation promotes phosphorylation and eventual degradation of inhibitory kappa B alpha (IκBα), nuclear translocation of NF-κB RelA/p50 dimers, and transcriptional stimulation of more than 100 target genes, including the iNOS [13–15]. Studies indicate that S-glutathionylation of IKKβ suppresses its kinase activity and prevents the degradation of IκBα and the DNA binding of RelA/p50 dimers; therefore, it is a critical mechanism for controlling NF-κB activity.
Glutaredoxin-1 (Grx1) is a cytosolic enzyme that catalyzes protein deglutathionylation [16]. Grx1 modulates inflammatory mediator synthesis by controlling S-glutathionylation-sensitive signaling pathways for example NF-κB [17]. Overexpression of Grx1 decreases IKKβ S-glutathionylation enhances NF-κB activation, and promotes inflammation mediator synthesis [18]. Grx1 knockdown decreases the generation of inflammatory mediators via controlling the S-glutathionylation-NF-κB signalling pathway [17]. These effects indicated that Grx1 is significant in controlling the activation of iNOS via the S-glutathionylation–NF-κB signaling pathway in NEC pathogenesis.

Considering the established involvement of NF-κB in controlling inflammation and the relationship between Grx1 and S-glutathionylation, we aimed to investigate the role of Grx1 in the control of NF-κB activation in the intestinal of NEC pups. Our study demonstrates that Grx1 has a crucial function in controlling NF-κB stimulation and producing pro-inflammatory mediators, which associate with the iNOS/NO signaling pathway.

**Materials And Methods**

**Animals**

For each experimental protocol in the investigation, we obtained approval from Chongqing Medical University's institutional animal care and usage committee. Prof. Jingyu Li generously provided Grx1−/− mice (C57BL/6J genetic background) (Sichuan University, Chengdu, China). The Research Animal Center of Chongqing Medical University provided wild-type (WT) mice (C57BL/6J) (Chongqing, China). While the controls were allowed to breastfeed with their mothers, the investigational offsprings (various gender) going through NEC induction were isolated from their mothers on neonatal day 5 (P5). The isolated pups (body weight: 3–4 g) were kept in 37 °C incubators on a 12:12-hour dark/light cycle and were offered bedding. NEC was generated in Grx1-knockout (KO) mice or in neonatal C57BL/6 mice through administration by the mouth of lipopolysaccharide (LPS) (4 mg/kg), hypoxia, and gavage feeding of hyperosmolar equation, from P5 to P9, as previously mentioned [19]. Controls were littermates that were breastfed. On P9, we sacrificed the pups and harvested the examination of the terminal ileum.

**Grx1 activity assay**

We measured Grx1 activity employing a glutaredoxin fluorescent kit (11536, Cayman) according to the guidelines of the supplier.

**GSH and GSSG determination**

Oxidized glutathione (GSH), known as GSSG, and reduced GSH levels were spectrophotometrically estimated by means of a GSSG and a kit for measuring GSH (S0053, Beyotime, China).

**Morphological and histological assessment**
At 4-μm intervals, we sectioned formalin-fixed terminal ileal specimens, stained them with hematoxylin and eosin, and inspected them under a microscope. We scored intestinal damage severity, applying a standard histological scoring system via two researchers blinded to the remedy cohorts [20].

**Western blot (WB) analysis**

We homogenized and centrifuged snap-frozen colon tissues and collected the centrifugation. The protein content was calculated by utilizing the bicinchoninic acid approach. SDS-PAGE was used to extract equal quantities of proteins (40 mg) by placing them into polyvinylidene difluoride films (IPFL00010; Millipore, Burlington, Massachusetts, USA) and treating them with PAs. We blocked the films, applying QuickBlock™ Western (Beyotime, Shanghai, China), and incubated them at 4°C overnight with the PAs β-actin (20536-1-AP; Proteintech), cleaved caspase 3 (Santa Cruz Biotechnology), IkBα (Cell Signaling Technology, Danvers, MA), Grx1 (ab45953, Abcam), IKKβ (ab124957, Abcam), RelA (ab32536, Abcam), p-RelA (ab76302, Abcam), and iNOS (ab178945, Abcam). We further incubated the films, using the suitable horseradish peroxidase-conjugated secondary antibodies for 1 h at ambient temperature to indicate the immunoreactivity bands. The optical density was evaluated by means of ImageJ software, and the band-relative intensities were scanned and stored by means of Kodak Scientific Imaging System (Kodak, Rockville, Maryland, USA) (National Institutes of Health, Bethesda, MD).

**Detection of protein S-glutathionylation**

An immunoprecipitation assay was carried out, as mentioned earlier [21]. To summarize, the protein was extracted using an SDS lysis buffer comprising 20 mM N-ethyl maleimide (NEM) (Sigma). We incubated 250 mg of protein with 1 mg/mL anti-PSSG antibody (Virogen, Watertown, Massachusetts) and recombinant protein G agarose beads and exposed it to Western blot (WB) analysis. Before immunoprecipitation, we incubated the selected reagent control specimens, administering 50 mM DTT for 30 mins, as previously stated [22].

**Nitric oxide and superoxide anion assessment**

We conducted O$_2$•– & NO analysis, as mentioned earlier, by means of the total NO assay kit (Beyotime Institute of Biotechnology, Shanghai) [23]. Briefly, we homogenized a weighted average of intestinal sections in sterile phosphate-buffered saline (PBS) (1 ml) and supernatants were obtained by centrifugation. For the O$_2$•– assay, we incubated a luminescent O$_2$•– test reagent, lucigenin (Sigma, St. Louis, MO) with the separated supernatants. We detected luminescence via a luminometer (Turner Biosystems, Sunnyvale, California), and it is stated as relative light units (RLU)/mg protein. Moreover, NEC pups were administered NO synthase suppressor, L-NAME before gavage feeding [24].

**Detection of malondialdehyde**
We collected, homogenized, and centrifuged freshly dissected intestinal tissues, following previously described instructions via the corresponding commercially available detection kits. We subjected the supernatants to subsequent determinations for the malondialdehyde (MDA) [23].

**Intestinal permeability**

According to the guideline for intestinal permeability [23], we administered fluorescein isothiocyanate (FITC)-conjugated dextran (70 kDa, Sigma, St. Louis, MO) (40 mg per 100 g body weight) intragastrically for 04 hr. Then, we sacrificed the pups and obtained the blood for serum fluorescence determination.

**Intestinal epithelial cell and peritoneal macrophage isolation and culture**

The isolation and culture of primary intestinal epithelial cells (IECs) from resected mouse small intestines have been previously documented [25]. Specifically, we washed the resected small intestine twice in sterile medium and cultivated it in ethylenediaminetetraacetic acid solution (1.25% trypsin, 0.5 mmol/L) at normal temperature and incubated intestinal crypts with media that contained 200 U/mL collagenase type IV. We washed the dissociated epithelial cells and incubated them with 5% CO₂ at 32°C. These essential enterocyte cultures were affirmed by immunological strategy, as described previously [26]. Peritoneal macrophages were gathered from mice through peritoneal lavage with 10 mL of super-cold PBS per animal. Cells were then turned down, resuspended in Dulbecco’s base fundamental medium (DMEM)/F-12 medium (Life Advances, Carlsbad, California), and adhered by plating on glass coverslips [23]. We treated the isolated IECs and peritoneal macrophages with NF-κB activation inhibitor SN-50 (25 mM, Calbiochem, San Diego, USA) for 30 min and then treated them further with saline or LPS (1 mg/ml) [27].

**Dihydroethidium staining for superoxide detection**

Staining was performed under the superoxide detection regime, as described in the foregoing section [23]. Specifically, we incubated IECs with the O₂•−-sensitive dihydroethidium (DHE) dye (red color) (5 μM; Thermo Fisher Scientific, Waltham, MA) at 37 °C for 30 min. We used 4′,6-diamidino-2-phenylindole (blue) to stain the nuclei (DAPI; Sigma). We visualized and recorded reactive oxygen species (ROS) production (O₂•−; 535 nm) with a confocal microscope (Leica TCS SP5). We used ImageJ software to do quantitative analysis on the confocal images.

**Migration and proliferation of Intestine epithelial tissue**

To evaluate IEC proliferation and migration, the subjects underwent intraperitoneal injection with bromodeoxyuridine. After 1080 minutes, we sacrificed the subjects and performed immunofluorescence staining on the terminal ileum following the prescribed quantification standard [28].

**Assay for enzyme-linked immunosorbent**
The enzyme-linked immunosorbent assay (ELISA) was used; Duoset ELISA kits were used to detect GM-CSF and CCL-20 in homogenized intestinal centrifugation as directed by the supplier (R&D Systems, Minneapolis, MN).

**RT-PCR**

We extracted total RNA from the segments of mouse intestines with an RNA assay kit in accordance with the vendor guidelines (AG, China). RNA quantity was evaluated, Using a reverse transcription reagent kit, the RNA was converted to cDNA (AG, China), and SYBR Premix Ex TaqTM (AG, China) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) was employed for amplification. We calculated the relative expression of mRNA and normalization by means of the delta-delta ($\Delta \Delta Ct$) approach, following β-actin gene expression. Table 1 shows the arrangements of the selected primers (AG, China).

**Table 1**

<table>
<thead>
<tr>
<th>Primer sequences</th>
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<tr>
<td><strong>Gene</strong></td>
<td></td>
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<tr>
<td>β-actin</td>
<td>CATCCGTAAAGACCTCTATGCCAAC</td>
</tr>
<tr>
<td>iNOS</td>
<td>TGACCACATGGACCACACCAC</td>
</tr>
<tr>
<td>TLR4</td>
<td>TTTATTCAGAGCCGGTGTG</td>
</tr>
<tr>
<td>TNF-α</td>
<td>CATCTTCTCAAATTCGAGTGACAA</td>
</tr>
<tr>
<td>IL-6</td>
<td>GGCTAAGGACCAAGACCATCCAA</td>
</tr>
</tbody>
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**Statistical assessment**

GraphPad Prism software, version 4, was used for data processing. The outcomes are expressed as mean ± SEM, considering the normal distribution of data. For comparison, a one-way ANOVA with post-hoc Tukey analysis was employed. The log-rank test was used to evaluate survival curves. $P<0.05$ was regarded as statistically significant.

**Results**

**Grx1 activity and oxidative stress induction in the pathogenesis of NEC**

Glutathione (GSH) is the important antioxidant and redox controller in fighting cellular oxidation, and conjugated glutathione can be released by Grx1 from target proteins that are involved in the pathogenic process of NEC. To find out whether Grx1 activity was responsible for the development of NEC, we exposed mice to NEC stress and evaluated the Grx1 content. Grx1 activity was shown to be significantly
lower in NEC intestinal tissue when compared to non-NEC tissue (Fig. 1A). Similarly, reduced Grx1 protein concentration was detected by WB assay in intestine tissues (Fig. 1B). Then, to assess oxidative stress in animals with NEC, we measured GSH and GSSG concentrations. The findings indicate a considerable decrease in GSH levels and a rise in GSSG in experimental NEC intestinal tissue (Fig. 1C, D). The existence of oxidative stress throughout the development of NEC was shown by an increase in the GSSG/GSH ratio in experimental NEC. Moreover, in Grx1−/− mice, the GSSG/GSH ratio increased more robustly compared with the WT mice when subjected to NEC stress (Fig. 1E).

Increased MDA content, a symbol for lipid peroxidation, was detected in the NEC pups' intestinal tissue, and it was further increased by Grx1 ablation (Fig. 1F). NEC stress decreased the production of the antioxidant enzyme superoxide dismutase (SOD), which was further decreased by Grx1 ablation (Fig. 1G). These results indicate that the increased oxidative stress in the development of NEC and Grx1 is implicated in the pathological mechanism of NEC.

**Grx1 ablation impedes the development of NEC**

To examine whether Grx1 increases the progression of NEC, we produced NEC in Grx1-KO and WT mice and compared them to the respective controls. All groups' body weights were measured; although mice in both NEC groups lost a lot of weight, the KO+NEC group lost body weight more slowly than the WT+NEC group (Fig. 2A). The mortality rates were also investigated. Survival curves showed that NEC treatment decreased postpartum survival rates significantly; however, Grx1 ablation slowed this progression (Fig. 2B).

In terms of histological alterations, WT+NEC animals had more severe loss of epithelial cells, necrosis of the whole villus, and transmural necrosis than KO+NEC mice (Fig. 2D). After NEC stress, the NEC score in KO mice was considerably lower than in WT mice (Fig. 2C). As a result, necrosis in the intestinal epithelium, as evidenced by Sytox Green staining, was enhanced in NEC and considerably decreased by Grx1 ablation (Fig. 2E). These results confirmed that Grx1 is critical in the development of NEC.

**Grx1 ablation increases S-glutathionylation of IKKβ, which inhibits NF-κB activation**

Considering the function of Grx1 in controlling NF-κB and pro-inflammatory cytokine secretion, as well as in protein deglutathionylation catalysis, we investigated whether S-glutathionylation of IKKβ (IKKβ-SSG) was reliant on Grx1. To investigate GSH-protein adducts in NEC mice, anti-GSH antibody was used for WB assay on intestinal GSH-proteins under nonreducing conditions. Under rest conditions, the amount of intestinal GSH protein adducts increased slightly in Grx1-KO mice compared with WT mice. NEC treatment increased intestinal GSH levels in both types of mice; however, the increase was significantly higher in Grx1-KO mice (Fig. 3A). A small increase in IKKβ-SSG was detected following NEC treatment; however, NEC-activated S-glutathionylation of IKKβ was significantly increased by Grx1 ablation (Fig. 3B). Increases in S-glutathionylation of IKKβ were associated with an increase in IkBa and decrease in phosphor-RelA, which is indicative of protracted IKK inactivation (Fig. 3B).
Assuming that S-glutathionylation of IKK limits its kinase activity, we then explore potential function of Grx1 in controlling NF-κB activation and downstream cytokines. Intestinal RelA content was noticeably increased following NEC treatment, whereas RelA was decreased following Grx1 ablation (Fig. 3C). In experimental NEC mice, the concentration of CCL-20 and GM-CSF, two cytokines regulated by NF-κB, also decreased following Grx1 ablation (Fig. 3D and E). These outcomes reveal that Grx1 ablation leads to impaired activity of NF-κB and consequent decreases in downstream mediators following NEC treatment. Endogenous Grx1 is important for the NEC-induced NF-κB activation.

**Grx1 ablation decreases the production of iNOS and NO**

Given that Grx1 regulates NF-κB binding to the iNOS promoter and iNOS gene transcriptional activation, we then explore the impact of Grx1 ablation on iNOS expression. Endogenous iNOS protein levels were low or undetectable in Grx1^{-/-} and WT mice. NEC stress induced a marked increase in iNOS, and Grx1 ablation significantly decreased the induction of iNOS (Fig. 3C).

iNOS is accountable for the high levels of NO that exert detrimental effects during inflammation. We determined the production of NO in NEC mice. An increase in NO and \( O_2^{•−} \) production after NEC were detected in the isolated intestine (Fig. 4A and B). NO production was found to be lower in Grx1^{-/-} mice at baseline. For NEC treatment, Grx1 ablation significantly decreased NO production (Fig. 4A). Furthermore, Grx1^{-/-} pups produced considerably less \( O_2^{•−} \) than the WT controls after NEC stress (Fig. 4B). The iNOS inhibitor NG-monomethyl-L-arginine, monoacetate salt (L-NMMA) suppressed NO signaling in NEC, implying that the source of NO is reliant on iNOS (Fig. 4A). Therefore, Grx1 deficiency was associated with the expression of iNOS, with a significant reduction in NO generation in NEC mice.

We then examined \( O_2^{•−} \) generation in single-cell suspensions of IECs using confocal microscopy to identify the fluorescence generated by oxidized dihydroethidium (DHE). Exposure of IECs to LPS (100 nmol/L, 1 hr) improved the DHE-derived fluorescence signals significantly, which was attenuated by Grx1 ablation (Fig. 4E). A reduction in DHE fluorescence by DTT in cells supported the switching from NO to \( O_2^{•−} \) (Fig. 4E).

The NF-κB activation inhibitor SN-50 was used to assess whether expression of iNOS and production of NO were mediated by NF-κB. Treatment of IECs with SN-50 prevented LPS-induced NO synthesis and iNOS activation (Fig. 4C, D), suggesting that NF-κB mediates the regulation of iNOS in IECs.

**Grx1 ablation reduces pro-inflammatory cytokines associated with iNOS signaling**

We next investigated whether Grx1-mediated iNOS could modulate pro-inflammatory cytokine activation in the pathophysiology of NEC, as iNOS-derived NO promote inflammation in the intestine. We first performed an RT-PCR to evaluate the expression of iNOS, TLR4, TNF-α, and the pro-inflammatory cytokine IL-6 on intestinal tissue. NEC stress greatly improved the production of iNOS, TLR4, TNF-α, as well as IL-6 mRNA in intestinal tissue; however, L-NAME administration or Grx1 ablation reduced this expression (Fig.
Moreover, NO donor sodium nitroprusside (SNP) abrogated this anti-inflammation effect of Grx1 ablation (Fig. 5A).

We then isolated IECs and peritoneal macrophages from WT and Grx1-/- mice. LPS exposure increased the expression of iNOS, TNF-α, TLR4, and IL-6 in both IECs and peritoneal macrophages from WT mice; however, this was attenuated by Grx1 ablation (Fig. 5B, C).

Moreover, SNP significantly promoted TNF-α, TLR4, and IL-6 messaging in IECs and peritoneal macrophages from mice; L-NAME inhibited this messaging, indicating the pro-inflammatory effects of NO (Fig. 5B, C).

Together, these data suggest that Grx1 functions as a pro-inflammatory mediator involved in iNOS signalling.

Grx1 ablation ameliorates iNOS-mediated apoptosis and intestinal barrier damage

We next evaluated whether Grx1 mediate iNOS and so impact intestinal cell apoptosis in the development of NEC. Grx1 ablation reduced intestinal apoptosis in NEC pups, as shown by SDS-PAGE analysis of caspase 3 and cleaved caspase3 (Fig. 6A). The mucosal barrier was disrupted during NEC stress, as evidenced by SIgA elevation (Fig. 6B), β-defensin-2 suppression (Fig. 6C), as well as FITC-dextran exudation (Fig. 6D); Grx1 ablation attenuated this gut barrier injury. In addition, the changes in MPO activity, indicating neutrophil infiltration reduction (Fig. 6E). We also investigated whether Grx1 affects the magnitude of bacterial translocation. NEC stress increased bacterial translocation over the intestinal barrier into the mesenteric lymph nodes (Fig. 6F), liver (Fig. 6G), and spleen (Fig. 6H). This extent was ameliorated by Grx1-/- ablation or L-NAME administration; however, SNP abrogated this protective effect of Grx1-/- ablation. These data suggest that Grx1 ablation ameliorates intestinal apoptosis and intestinal barrier damage in NEC through iNOS/NO signaling.

Grx1 ablation promotes IEC migration and proliferation via NO signaling

We then investigated whether Grx1 influences IEC proliferation and migration in the intestinal, as both of these mechanisms are important in NEC pathogenesis. IECs were identified in intestinal tissue slices using BrdU immunostaining (Fig. 7A). In the mouse model, NEC treatment severely impaired IEC migration, and Grx1 ablation significantly improved this deficit (Fig. 7B, C). BrdU-positive cells were used to evaluate IEC proliferation; NEC stress significantly reduced IEC proliferation, which was significantly attenuated by Grx1 ablation (Fig. 7D). Moreover, inhibiting NO generation with L-NAME promoted IEC migration and proliferation in NEC mice; SNP administration abrogated the protect effect of Grx1 ablation on IEC (Fig. 7B, C, D). These findings suggest that Grx1 ablation contribute to IEC migration and proliferation, which is associated with iNOS/NO signaling in the development of NEC.

Discussion
To present, the possible role of Grx1 in intestinal damage during NEC has not been well investigated. We investigated the role of Grx1 in controlling NF-κB activation, iNOS expression, S-glutathionylation, NO generation, and pro-inflammatory cytokine production in NEC mice. In an experimental animal model of NEC, we found that Grx1 ablation successfully reduced the severity of NEC and increased survival rate. Mechanically, Grx1 deletion increased IKKβ S-glutathionylation while decreased NF-κB activation and transcription (Fig. 8). Because of decreased binding of NF-κB to the iNOS promoter, that significantly restricts iNOS expression [27]. In our study, iNOS inhibition reduced NO production, which was associated with reduced pro-inflammatory cytokine. Together, these results shown the vital function of Grx1 in NEC pathogenesis.

S-glutathionylation (PSSG) has arisen as a critical oxidative thiol modification that controls signalling molecules and transcription factors [29, 30]. Grx1, a ubiquitous cytosolic enzyme that catalyzes protein deglutathionylation. GSH adducts influence the function of several proteins [31–33]. Grx1 levels were shown to be lower in NEC mice in prior research, and Grx1 depletion alleviated the severity of experimental NEC [34, 23]. We provided additional evidence that NEC stress decreases Grx1 levels in intestinal tissues and that Grx1 ablation relieves the severity of intestinal morphology and increases survival in experimental NEC. Grx1 ablation significantly increased oxidative stress, represented by an increase in the GSSG/GSH ratio, due to the increased GSSG and decreased GSH in NEC stress combined with the increased MDA and decreased SOD. Our data indicate that Grx1 ablation protected NEC by boosting GSH adducts. As a result, lower levels of Grx1 in the intestines of WT/NEC mice may represent evidence of a pathophysiological response to detrimental consequences of NEC stress rather than merely a sign of oxidative stress.

NO is recognized to serve a paradoxical function in gut physiology. Endothelial NOS (eNOS) and inducible NOS (iNOS) are the major NO synthases that catalyze arginine to produce NO in the gut. eNOS is predominantly expressed in intestinal microcapillaries and is responsible for the low background NO that essential for the preservation of mucosal capillaries and mucosal homeostasis [35, 36]. The high levels of NO are mainly induced by iNOS in the intestinal. Enhanced iNOS expression has been seen in IECs from surgically removed NEC tissue samples [37]. In a NEC rat model, enhanced iNOS mRNA and protein expression, enterocyte apoptosis in the intestinal epithelium [38]. Increased NO concentrations during inflammation have a detrimental impact on the intestinal barrier via several pathways [39–42]. In high concentrations, NO reacts with superoxide to form its reactive nitrogen derivative, peroxynitrite, which is highly toxic to epithelial cells [12]. NO and peroxynitrite could influence intestinal barrier permeability by promoting apoptosis and necrosis in enterocytes [43]. NO and metabolites also may impair epithelial restoration, including enterocyte proliferation and migration [44].

In line with previous reports, we found that NEC stress increased iNOS and NO production, induced enterocyte apoptosis, attenuated enterocyte proliferation and migration, and increased gut barrier permeability and bacterial translocation. Additionally, Grx1 deletion decreased iNOS and NO production in NEC mice and attenuated NEC stress-induced adverse effects. Furthermore, a switch from NO to O_2^- generation in the intestines of pups with NEC was attenuated by Grx1 ablation, suggesting the
association of Grx1 with oxidative injury in the pathogenesis of NEC and verifying the important role of Grx1 in NO metabolism during oxidative stress through iNOS signaling. Therefore, iNOS-derived NO is essential for intestinal homeostasis, and excessive NO lead to NEC epithelial injury. In addition, Grx1 ablation alleviated NEC stress-induced inflammatory injury, improved IEC migration and proliferation, and improved intestinal barrier function, including bacterial invasion susceptibility and mucosal permeability. We observed that administration of the NO donor SNP abrogated the protective effect conferred by Grx1 ablation, indicating that Grx1 regulates intestinal function through iNOS/NO signaling in NEC.

The most extensively research has been done on the modulation of the iNOS gene in reaction to pathogen-associated molecular patterns in neutrophils and macrophages. This induction involves signaling via activation of the transcription factor NF-κB that binding to the iNOS promoter and transcriptional activation of the iNOS gene [45,14]. The activity of NF-κB transcription factors can be inhibited by S-glutathionylation. According to research, Grx1 inhibition increased GSSG content, which creates ideal circumstances for S-glutathionylation. The S-glutathionylation of IKKβ inhibits the NF-κB pathway, which can be reversed by Grx1 [32]. S-glutathionylation of IKKβ was increased in Grx1 knockout cells, and following Grx1 overexpression, S-glutathionylation of IKKβ was reduced, then NF-κB activation was increased [17]. Grx1-overexpressing transgenic mice enhance endothelial NF-κB activity and inflammation [46]; therefore, Grx1 deletion may inactivate NF-κB by PSSG of IKKβ [32,33].

In line with a previous study, we found that Grx1 ablation improved the PSSG content in the intestine of NEC mice. The increased PSSG promoted IKKβ-SSG, which inhibited the phosphorylation and degradation of IκBα and reduced the translocation of RelA/p50 dimers into the nucleus, preventing NF-κB activation from regulating iNOS production. A study indicated the iNOS induction was suppressed in aortic endothelium and hearts of Grx1-KO mice [47]. Furthermore, we found that iNOS expression is an NF-κB-dependent process; the lower iNOS and NO production that we observed may be accredited to the inactivation of the NF-κB pathway by S-glutathionylation of IKKβ.

Earlier research has shown that cysteine 189 of IκBα can be S-glutathionylated, which reduces phosphorylation by IKK and attenuates ubiquitination in vitro [48], involve in its degradation and consequent activation of NF-κB [49]. Future research should focus on determining the relationships between Grx1 and IκBα are connected to S-glutathionylation of IκBα, and whether these processes affect IKK signalosome activation and/or assembly in NEC pathogenesis. Grx1 is found ubiquitously; on the other hand, it remains unclear which types of occupant cells are important in the Grx1 KO. More research is needed to determine the conditions in which Grx1 ablation is applied across distinct target cell types in the NEC pathogenesis.

In conclusion, Grx1 ablation inhibited iNOS/NO production to protect NEC through GSH adducts on NF-κB signaling. As a result, down-regulation of Grx1 might be a potential treatment target to alleviate NEC stress-induced intestinal injury.

Declarations
Acknowledgements

All experiments were approved by the animal care and use committee of Chongqing Medical University. All methods performed in the study were carried out in accordance with the approved guidelines and regulations. The study is reported in accordance with the ARRIVE guidelines.

Data availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Author Disclosure Statement

No potential conflicts of interest relevant to this article are reported.

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References


Figures
Figure 1

**Evaluation of oxidative stress and Grx1 activity in experimental NEC.**

Grx1 activity was measured in intestinal homogenates (B) The WB approach was used to analyze Grx1 proteins in intestinal of control and NEC mice; Right panel: Densitometry analysis of Grx1 normalized by β-actin. (C, D, E) Levels of GSSG, GSH, and the GSH/GSSG ratio in the intestinal homogenates.

glutaredoxin-1, Grx1; glutathione, GSH; oxidized GSH, GSSG; necrotizing enterocolitis, NEC; Western blot, WB. The data represent three separate experiments. Two-sided one-way ANOVA was utilized for data comparison with post-hoc Tukey test (n = 6–8 mice per group, data are presented as means ± SEM).

**P<0.01, ***P<0.001, ****P<0.0001, vs. wild-type (WT)/dam-fed (DF) mice; #P<0.05, ##P<0.01, ###P<0.001, ####P<0.0001 vs. WT/NEC.
Figure 2

**Grx1 deficiency decreases the severity of experimental NEC.**

(A) Weight changes among different groups. (B) The Kaplan-Meier method was used to compare survival rates in different experimental groups. (C) Severity scores were computed based on morphological changes. (D) Terminal ileum specimens were stained with hematoxylin and eosin. Scale bars: 100 μm. (E) Sytox Green, a necrosis marker, was used to detect cell death in the ileal epithelium based on its binding property to cellular nucleic acids present only in dead cells. Comparison was made between groups. Scale bars: 100 μm. F. Groups were compared in terms of necrotic cells, a two-sided one-way ANOVA with a Tukey post-hoc test (n = 10–15 mice per group, data are presented as means ± SEM). *P<0.05, **P<0.001, ****P<0.0001, vs. WT/DF mice; #P<0.05, ##P<0.01, ####P<0.0001 vs. WT/NEC.
**Figure 3**

**Assessment of NF-κB activation.**

WB analysis of S-glutathionylation of intestinal tissue proteins in WT and Grx1-/- mice following with dam feed or NEC stress. (B) S-glutathionylated protein immunoprecipitation utilizing an anti-GSH antibody to analysis of S-glutathionylation of IKKβ in intestinal tissue lysates of WT and Grx1-/- mice following with dam feed or NEC stress; Bottom panels: WB analysis of whole-cell lysates (WCLs) for total IKKβ, IκBα, as well as phosphorylated RelA. (C) The WB assay was used to analyze RelA and iNOS proteins in intestinal of WT and Grx1-/- mice following with dam feed or NEC stress; Down panel: Densitometry analysis of RelA and iNOS normalized by β-actin. (D) Evaluation of NF-κB luciferase activity. (E, F) Evaluation of CCL-20 and GM-CSF concentrations in intestinal tissues using ELISA. For data comparison, we used post-hoc Tukey test two-sided one-way ANOVA (n = 6–8 mice per group. Date are presented as means ± SEM). ****P<0.0001, vs. WT/DF mice; ##P<0.01, ###P<0.001 vs. WT/NEC.
Figure 4

(A) Evaluation of nitric oxide and superoxide generation in intestinal homogenates.

(A) (A, B) Comparison between the groups with and without the NOS inhibitor L-NMMA in terms of NO and O$_2^{•–}$ production ***P<0.001, ****P<0.0001, vs. WT/DF mice; ###P<0.001, ####P<0.0001 vs. WT/NEC. (C) NO production was assessed in the IECs with and without NF-κB activation inhibitor SN-50. (D) WB was performed for iNOS expression assessment on IECs treated with saline, LPS or LPS+SN50; Right panel: Densitometry analysis of Grx1 normalized by β-actin. ****P<0.0001, vs. saline; ##P<0.01 vs. LPS. (E) Representative fluorescence micrographs with DHE labelling for intracellular ROS detection. Two-sided one-way ANOVA was utilized for data comparison with post-hoc Tukey test (n = 6–8 mice per group, data are given as means SEM).
Figure 5

(A) iNOS, TLR4, TNF-α, and IL-6 mRNA were detected in the intestinal tissue by RT-PCR. IECs (B) as well as peritoneal macrophages (C) were detached from mice and treated as directed. The assessment was made of the mRNA expression level of iNOS, TLR4, TNF-α, and IL-6, which are expressed in relation to β-actin. Two-sided one-way ANOVA was utilized for data comparison with post-hoc Tukey test (n = 6–8 mice per group, data are presented as means ± SEM). ***P<0.001, ****P<0.0001, vs. WT/DF mice; ##P<0.01, ###P<0.001 vs. WT/NEC; ^^P<0.01, ^^^P<0.001, ^^^^^P<0.0001 vs. glutaredoxin 1 (Grx1)^{−/−}/NEC.

Grx1 influences the expression of pro-inflammatory cytokines in NEC mice.
Figure 6

Effects of Grx1 depletion on apoptosis and intestinal barrier injury in NEC.

A. Immunoblotting analysis of caspase 3 and cleaved caspase3 proteins in intestinal of WT and Grx1-/mice following with dam feed or NEC stress. As a loading control, β-actin was employed. Right panels: Densitometry analysis of left panels image. B. The convergence of SIgA was estimated from the terminal ileum of pups treated as indicated. C. The centralization of β-defensin 2 was estimated in the distal ileum of mice. D. Serum FITC-dextran concentrations in groups was detected. E. MPO activity was assessed in ileum from indicated groups. Bacterial growth from mesenteric lymph nodes were quantified (F), liver (G), and spleen (H) of mice under the designated circumstances. The data represented three independent experiments. For data comparison, two-sided one-way ANOVA was utilized with post-hoc Tukey test (n = 6–8 mice per group was adopted, and data are presented as means ± SEM). ***P<0.001, ****P<0.0001, vs. WT/DF mice; ###P<0.001, ##P<0.01, vs. WT/NEC; ^^P<0.01, ^^^P<0.001, ^^^^^P<0.0001 vs. Grx1^-/-/NEC.
Figure 7

Grx1 regulates intestinal epithelial cell migration.

(A) Representative pictures of BrdU immunostaining of the digestive tract. Bar scale: 100 µm. (B) IEC movement rate (characterized as FLE/18). (C) IEC movement (characterized as FLE/complete thickness of the mucosa × 100%). (D) IEC multiplication (characterized as BrdU-positive cells/high-power field). n = 6 animals per group, 6 fields/animal. For data comparison, two-sided one-way ANOVA was utilized with post-hoc Tukey test (n = 6–8 mice per group, and data are presented as means ± SEM). **P<0.01, ***P<0.001, ****P<0.0001, vs. WT/DF mice; #P<0.05, ##P<0.01, ###P<0.001 vs. WT/NEC; ^^P<0.01 vs. Grx1−/−/NEC.
Figure 8

Glutaredoxin-1 regulates NF-κB activation.