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## Heme oxygenase-1 induction contributes to renoprotection by G-CSF during rhabdomyolysis-associated acute kidney injury

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### Abstract

Granulocyte colony-stimulating factor (G-CSF) is renoprotective during acute kidney injury (AKI) induced by ischemia and cisplatin nephrotoxicity; however, the underlying mechanism is not entirely clear. Rhabdomyolysis is another important clinical cause of AKI, due to the release of nephrotoxins (e.g., heme) from disrupted muscles. The current study has determined the effects of G-CSF on rhabdomyolysis-associated AKI using *in vivo* and *in vitro* models. In C57BL/6 mice, intramuscular injection of glycerol induced AKI, which was partially prevented by G-CSF pretreatment. Consistently, glycerol-induced renal tissue damage was ameliorated by G-CSF. In addition, animal survival following the glycerol injection was improved from 30 to 70% by G-CSF. In cultured renal tubular cells, hemin-induced apoptosis was also suppressed by G-CSF. Interestingly, G-CSF induced heme oxygenase-1 (HO-1, a critical enzyme for heme/hemin degradation and detoxification) in both cultured tubular cells and mouse kidneys. Blockade of HO-1 with protoporphyrin IX zinc(II) (ZnPP) could largely diminish the protective effects of G-CSF. Together, these results demonstrated the renoprotective effects of G-CSF in rhabdomyolysis-associated AKI. Notably, G-CSF may directly protect against tubular cell injury under the disease condition by inducing HO-1.

**Keywords:** renal tubular cell injury, apoptosis, glycerol-induced rhabdomyolysis

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RHABDOMYOLYSIS IS A CLINICAL condition initiated by acute disruption of skeletal muscle due to physical or chemical damage from crush injury, exhausting exercise, surgery, or toxins (10). A notable complication associated with rhabdomyolysis is acute kidney injury (AKI), resulting in rapid deterioration of renal function or acute renal failure (35, 41). Rhabdomyolysis-associated AKI involves the nephrotoxicity of

heme, which is released from myoglobin in a pathogenic quantity. Experimentally, rhabdomyolysis-associated AKI is modeled in vivo in animals by glycerol disruption of skeletal muscle and in vitro by hemin/heme treatment of renal tubular cells (7, 19, 20). These studies have not only characterized rhabdomyolysis-associated tissue damage and renal cell injury but have also led to the discovery of heme oxygenase-1 as a critical renoprotective molecule.

Heme oxygenases are the rate-limiting enzymes that catalyze the degradation of heme, releasing biliverdin, iron, and carbon monoxide (4, 13, 14, 18, 29). Among the three isoforms, heme oxygenase-1 (HO-1) is inducible in response to cellular stress. Importantly, upon induction, HO-1 protects against tissue and cell injury. In kidneys, the protective effects of HO-1 have been demonstrated in a variety of renal injury models including ischemia-reperfusion, glycerol-induced rhabdomyolysis, cisplatin nephrotoxicity, and endotoxemia (19, 20, 26–28, 36).

Granulocyte colony-stimulating factor (G-CSF) is a pleiotropic cytokine which is well known for its ability to stimulate the mobilization, proliferation, and probably differentiation of bone marrow cells. In kidneys, ischemia-reperfusion induces G-CSF in serum and renal tissues (42). Interestingly, depending on the dosage and time of application, G-CSF has been shown to protect against AKI in experimental models of ischemia-reperfusion and nephrotoxins (5, 12, 16, 21, 31, 33). Despite these observations, the mechanism(s) underlying the renoprotective effects of G-CSF is largely unclear (22). The beneficial effects of G-CSF may be related to bone marrow cell mobilization into renal tissues. However, during AKI the mobilized bone marrow cells do not mainly function to differentiate into renal tubules (3, 11, 17); instead, they may produce tropic factors, contributing to renal tissue repair or regeneration (1, 32, 34). In addition, G-CSF may prevent tissue damage by directly acting on parenchymal cells. It has been reported recently that G-CSF can protect cardiomyocytes from oxidative stress injury and neurons against glutamate toxicity injury (8, 23). Whether G-CSF has directly cytoprotective effects in renal cells or tissues is unclear (16, 30).

In the current study, we have demonstrated evidence for the renoprotective effects of G-CSF in experimental models of rhabdomyolysis-associated AKI. G-CSF mobilizes and recruits bone marrow cells into renal tissues, but the cells do not seem to differentiate into renal tubules. Notably, G-CSF induces HO-1, a well-recognized renoprotective protein. Inhibition of HO-1 attenuates the renal-protective effects of G-CSF, suggesting an important role for HO-1 in tissue protection by G-CSF.

## MATERIALS AND METHODS

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**Mouse model of rhabdomyolysis-associated AKI.** C57BL/6 mice (male, 8–10 wk) were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained in the animal facility of the Charlie Norwood Veterans Affairs (VA) Medical Center (Augusta, GA). The animals were housed with a 12:12-h light-dark cycle and food and water available ad libitum. To induce rhabdomyolysis, the animals were injected with 7.5 ml/kg 50% glycerol intramuscularly to the two hindlegs or injected with saline as a control (20). To study the effects of G-CSF, 200 mg·kg<sup>-1</sup>·day<sup>-1</sup> recombinant human G-CSF (Amgen, Thousand Oaks, CA) or the same volume of saline was administered intraperitoneally to the animals for 5 consecutive days before a glycerol injection. To inhibit HO-1, 20 mg/kg protoporphyrin IX Zn(II) (ZnPP) was administered intraperitoneally to the mice 2 h before the glycerol injection, while saline was given to the no-ZnPP animals. All animal experiments were performed according to a protocol approved by the Institutional Animal Care and Use Committees of the Charlie Norwood VA Medical Center.

**Analysis of renal function and histology.** Renal function and histology were examined as previously (2,

[38–40](#)). Blood urea nitrogen (BUN) was monitored to indicate renal function. Briefly, blood samples were collected to obtain serum following clotting and centrifugation. BUN was measured with a kit from Biotron Diagnostics (Hemet, CA). To analyze renal histology, kidneys were harvested from the animals, fixed with 4% paraformaldehyde, and paraffin-embedded. Tissue sections of 4  $\mu\text{m}$  were subjected to hematoxylin-eosin (H&E) staining and examined by microscopy. Tubular damage was indicated by necrotic lysis, tubular dilation, cast formation, and sloughing of cellular debris into the tubular lumen. Tubular injury scores were determined by the percentage of renal tubule injury: 0, no damage; 1, <25%; 2, 25–50%; 3, 50–75%; and 4, >75%. Images of representative fields were also recorded.

**Terminal transferase-dUTP nick-end labeling assay.** Terminal transferase-dUTP nick-end labeling (TUNEL) assay was performed to detect apoptosis using the in situ Cell Death Detection kit from Roche Applied Sciences (Indianapolis, IN) as described previously ([38](#), [39](#)). Briefly, cells and kidney tissues were fixed in 4% paraformaldehyde and permeabilized in sodium citrate, pH 6.0. The samples were then incubated with TM red-labeled dUTP, and TUNEL-positive nuclei were identified by fluorescence microscopy.

**Immunohistochemical staining of HO-1 in renal tissues.** Renal tissues were fixed with 4% paraformaldehyde, paraffin-embedded, and cut into 4- $\mu\text{m}$  sections. The tissue sections were then deparaffinized and permeabilized by 2 h of incubation at 65°C in 0.1 M sodium citrate, pH 6.0. The tissues were subsequently incubated for 1 h with 0.1% phenalhydrazine and 0.03%  $\text{H}_2\text{O}_2$  at 37°C to block the endogenous peroxidase activity. After that, the tissues were incubated sequentially with a blocking buffer (2% BSA, 0.2% nonfat dry milk, 0.8% Triton X-100, and 2% normal donkey serum) and the anti-HO-1 antibody (1:200 dilution, Stressgen, Victoria, BC) in the blocking buffer. After incubation with biotin-labeled secondary antibody, the color was developed with an ABC kit from Vector Laboratories (Burlingame, CA). The staining was evaluated in a blinded manner.

**Hemin-induced tubular cell apoptosis.** Rat kidney proximal tubular epithelial cells (RPTC) were originally obtained from Dr. U. Hopfer at Case Western Reserve University (Cleveland, OH). The cells were maintained and plated for experiments as described previously ([24](#), [37](#)). For G-CSF treatment, indicated concentrations of G-CSF were added to the culture medium, and the medium was changed every day for up to 3 days. After G-CSF pretreatment, 50  $\mu\text{M}$  hemin (Fisher Scientific, Pittsburgh, PA) was added to the culture medium with 0.1% fetal bovine serum to induce apoptosis. To inhibit HO-1 activity, 2  $\mu\text{M}$  ZnPP (Sigma-Aldrich, St. Louis, MO) was added in the culture medium with 0.1% fetal bovine serum for 2 h before hemin treatment. Typical apoptotic morphology including cellular shrinkage and formation of apoptotic bodies was examined by phase-contrast microscopy.

**Caspase activity assay.** The activity of caspases was measured as described previously ([2](#), [38](#), [39](#)). Briefly, cells were extracted with 1% Triton X-100 and added to (25  $\mu\text{g}$  of protein/sample) an enzymatic reaction containing 50  $\mu\text{M}$  carbobenzoxy-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (DEVD.AFC), a fluorogenic substrate for caspase-3, -6, and -7. Fluorescence at 360-nm excitation/530-nm emission was monitored. For each measurement, a standard curve was constructed using free AFC. Based on the standard curve, the fluorescence reading from each enzymatic reaction was translated into nanomolar free AFC  $\cdot \mu\text{g}$  protein<sup>-1</sup>  $\cdot$  h<sup>-1</sup> to indicate caspase activity.

**Immunoblot analysis.** Whole cell lysate and tissue lysate were collected in a buffer containing 2% SDS. Reducing gel electrophoresis and protein transferring/blotting were conducted by standard procedures. The blots were incubated with 5% fat-free milk for blocking and then exposed to specific primary antibodies overnight at 4°C. Finally, the blots were exposed to the horseradish peroxidase-conjugated secondary

antibody, and antigens on the blots were revealed using an enhanced chemiluminescence (ECL) kit (Pierce, Rockford, IL). Polyclonal anti-HO-1 from Stressgen, anti-G-CSF receptor from Santa Cruz Biotechnology (Santa Cruz, CA), and monoclonal anti- $\beta$ -actin (Sigma) antibodies were used at 1:2,000, 1:200, and 1:5,000 dilutions, respectively, for immunoblotting in this study.

**RT-PCR analysis of mRNA expression.** Total RNA were extracted with TRI reagent (Sigma-Aldrich). RT-PCR was conducted by standard methods using the Superscript First-strand synthesis system (Invitrogen, Carlsbad, CA). The primers for amplification of HO-1 were 5'-CAGTCTATGCCCCGCTCTAC-3' (forward) and 5'-ACCAGCAGCTCAGGATGAGT-3' (reverse). The primers for  $\beta$ -actin gene were 5'-GTGGGGCGCCCAAGGCACCA-3' (forward) and 5'-CTCCTTAATGTCACGCACGATTTC-3' (reverse).

**Mouse proximal tubular cell isolation.** Proximal tubular cells were isolated as described previously (38, 39). Briefly, the renal cortical part from C57BL/6 mice was minced and digested with 0.75 mg/ml collagenase 4 (Worthington, Lakewood, NJ). Then, the cells were centrifuged with 32% Percoll in DMEM/F-12 medium at 2,000 *g* for 10 min to collect the proximal tubules.

**Chimeric mouse model.** Bone marrow transplantation was performed as described by Hess et al. (9). Briefly, C57BL/6 male recipient mice were irradiated with  $^{137}\text{Cs}$  for bone marrow ablation. Donor bone marrow cells from female C57BL/6-TgN(ACTbEGFP)10sb mice were introduced into the retro-orbital sinus of recipient mice within 24 h after irradiation.

**Statistics.** Kaplan-Meier analysis was conducted using GraphPad Prism software to compare the animal survival rates. Other statistical differences between two groups were calculated by Student's *t*-test (Microsoft Excel).  $P < 0.05$  was considered significantly different.

## RESULTS

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**G-CSF pretreatment ameliorates rhabdomyolysis-associated AKI.** To examine the effects of G-CSF on rhabdomyolysis-associated AKI, male C57BL/6 mice were pretreated for 5 consecutive days with G-CSF or saline as a control. The animals were then injected with 7.5 mg/kg 50% glycerol in the hindleg muscle. Serum samples were collected from the animals to measure BUN to indicate renal function. As shown in [Fig. 1A](#), without a glycerol injection both saline- and G-CSF-treated animals showed low baseline levels of BUN (*day 0*). One day after the glycerol injection, BUN was increased from  $34.9 \pm 6.3$  to  $187.3 \pm 27.2$  mg/dl ( $n = 10$ ) in the saline-pretreated group and further elevated to  $198.9 \pm 30.3$  mg/dl ( $n = 6$ ) at *day 2*. The glycerol-induced BUN elevation was partially but significantly ameliorated in the animals pretreated with G-CSF. As a result, the BUN values in these animals were  $145.8 \pm 23.9$  and  $135.6 \pm 57.0$  mg/dl ( $n = 7$ ) at *days 1* and *2* of glycerol treatment, respectively ([Fig. 1A](#)).

The renoprotective effect of G-CSF following the glycerol injection was also confirmed by the examination of renal histology. As shown in [Fig. 1B](#), in the absence of a glycerol injection, renal tissues showed a normal healthy histology regardless of G-CSF pretreatment. However, 2 days after the glycerol injection, widespread tissue damage was noticed in the kidneys. Tissue damage was shown in both the cortex and renal medulla. The injured tubules showed the loss of microvilli, necrotic lysis, tubular dilation, cast formation, or sloughing of cellular debris into the tubular lumen. However, it is noteworthy that severe damage including necrotic lysis was mainly in the outer stripe of outer medulla and cortex, whereas the injurious changes in the inner medulla were mainly cast formation and tubular dilation. In mice pretreated with G-CSF, glycerol-induced renal tissue damage was partially ameliorated ([Fig. 1B](#)). We further graded the renal tissue damage in three separate experiments. A summary of the tubular damage scores is shown in

[Fig. 1C](#) ( $n = 6$ ). Clearly, G-CSF could partially but significantly attenuate renal tissue damage during glycerol-induced rhabdomyolysis. Of note, the protective effect of G-CSF in the outer medulla was not as evident as that in the cortex and inner medulla. One explanation is that the S3 proximal tubules in the outer stripe of the outer medulla were severely injured and harder to protect. We also examined apoptosis in the tissues by TUNEL assay. Glycerol induced  $7.1 \pm 3.6$  apoptotic cells/mm<sup>2</sup> tissue, which was reduced to  $4.5 \pm 3.2$  cells/mm<sup>2</sup> by G-CSF pretreatment ( $n = 5$ ).

**Beneficial effects of G-CSF on animal survival following glycerol-induced rhabdomyolysis.** We further monitored animal death/survival following the glycerol injection. As shown in [Fig. 2](#), animal death started in the saline-pretreatment group after 2 days of glycerol treatment and, by the end of 10 days, only 30% animals were alive ( $n = 14$ ). In contrast, G-CSF-pretreated animals ( $n = 7$ ) did not die until *day 6* after the glycerol injection. This group of animals had a >70% survival rate during the whole observation period. The difference between the survival rates of these two groups is significant by Kaplan-Meier analysis ( $P < 0.05$ ). Thus G-CSF not only postponed animal death but also improved long-term survival following glycerol-induced rhabdomyolysis.

**G-CSF pretreatment inhibits hemin-induced apoptosis in cultured tubular cells.** A major mediator or inducer of renal injury during rhabdomyolysis is the free heme that is released from myoglobin from disrupted muscles. Thus hemin treatment of cultured tubular cells has been established to mimic the *in vivo* situation of rhabdomyolysis-associated renal injury ([7](#)). Using a similar model, we tested whether G-CSF could directly protect against hemin-induced apoptosis in cultured RPTC. As shown in [Fig. 3A](#), typical apoptosis was induced during hemin treatment, showing cellular shrinkage, nuclear condensation, and fragmentation. These cells were also positive for TUNEL staining ([Fig. 3A](#), *bottom*). By cell counting, 50  $\mu$ M hemin induced 30% apoptosis at 6 h, which increased to 50% at 10 h. Notably, the apoptosis was reduced by 500 ng/ml G-CSF pretreatment to 25 and 30% at 6 and 10 h, respectively ([Fig. 3B](#)). As a biochemical marker of apoptosis, we measured caspase activity. Hemin induced an over fivefold increase in caspase activity within 6 h, which was partially but significantly suppressed by G-CSF ([Fig. 3C](#)). The effect of G-CSF on caspase activity was not as obvious as that of apoptosis, suggesting that hemin may induce apoptosis via caspase-dependent and caspase-independent mechanisms.

**Induction of HO-1 by G-CSF in cultured tubular cells and mouse kidneys.** The direct protective effects of G-CSF in RPTC cells shown in [Fig. 3](#) were intriguing. To study the underlying mechanism, we initially focused on HO-1, the rate-limiting enzyme in heme degradation that has been shown to be cytoprotective in a variety of pathological conditions including acute kidney injury ([4](#), [13](#), [14](#), [18](#), [29](#)). In control RPTC cells and kidneys, HO-1 expression is low and after hemin or glycerol treatment, HO-1 is greatly induced (Supplemental Fig. S1; all supplemental material for this article is accessible online on the journal web site).

G-CSF treatment for 8–48 h did not change HO-1 expression in RPTC cells; however, at 72 h G-CSF induced HO-1 in a dose-dependent manner ([Fig. 4A](#)). Dose-dependent induction of HO-1 by G-CSF was further verified by densitometry analysis of the immunoblots from three separate experiments ([Fig. 4B](#)). In addition, RT-PCR analysis detected the induction of HO-1 mRNA by G-CSF in RPTC cells ([Fig. 4](#), *C* and *D*), suggesting a regulation at the gene transcription level. In RPTC and primary mouse kidney proximal tubular cells, we also detected the expression of the G-CSF receptor, which appeared as multiple bands in immunoblot analysis likely due to different degrees of glycosylation of the protein including the mature form ([6](#)). We further knocked down the G-CSF receptor in RPTC cells and determined G-CSF-induced

HO-1 expression. As shown in Supplemental Fig. S2, HO-1 induction by G-CSF was significantly attenuated in the cells with G-CSF receptor knockdown, suggesting the involvement of G-CSF receptor-mediated signaling in HO-1 induction.

Importantly, G-CSF could induce HO-1 expression in vivo in kidney tissues. This was first shown by immunoblot analysis of renal cortical tissues. As shown in [Fig. 5A](#), G-CSF treatment ( $200 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ ) of mice for 5 days led to a marked increase in HO-1 in the renal cortical tissue lysate. By immunohistochemical staining, we showed that HO-1 was mainly induced in cortical tubules and not in glomeruli ([Fig. 5B](#)).

**Inhibition of HO-1 by ZnPP attenuates the protective effects of G-CSF.** To determine whether HO-1 induction contributed to the observed renoprotective effects of G-CSF, we examined the effects of ZnPP, a pharmacological inhibitor of HO-1 ([15, 25](#)). RPTC cells were cultured for 3 days in the absence or presence of 500 ng/ml G-CSF for pretreatment. One group of dishes was then incubated with 2  $\mu\text{M}$  ZnPP for 2 h to inhibit HO-1 ([Fig. 6A](#)), while the other group received the vehicle solution. Both groups were finally treated with 50  $\mu\text{M}$  hemin for 8 h to induce apoptosis. As shown in [Fig. 6A](#), hemin treatment induced 50% apoptosis in RPTC cells without G-CSF pretreatment, which was decreased to 30% by G-CSF pretreatment. ZnPP alone did not induce much apoptosis; however, it significantly enhanced apoptosis during hemin treatment. Notably, ZnPP attenuated the protective effects of G-CSF and, as a result, the cells with or without G-CSF pretreatment showed similarly high levels of apoptosis, >70%, suggesting that HO-1 induction was critical to the renoprotective effects of G-CSF in vitro.

We further determined the effects of ZnPP in vivo in C57BL/6 mice. To this end, the animals were divided into three groups. *Group 1* received saline, while *groups 2* and *3* were pretreated with G-CSF for 5 days. Two hours before the glycerol injection, *group 3* was administered with 20 mg/kg ZnPP, whereas *groups 1* and *2* were given the vehicle solution. BUN was measured at *days 0, 1, and 2* after the glycerol injection to indicate renal injury. As shown in [Fig. 6B](#), following the glycerol injection the mice in *group 1* (No G-CSF) showed BUN increases at both *days 1* and *2*, to 110 mg/dl. The BUN increases were significantly suppressed by G-CSF pretreatment in *group 2* mice (G-CSF), to 65 mg/dl. However, in *group 3* the renoprotective effects of G-CSF were diminished by ZnPP ([Fig. 6B](#)).

## DISCUSSION

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The effects of G-CSF have been studied in experimental models of AKI induced by ischemia-reperfusion, folic acid, and cisplatin toxicity. In the majority of the studies, G-CSF was shown to mobilize bone marrow cells into renal tissues and ameliorate renal injury ([5, 12, 21, 31](#)). However, bone marrow cell mobilization-associated granulocytosis by G-CSF may also worsen renal injury as shown in a mouse model of renal ischemia-reperfusion injury ([33](#)). In the current study, we have determined the effects of G-CSF on rhabdomyolysis-associated AKI. Our results show that G-CSF protects against glycerol-induced AKI in vivo and hemin-induced apoptosis in vitro. Mechanistically, we have demonstrated the first evidence for HO-1 induction by G-CSF. HO-1, after being induced, contributes to the renoprotective effects of G-CSF. Thus the results have revealed a novel mechanism of tissue protection by G-CSF.

As a pleiotropic cytokine, G-CSF is well known for its ability to stimulate the mobilization and proliferation of bone marrow cells. During AKI induced by renal ischemia and cisplatin nephrotoxicity ([12, 30, 33](#)), G-CSF mobilizes bone marrow cells into injured tissues. Consistently, using a chimerical mouse model with transplanted GFP-positive bone marrow, we showed that G-CSF increased the number of bone marrow

cells in the renal cortex during glycerol-induced AKI (Supplemental Fig. S3). Despite these findings, it remains unclear whether and how the bone marrow cells contribute to renal injury and repair or regeneration. Although bone marrow stem cells may differentiate into renal tubular cells, recent studies indicate that bone marrow cell differentiation into or fusion with tubular cells is not frequent but a rare event (3, 11, 17, 30). In our study, GFP-labeled bone marrow was reconstituted into bone marrow-ablated recipient mice. Although GFP-labeled cells were detected in the renal tissues of the recipient mice, these cells were shown in the interstitium and not incorporated into renal tubules. G-CSF pretreatment increased the recruitment of bone marrow cells into renal tissues but did not induce the differentiation or incorporation of these cells into renal tubules for tissue repair (Supplemental Fig. S3).

On the other hand, bone marrow cells recruited into renal tissues may provide a protective, reparative, or regenerative microenvironment. This has been demonstrated recently for mesenchymal/stromal stem cells, a specific stem cell population in bone marrow. In an experimental model of renal ischemia-reperfusion, Togel et al. (34) showed that infused mesenchymal/stromal stem cells can protect against kidney injury by their vasculotropic, paracrine actions. In a separate study, Bi et al. (1) showed that intravenous infusion or intraperitoneal injection of bone marrow-derived stromal cells can enhance tubular cell proliferation and decrease tubular cell apoptosis, leading to renoprotection during cisplatin-induced AKI. Importantly, similar protective effects were demonstrated for conditioned media from cultured stromal cells (1). Together, these studies suggest that bone marrow-derived mesenchymal/stromal stem cells that are recruited into injured renal tissues may protect the kidneys by secreting protective and/or regenerative factors. In our study, G-CSF treatment led to significantly higher mobilization and recruitment of bone marrow cells into injured renal tissues, increasing a tropic cell population for renal protection and repair.

Interestingly, we have also suggested a direct cytoprotective action of G-CSF in renal tubular cells. In 2004, Gonzales-Michaca and colleagues (7) established an *in vitro* model to study rhabdomyolysis-associated AKI by hemin incubation of proximal tubular cells. In this model, hemin induced apoptosis as shown by cell morphology, TUNEL staining, and caspase activation (7). Using a similar model, we now demonstrate that G-CSF pretreatment can suppress hemin-induced apoptosis in RPTC cells (Fig. 3). While the mechanism underlying the direct cytoprotection remains unclear, hemin-induced caspase activation is suppressed, suggesting that the antiapoptotic effects of G-CSF result from the inhibition of caspase-activation pathways. In addition, our results show that RPTC cells express the G-CSF receptor (Fig. 4), although it remains to be determined whether the cytoprotective effects of G-CSF depend on G-CSF receptor-mediated signaling. A direct cytoprotective action of G-CSF has been reported in other cell types. For example, Harada et al. (8) showed that G-CSF can bind to its receptor on cardiomyocytes and induce phosphorylation and activation of the Jak 2/ Stat 3 pathway, leading to expression of the antiapoptotic protein Bcl-2 and myocardial protection. More recently, Nishio et al. (23) reported that G-CSF can protect against glutamate-induced cell death in cultured neurons.

Regarding the cytoprotective mechanisms of G-CSF, we have identified an intriguing induction of HO-1. HO-1 is the inducible form of heme oxygenase, the rate-limiting enzyme in heme degradation. It has now been increasingly appreciated that HO-1 has a much broader role in tissue injury and protection, including antiapoptotic and anti-inflammatory actions (4, 14, 18, 29). In our study, HO-1 was induced by G-CSF *in vitro* in cultured tubular cells and also *in vivo* in mouse kidneys. Notably, pharmacological inhibition of HO-1 by ZnPP could attenuate the renoprotective effects of G-CSF (Fig. 6), suggesting that HO-1 induction is important to G-CSF-mediated protection. We showed that G-CSF induced both HO-1 mRNA and protein, indicating the involvement of transcriptional mechanisms; however, further studies need to

gain more mechanistic insights about the regulation and identify the responsible transcription factors.

In conclusion, we have demonstrated the renoprotective effect of G-CSF in experimental models of rhabdomyolysis-associated AKI. The protection may not only be related to bone marrow cell mobilization but also to direct cytoprotective effects of G-CSF. A novel cytoprotective mechanism of G-CSF revealed by our current study is the induction of HO-1.

## GRANTS

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## DISCLOSURES

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No conflicts of interest, financial or otherwise, are declared by the authors.

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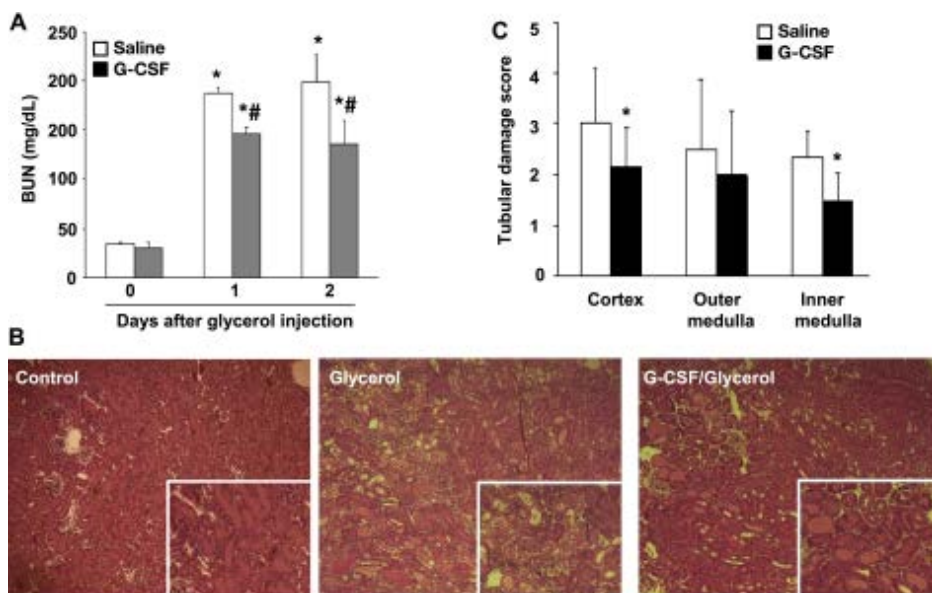
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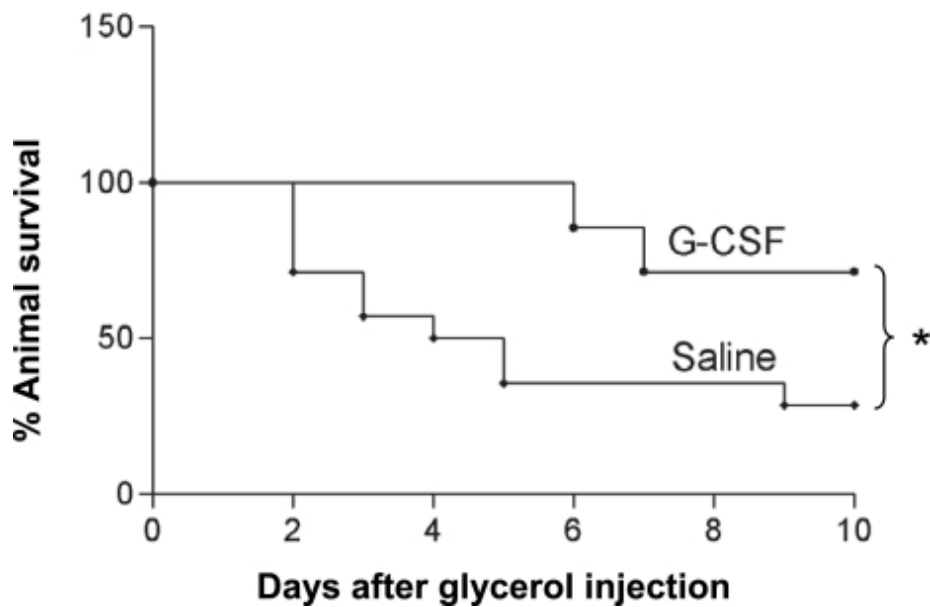
## Figures and Tables

**Fig. 1.**



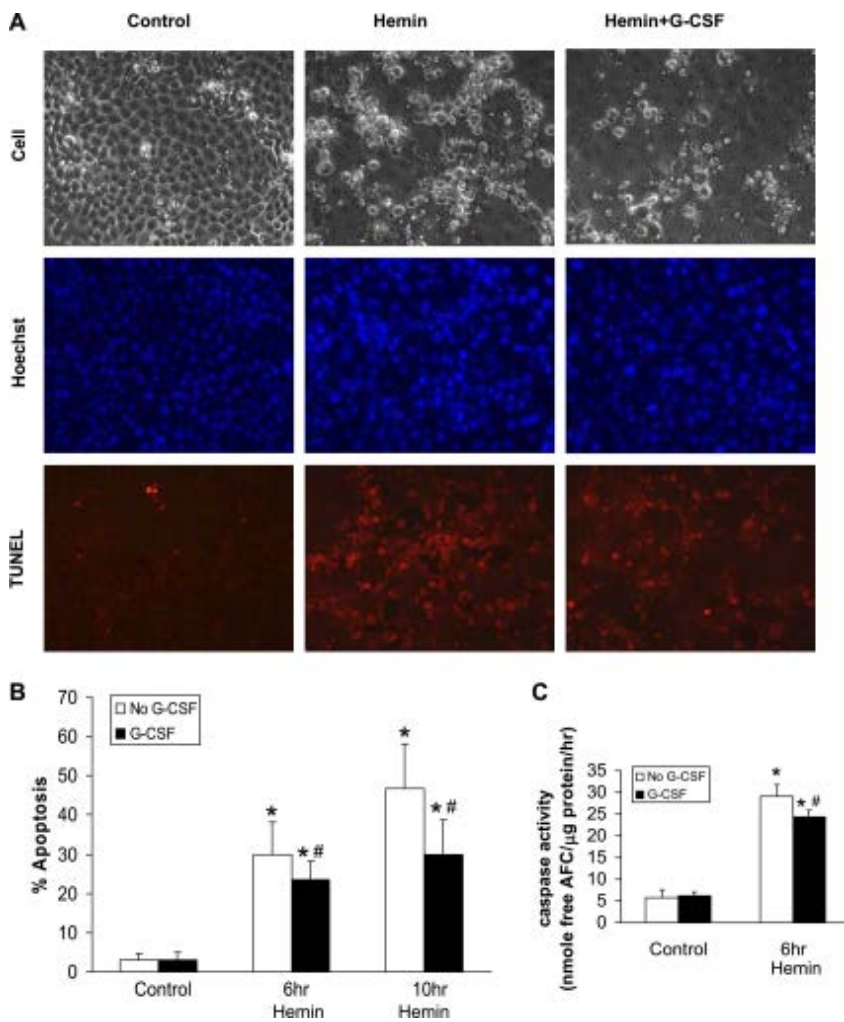
Effects of granulocyte colony-stimulating factor (G-CSF) on glycerol-induced kidney injury. C57BL/6 mice (male, 8–10 wk) were pretreated for 5 days with  $200 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$  G-CSF or saline. The animals were then injected with  $7.5 \text{ ml/kg}$  50% glycerol in the hindleg muscles. **A:** blood urea nitrogen (BUN) levels. Blood samples were collected just before glycerol injection or 1–2 days after glycerol injection to determine BUN. \*Significantly different from *day 0* ( $P < 0.05$ ). #Significantly different from saline-pretreated groups ( $P < 0.05$ ). **B:** representative renal histology. Renal tissues were collected from animals without or with 2 days of glycerol treatment, which had been pretreated with saline or G-CSF. The tissues were fixed and processed for hematoxylin and eosin (H&E) staining. *Insets:* histology at a higher magnification. **C:** tissue damage score. The percentage of proximal tubules in renal cortex and outer medulla that showed necrotic lysis or debris was evaluated to determine tissue damage score: 0, none; 1, <25%; 2, 25–50%; 3, 50–75%; 4, >75%. Values are means  $\pm$  SD ( $n = 6$ ). The results show partially but significant renoprotective effects of G-CSF during glycerol-induced acute kidney injury (AKI). \*Significantly different from saline-pretreated group ( $P < 0.05$ ).

**Fig. 2.**



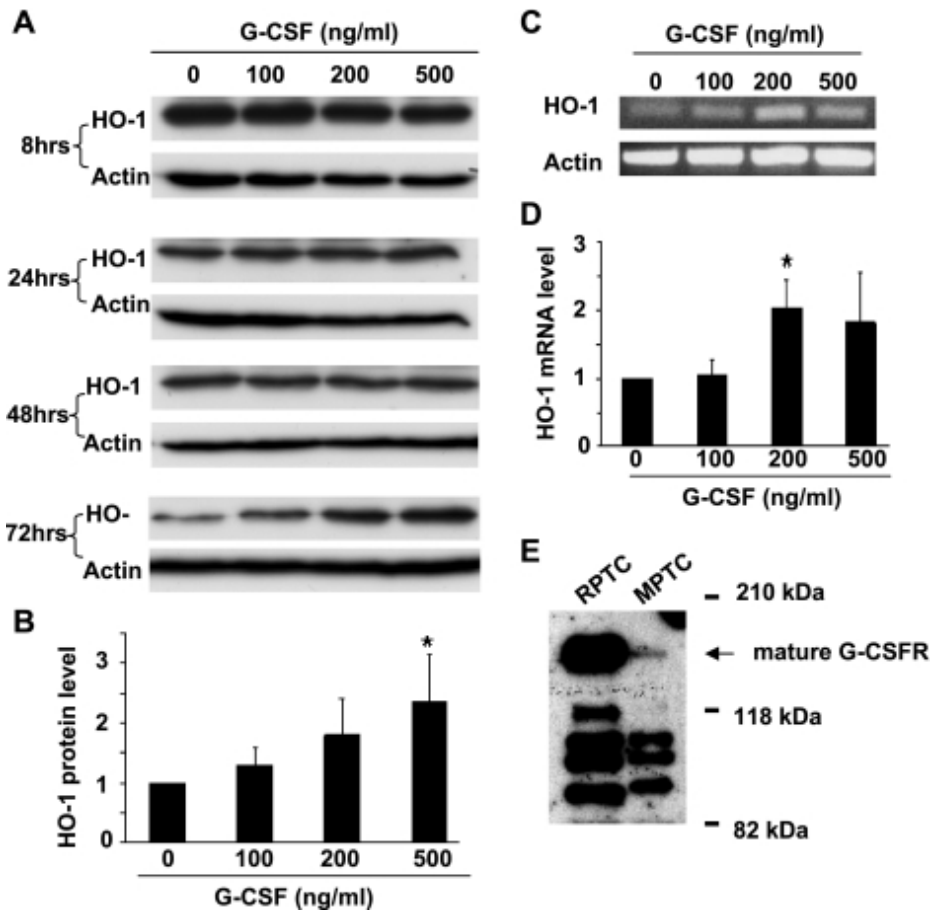
Effects of G-CSF on animal survival following glycerol injection. C57BL/6 mice (male, 8–10 wk) were divided into 2 groups. One group was pretreated for 5 days with  $200 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$  G-CSF ( $n = 7$ ), and the other group was pretreated with saline ( $n = 14$ ). Both groups of animals were then injected with  $7.5 \text{ ml/kg}$  50% glycerol in hindleg muscles. Animal death and survival in these 2 groups were recorded for 10 days following glycerol injection. The results show a beneficial effect of G-CSF on animal survival following glycerol-induced rhabdomyolysis in mice. \*Significant difference between the survival rates of the 2 groups ( $P < 0.05$ ).

**Fig. 3.**



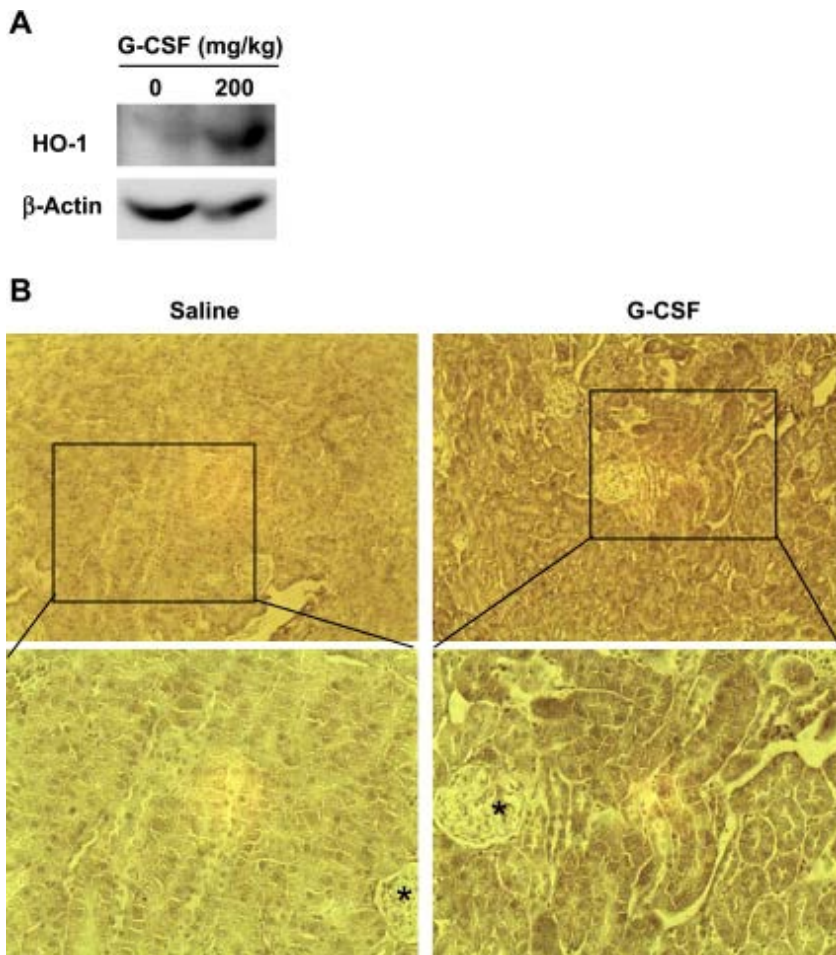
Effects of G-CSF on hemin-induced apoptosis in rat proximal tubule cells (RPTC) cells. RPTC cells were preincubated for 3 days with or without 500 ng/ml G-CSF. The cells were then treated with 50  $\mu$ M hemin for 6 or 10 h, or left untreated as control. **A**: representative cell morphology after 6 h of treatment. Cells were subjected to Hoechst 33342 or TUNEL staining and then examined by phase-contrast and fluorescence microscopy. **B**: % apoptosis. The cells with typical apoptotic morphology (cellular shrinkage, formation of apoptotic bodies, and nuclear fragmentation) were counted to determine the percentage of apoptosis. **C**: caspase activity. Cell lysate was collected to determine caspase activity by an enzymatic assay as described in MATERIALS AND METHODS. Values are means  $\pm$  SD ( $n = 3$ ). The results demonstrate cytoprotective effects of G-CSF on hemin-induced tubular cell apoptosis. \*Significantly different from the control groups without hemin treatment ( $P < 0.05$ ). #Significantly different from the hemin-treated groups without G-CSF pretreatment ( $P < 0.05$ ).

**Fig. 4.**



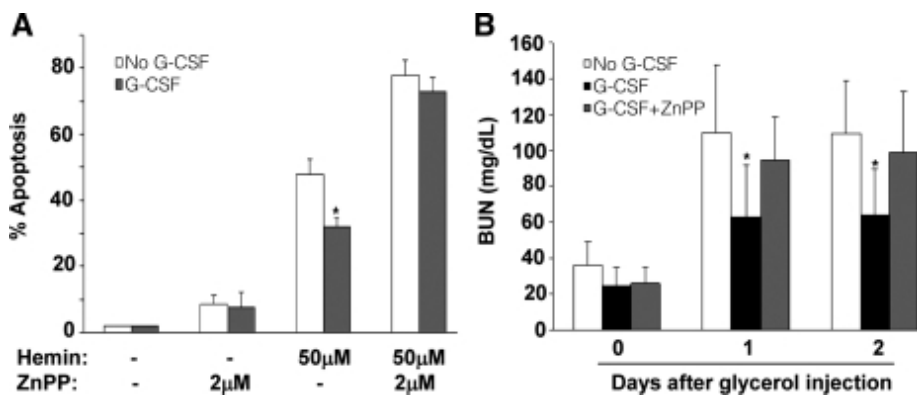
Heme oxygenase (HO)-1 induction by G-CSF in RPTC cells. *A*: immunoblot analysis of HO-1. RPTC cells were incubated with 0, 100, 200, or 500 ng/ml G-CSF for 8, 24, 48, or 72 h to collect whole cell lysates for immunoblot analysis. The blots were reprobed for  $\beta$ -actin as a protein loading/transferring control ( $P < 0.05$ ). *B*: densitometry of HO-1 immunoblots from 3 separate experiments in which RPTC cells were incubated with 0–500 ng/ml G-CSF for 72 h. The control value was arbitrarily set as 1 to normalize the signals of other lanes. \*Significantly different from control ( $P < 0.05$ ). *C*: RT-PCR analysis of HO-1 mRNA. RPTC cells were treated for 72 h with 0, 100, 200, or 500 ng/ml G-CSF. Total RNA was extracted for semiquantitative analysis of HO-1 mRNA by RT-PCR as described in MATERIALS AND METHODS.  $\beta$ -Actin mRNA was also analyzed as a control. *D*: densitometry of HO-1 mRNA RT-PCR analysis from 3 separate experiments. The control value was arbitrarily set as 1 to normalize the signals of other lanes. \*Significantly different from control ( $P < 0.05$ ). *E*: immunoblot analysis of G-CSF receptor in RPTC cells and isolated mouse proximal tubular cells.

**Fig. 5.**



HO-1 induction by G-CSF in mouse kidneys. *A*: immunoblot analysis of HO-1 in mouse kidney cortex. C57BL/6 mice were treated with 200 mg·kg<sup>-1</sup>·day<sup>-1</sup> G-CSF or saline for 5 days. Renal cortical tissues were collected and homogenized for immunoblot analysis of HO-1 and  $\beta$ -actin. *B*: immunohistochemical staining of HO-1 in mouse kidneys. C57BL/6 mice were treated with 200 mg·kg<sup>-1</sup>·day<sup>-1</sup> G-CSF or saline for 5 days to collect kidney tissues for fixation with paraformaldehyde for immunohistochemistry. The *bottom* micrographs are magnified images of the boxed areas in the *top* panels. \*, Glomeruli. The results show that HO-1 is mainly induced by G-CSF renal tubules.

**Fig. 6.**



Attenuation of the renoprotective effects of G-CSF by protoporphyrin IX zinc(II) (ZnPP). *A*: attenuation of the cytoprotective effects of G-CSF in RPTC cells. RPTC cells were pretreated with or without 500 ng/ml G-CSF for 3 days. One group of cells was then incubated with 2  $\mu$ M ZnPP for 2 h to inhibit HO-1. The cells were finally treated with 50  $\mu$ M hemin for 8 h. Apoptosis was determined by morphological criteria. Values are means  $\pm$  SD ( $n = 3$ ). \*Significantly

different from the hemin-treated no G-CSF group ( $P < 0.05$ ). *B*: attenuation of the renoprotective effects of G-CSF in mouse. C57BL/6 mice were randomly divided into 3 groups ( $n = 5$  in each group). *Group 1* received saline, while *groups 2* and *3* were pretreated with G-CSF for 5 days. Then, *group 3* was administered 20 mg/kg ZnPP for 2 h, while *groups 1* and *2* were given the vehicle solution. Finally, all mice were injected with 7.5 ml/kg glycerol to induce rhabdomyolysis. Serum samples were collected just before glycerol treatment (*day 0*) or 1 and 2 days after glycerol injection to indicate renal injury. Values are means  $\pm$  SD ( $n = 5$ ). The results show that the renal protective effects of G-CSF are diminished by ZnPP. \*Significantly different from the No G-CSF group ( $P < 0.05$ ).

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