Disruption of Guanylyl Cyclase-G Protects against Acute Renal Injury

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ABSTRACT

The membrane forms of guanylyl cyclase (GC) serve as cell-surface receptors that synthesize the second messenger cGMP, which mediates diverse cellular processes. Rat kidney contains mRNA for the GC-G isoform, but the role of this receptor in health and disease has not been characterized. It was found that mouse kidney also contains GC-G mRNA, and immunohistochemistry identified GC-G protein in the epithelial cells of the proximal tubule and collecting ducts. Six hours after ischemia-reperfusion (I/R) injury, GC-G mRNA and protein expression increased three-fold and remained upregulated at 24 h. For determination of whether GC-G mediates I/R injury, a mutant mouse with a targeted disruption of the GC-G gene (*Gucy2g*) was created. At baseline, no histologic abnormalities were observed in GC-G$^{-/-}$ mice. After I/R injury, elevations in serum creatinine and urea were attenuated in GC-G$^{-/-}$ mice compared with wild-type controls, and this correlated with less tubular disruption, less tubular cell apoptosis, and less caspase-3 activation. Measures of inflammation (number of infiltrating neutrophils, myeloperoxidase activity, and induction of IL-6 and P-selectin) and activation of NF-κB were lower in GC-G$^{-/-}$ mice compared with wild-type mice. Direct transfer of a GC-G expression plasmid to the kidneys of GC-G$^{-/-}$ mice resulted in a dramatically higher mortality after renal I/R injury, further supporting a role for GC-G in mediating injury. In summary, GC-G may act as an early signaling molecule that promotes apoptotic and inflammatory responses in I/R-induced acute renal injury.

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Acute renal failure represents a common clinical problem associated with a high morbidity and mortality.[1] Renal ischemia-reperfusion (I/R) is the major cause of acute renal failure in the native[2] and transplanted kidneys.[3] It has been well documented that apoptosis and inflammation are key mechanisms leading to organ damage during renal I/R[4]; however, many upstream signal events are still unclear.

Intracellular second-messenger cGMP synthesized by soluble or receptor guanylyl cyclases (GC), has been implicated in a broad spectrum of (patho-)physiologic processes, such as regulation of BP, endothelial dysfunction, platelet aggregation, fluid/electrolyte homeostasis, and phototransduction.[5,6] Recent pharmacologic studies suggested that soluble GC are involved in a chronic model of glomerulonephritis[7,8]; however, little is known about the role of receptor GC in renal diseases.

In mammals, seven isoforms of receptor GC

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have been identified: GC-A through GC-G.[5,6] GC-A and GC-B function as receptors for atrial natriuretic peptide (ANP) and B-type natriuretic peptide, and GC-C mediates the effects of guanylin and uroguanylin on intestinal electrolyte and water transport.[5,6] Endogenous ligands for GC-D, -E, -F, or -G have yet to be identified; therefore, these receptors remain as orphans. Despite being an orphan receptor, GC-E has been clearly shown by gene targeting to have a role in maintaining normal retinal structure and function[9]; the study led to the discovery of numerous mutations in the GC-E gene responsible for several forms of retinal disorders in humans.[10]

We cloned from the mouse testis the full-length cDNA for GC-G,[11] which seems to be the last member of the receptor GC family identified thus far; however, its protein distribution in other tissues and its function are largely unknown. Here, we demonstrate the exclusive expression of GC-G in tubular epithelial cells in the kidney and define its pathologic role during I/R-induced acute renal failure by using a new genetic mouse model with targeted disruption of the GC-G gene (*Gucy2g*). Our study is the first demonstration that GC-G–deficient ($GC\text{-}G^{-/-}$) mice are protected against ischemic renal failure and tissue damage and suggests that the induction/activation of GC-G during I/R is detrimental and contributes to tubular damage and renal failure through apoptosis and inflammation.

RESULTS

Renal Expression of GC-G under Normal and I/R Conditions

Previous studies showed that GC-G mRNA is expressed in rat kidney,[12–14] and we found GC-G mRNA expressed in the mouse kidney by reverse transcriptase–PCR (RT-PCR) analysis with three independent pairs of mouse GC-G–specific oligonucleotide primers (Figure 1A). To verify further the cell type expression of GC-G, we performed immunohistochemistry using an anti–GC-G–specific antibody in mouse kidney sections. GC-G immunoreactivity was observed in the epithelial cells of the proximal tubules and cortical and medullary collecting ducts (Figure 2, E and G, and Supplemental Figure 1).

Because GC-G is expressed in renal epithelial cells and because these cells are particularly susceptible to I/R injury, we then examined the renal expression of GC-G in a mouse I/R

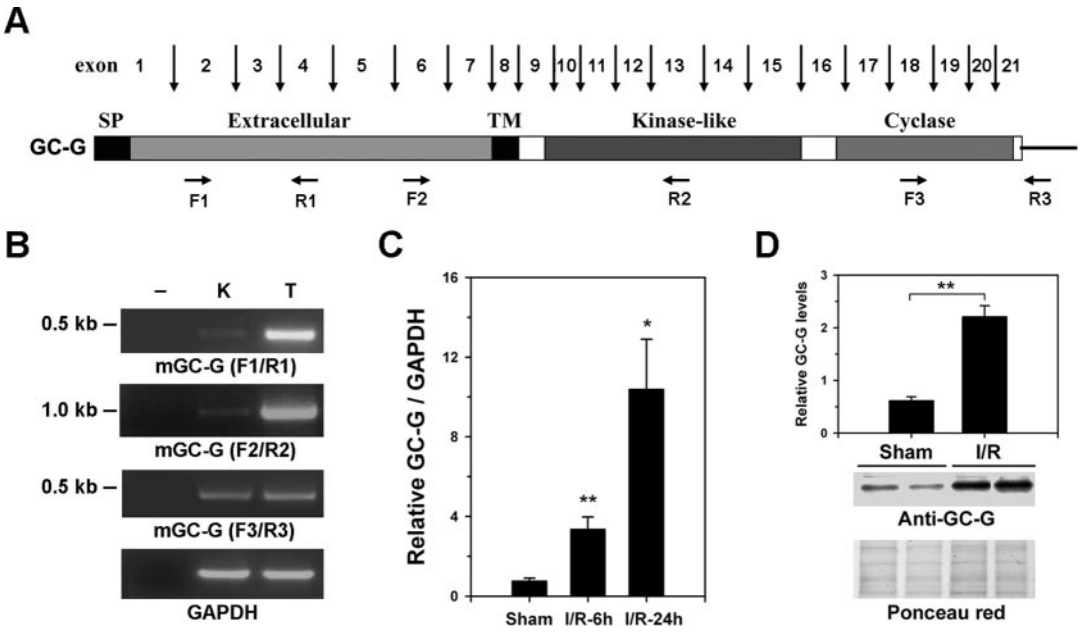

Figure 1. Expression of GC-G mRNA under normal or renal I/R conditions. (A) Locations of GC-G–specific oligonucleotide primers used for RT-PCR. Exon boundaries are marked over the domain organization of GC-G.[11] Note that three pairs of primers used for RT-PCR analysis span introns to avoid genomic amplification. (B) GC-G expressed in the mouse kidney. Mouse kidney (K) or testis (T) cDNA underwent PCR with three independent pairs of GC-G–specific primers (see A). –, water control; GAPDH, glyceraldehyde-3 phosphate dehydrogenase. (C) Quantitative RT-PCR analysis of expression of GC-G mRNA after renal I/R injury. After renal reperfusion at 6 or 24 h, the kidneys were harvested, and total RNA was extracted and converted to cDNA. Expression levels were normalized to GAPDH. Experiments were performed twice in duplicate with similar results ($n = 5$ mice in each group). *$P < 0.05$ (I/R-24 h *versus* sham surgery); **$P < 0.01$ (I/R-6 h *versus* sham surgery). (D) Western blot analysis of GC-G protein expression in I/R kidney. Protein extracts (30 μg) from the control (sham) and I/R kidneys in duplicate were subjected to Western blot analysis using the anti–GC-G–specific antibody. Ponceau red staining of the polyvinylidene difluoride membrane confirmed the relative loading of total protein for each sample (bottom). (Top) Quantification of results by densitometric scanning and normalized by the total amount of protein loading. **$P < 0.01$.

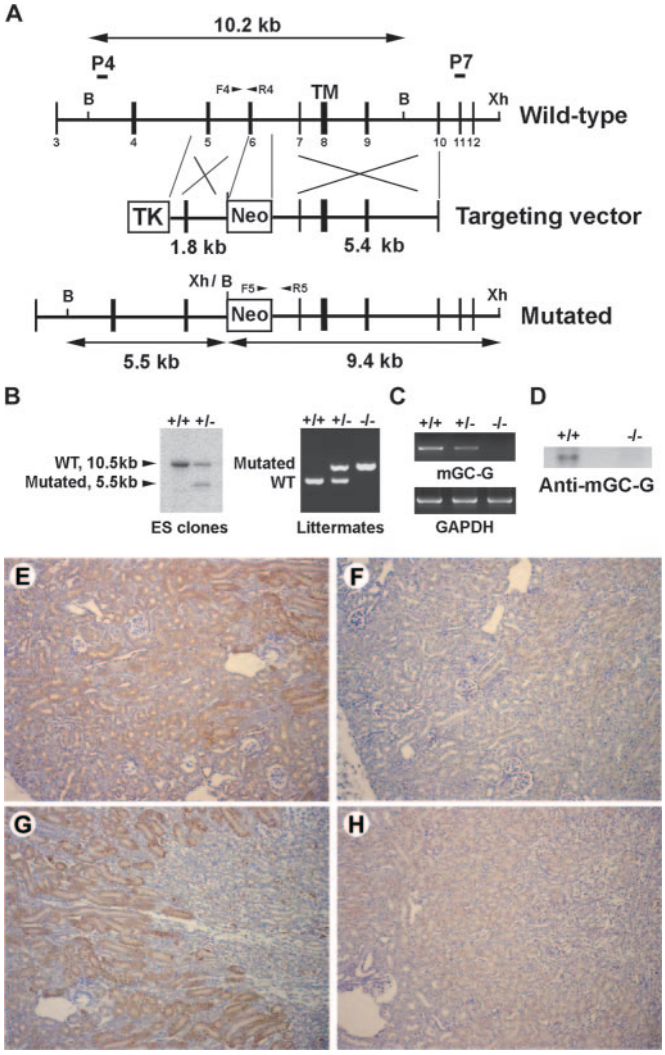

Figure 2. Targeted disruption of GC-G gene and its renal expression in mice. (A) Maps of the WT GC-G locus, the targeting vector, and the mutated locus. The coding exons of the GC-G gene are shown as a box. The map of the targeting vector shows the replacement of exon 6 coding for a portion of the extracellular domain with neomycin-resistance gene (Neo). One copy of the thymidine kinase (TK) gene was placed at the 5′ end of the targeting vector for negative selection. The expected sizes of *BglII*- or XhoI-generated fragments from the WT and disrupted (mutated) GC-G genes detected with a 5′ probe (P4) or 3′ probe (P7) are indicated. TM, transmembrane domain encoded in exon 8; B, *BglII*; Xh, XhoI. (B) Southern blot analysis and PCR genotyping of genomic DNA from ES cell clones or tail genomic DNA from littermates. Hybridization with 5′ probe yielded a 10.5- or 5.5-kb band from the WT or mutant allele, respectively, as indicated. Probing with 3′ probe confirmed the corrected targeting event as well (data not shown). Primers specific for WT (F4/R4) and mutated allele (F5/R5) were used for genotyping analysis of the littermates. (C) Kidney cDNA derived from WT (+/+), heterozygous (+/−), and homozygous null (−/−) mice underwent RT-PCR amplification with the GC-G–specific oligonucleotide primers (F2/R2 in Figure 1). (D) Western blot analysis of sperm extracts from WT (+/+) and homozygous null (−/−) mice with the anti–GC-G–specific antibody. (E through H) GC-G immunohistochemistry of kidneys of WT and GC-G$^{-/-}$ mice. The kidney sections from WT (E and G) and GC-G$^{-/-}$ mice (F and H) were probed with anti–GC-G–specific antibody. GC-G protein expression was present in renal tubular epithelial cells within the cortex (brown, E) and outer medulla region (brown, G) of WT kidneys but was absent from GC-G$^{-/-}$ kidneys (F, cortex; H, medulla). Magnification ×200.

model under bilateral renal ischemia for 45 min followed by reperfusion up to 24 h. After 6 h of reperfusion, the renal GC-G mRNA level was approximately three times higher than that in sham-operated mice and continued to be upregulated 24 h after reperfusion (Figure 1C), which is consistent with renal GC-G protein expression determined by Western blot analysis (Figure 1D).

Generation of GC-G$^{-/-}$ Animals

Because GC-G is expressed by renal tubular epithelial cells and is rapidly upregulated after I/R injury, we hypothesized that GC-G might play important role in the development of I/R injury. To determine the involvement of GC-G in renal injury *in vivo*, we generated a new genetically altered mouse model lacking the functional GC-G gene. The mouse GC-G gene (*Gucy2g*) was disrupted by replacing a portion of exon 6, which codes for a region of the extracellular domain, with a neomycin-resistance gene cassette (Figure 2A). The GC-G$^{-/-}$ mice developed normally and did not show apparent defects in renal histologic features or renal function. The targeted allele was further maintained in a C57BL/6 genetic background by backcrossing for at least five generations. The homozygous null mice completely lacked the expression of functional GC-G at both the mRNA (Figure 2C) and protein (Figure 2, F and H) levels in the kidney. Consistently, a 50% decrease in the total cGMP level was observed in GC-G$^{-/-}$ kidney compared with wild-type (WT; Figure 3A).

Preservation of Renal Function and Structure after Renal I/R in GC-G$^{-/-}$ Mice

To study the role of GC-G in I/R injury, we subjected WT and GC-G$^{-/-}$ mice to bilateral I/R and compared renal function. As compared with sham-operated mice, WT mice showed increased serum level of urea and creatinine on I/R injury, which suggests renal dysfunction (Figure 3B); however, the level of serum urea and creatinine in GC-G$^{-/-}$ mice under I/R was significantly lower than that observed in WT littermates, which suggests a marked prevention of the renal failure associated with renal I/R injury. Furthermore, the magnitude of renal failure, as determined by increased serum urea or creatinine level, was protected, by >74 or 86%, respectively, in GC-G$^{-/-}$ mice (Figure 3B). Sham-operated WT and GC-G$^{-/-}$ mice showed healthy and histologically comparable tubular systems, in accordance with renal function (Figure 4, A and B); however, WT mice showed substantial histopathologic changes, such as tubular necrosis, tubular dilation, protein

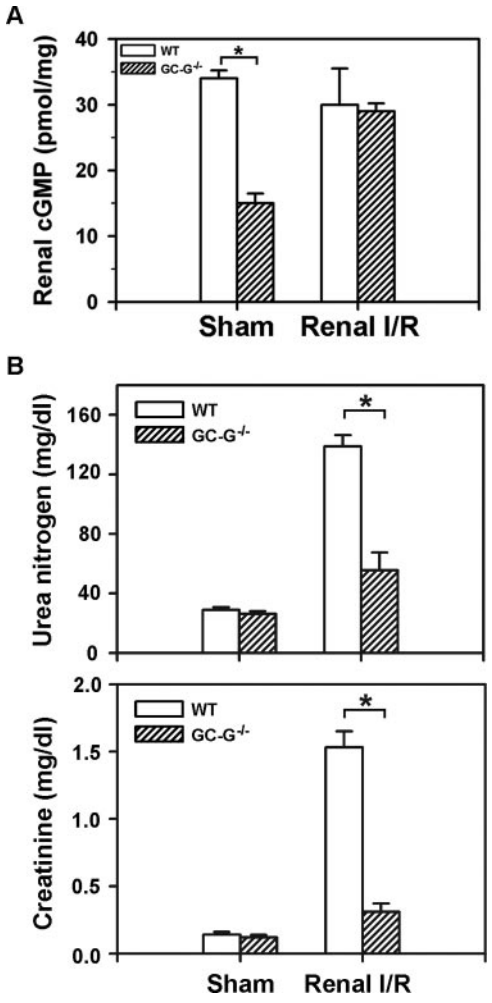

Figure 3. Effect of I/R on renal total cGMP levels and kidney function in WT and GC-G$^{-/-}$ mice. (A) Total cGMP levels in WT and GC-G$^{-/-}$ kidneys under sham or I/R conditions. Kidneys that were obtained from WT and GC-G$^{-/-}$ mice that underwent a sham operation or I/R injury were subjected to EIA assays for cGMP concentrations. Whereas GC-G$^{-/-}$ kidneys showed a marked reduction in the total cGMP concentrations by approximately 50% as compared with WT mice under sham conditions, a similar level of overall renal cGMP concentrations seen under renal I/R is probably due to I/R-induced upregulation of soluble GC $\alpha1/\beta1$ subunit mRNA in GC-G$^{-/-}$ mice compared with WT controls (data not shown). *$P < 0.01$ ($n = 5$ in each group). (B) Effect of I/R on renal function in WT and GC-G$^{-/-}$ mice. Mice underwent bilateral renal arterial clamping for 45 min. Serum urea nitrogen (top) and creatinine (bottom) levels were measured 24 h after reperfusion or sham surgery. GC-G$^{-/-}$ mice showed less severe renal failure as compared with sham-operated WT mice. Data are means ± SEM ($n = 5$ mice in each group). *$P < 0.01$, WT *versus* GC-G$^{-/-}$.

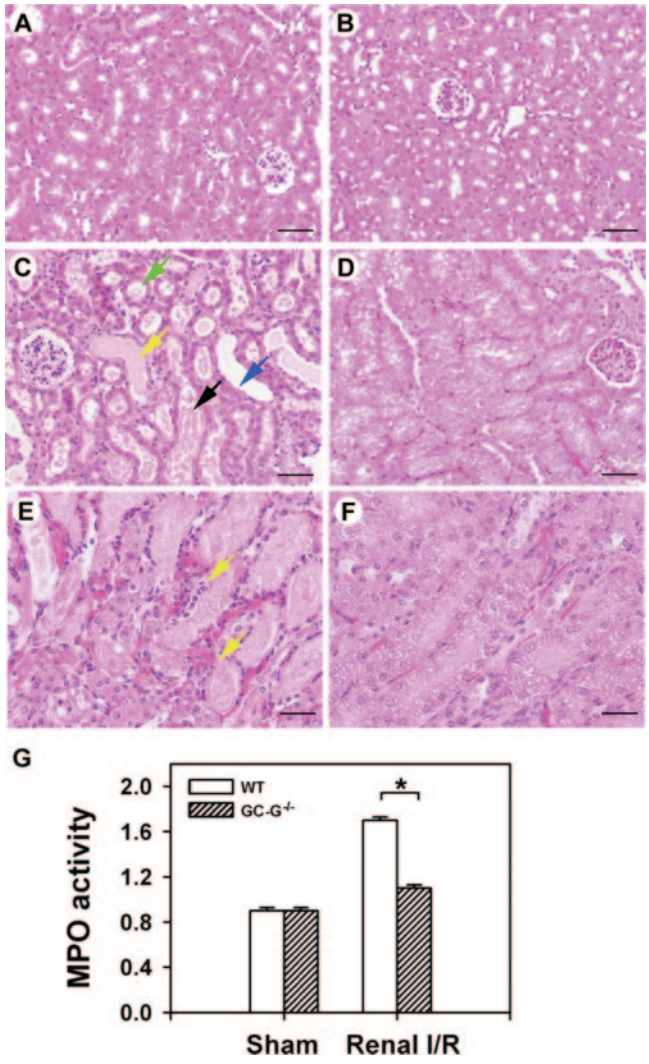

Figure 4. Effect of I/R on renal tubular disruption, PMN infiltration number, and MPO activity in WT and GC-G$^{-/-}$ mice. WT and GC-G$^{-/-}$ mice underwent a sham operation (A and B) or renal I/R injury (C and D). Renal sections were stained with hematoxylin and eosin for histologic examination. WT kidneys showed marked degree of renal injury (C), including extensive loss of brush border (green arrow), tubular necrosis (black arrow), tubular dilation (blue arrow), and cast formation (yellow arrow), whereas GC-G$^{-/-}$ kidneys showed a marked reduction in the severity of these features (D). No apparent differences were noticed in renal histology between sham controls of WT (A) and GC-G$^{-/-}$ (B) mice. Hematoxylin and eosin staining of kidneys from WT (E) and GC-G$^{-/-}$ (F) mice obtained after renal I/R. A significant number of infiltrating PMN (yellow arrows) accumulated in WT mice (E), with a fewer number in the GC-G$^{-/-}$ kidney (F). (G) MPO activity in renal tissue samples obtained from WT and GC-G$^{-/-}$ mice after I/R. MPO activity is expressed as ΔOD_{460}/min per mg protein, which was decreased significantly in the kidney of GC-G$^{-/-}$ mice as compared with WT mice. *$P < 0.01$, WT *versus* GC-G$^{-/-}$. Figures are representative of three experiments performed on different days ($n = 5$ mice in each group). Bar = 50 μm.

casts, and loss of brush border, after I/R injury (Figure 4C). In contrast, in GC-G$^{-/-}$ mice under I/R injury, most of the tubular structure and integrity were maintained, and histologic damage was milder as compared with WT mice (Figure 4D).

Decreased Number of Polymorphonuclear Cells and Renal Myeloperoxidase Activity in GC-G$^{-/-}$ Mice

One of the critical events of reperfusion injury is the accumulation of polymorphonuclear cells (PMN) within the postischemic renal tissue.[4] When examined under high magnification, the renal outer medulla of WT mice under renal I/R showed a large number of infiltrating PMN in peritubular capillaries (Figure 4E, yellow arrows), but kidneys of GC-G$^{-/-}$ mice after 24 h of reperfusion showed significantly fewer PMN (Figure 4F). In line with the increased PMN infiltration number, myeloperoxidase (MPO) activity, an abundant constituent of neutrophils, was increased in the kidneys of WT mice subjected to I/R but was much lower in level in GC-G$^{-/-}$ mice (Figure 4G). Taken together, these results suggest that GC-G$^{-/-}$ mice were protected against the renal I/R injury and that GC-G could have a role in the development of ischemic renal failure in this experimental model.

Attenuation of Tubular Epithelial Cell Apoptosis during Renal I/R in GC-G$^{-/-}$ Mice

Because apoptosis has been suggested in the pathogenesis of renal I/R,[4,15,16] we further evaluated the role of GC-G in tubular epithelial cell apoptosis after renal ischemia by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay. TUNEL-positive cells were virtually undetectable in the kidneys of sham-operated WT and GC-G$^{-/-}$ mice (0.001 ± 0.0001 *versus* 0.0019 ± 0.0002%; NS; Figure 5, A and B). After I/R injury, significant numbers of TUNEL-positive cells were observed in WT mice (Figure 5C). In agreement with previous reports,[4,15,17] TUNEL-positive cells were mainly identified in renal proximal and distal tubules. Importantly, substantially fewer TUNEL-positive cells were identified in GC-G$^{-/-}$ mice after I/R insult (Figure 5D). The percentage of TUNEL-positive cells in the GC-G$^{-/-}$ mice was 78.1% lower than that in WT mice (0.06 ± 0.01 *versus* 0.274 ± 0.015%, respectively; $P < 0.01$; Figure 5E).

Caspases, a family of cysteine proteases acting as the molecular executioners for programmed cell death, are induced and activated under renal I/R conditions.[15,16,18] In addition, two main pathways of death receptor–dependent and mitochondria-mediated apoptosis have been implicated in I/R-induced apoptosis, and both pathways activate downstream effector caspase-3.[4] We thus further examined caspase-3 activation in kidneys of WT and GC-G$^{-/-}$ mice by Western blot analysis using an antibody that specifically reacted with the active, cleaved forms of caspase-3. Consistent with TUNEL assay results, GC-G$^{-/-}$ mice under I/R injury exhibited much less caspase-3 activation, as reflected by the total amount of cleaved caspase-3 (17/19 kD) products, as compared with WT mice (Figure 5F). These results suggest that GC-G is involved in tubular cell apoptosis during renal I/R.

Suppression of P-Selectin and IL-6 Expression in GC-G$^{-/-}$ Mice

I/R-induced local expression of adhesion molecules and proinflammatory cytokines plays a central role in amplifying ischemic tissue injury.[19–24] For example, expression of adhesion molecule P-selectin and proinflammatory cytokine IL-6 is induced in ischemic renal tissues, and its inhibition protects against I/R-induced renal failure in mice.[22–24] We thus compared the expression of P-selectin and IL-6 in kidneys of WT and GC-G$^{-/-}$ mice subjected to renal I/R. GC-G$^{-/-}$ mice showed marked reduction in P-selectin and IL-6 mRNA level as compared with WT controls (Figure 6A). Likewise, the protein level of P-selectin was reduced in renal tissues of GC-G$^{-/-}$ mice as compared with that of WT mice, as confirmed by immunohistochemistry (data not shown). Thus, in response to renal I/R, mice lacking functional GC-G show reduced expression of adhesion molecules and inflammatory cytokines.

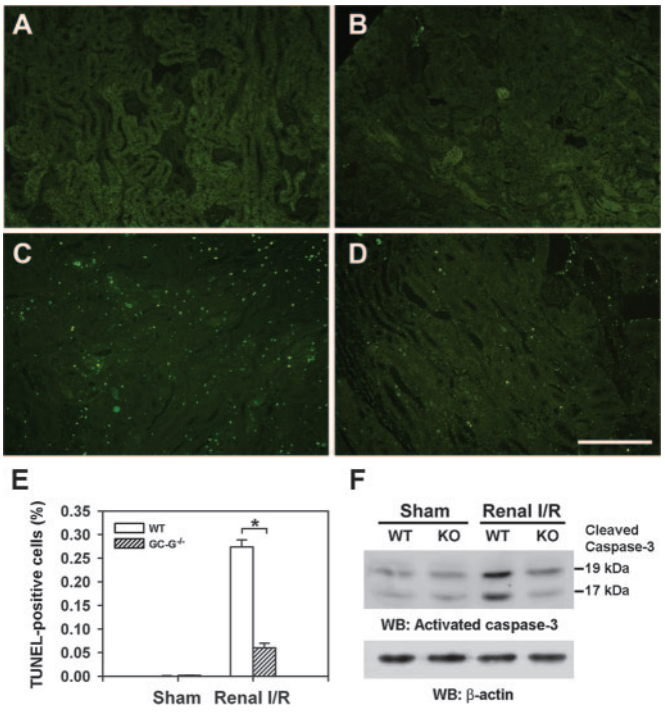

Figure 5. Effect of I/R on renal tubular epithelial cell apoptosis in WT and GC-G$^{-/-}$ mice. WT (A and C) and GC-G$^{-/-}$ (B and D) mice underwent a sham operation (A and B) or 45 min of renal ischemia followed by 24 h of reperfusion (C and D). TUNEL staining of representative kidney sections from each experimental group is shown. Bar = 200 μm. (E) Quantitative analysis of TUNEL-positive renal epithelial nuclei per total nuclei in WT and GC-G$^{-/-}$ mice subjected to sham operation or I/R injury ($n = 5$ mice per group). (F) Active caspase-3 protein expression. Renal lysates derived from WT or GC-G$^{-/-}$ mice subjected to sham operation or renal I/R were probed with specific antibody against the cleaved, active form of caspase-3. Experiments were performed twice with similar results.

Reduced NF-κB Activity in GC-G$^{-/-}$ Mice

NF-κB as a transcription factor plays a critical role in inflammatory responses by regulating the expression of chemokines, growth factors, immunoreceptors, cytokines, and cell adhesion molecules.[25] Because the P-selectin and IL-6 genes contain

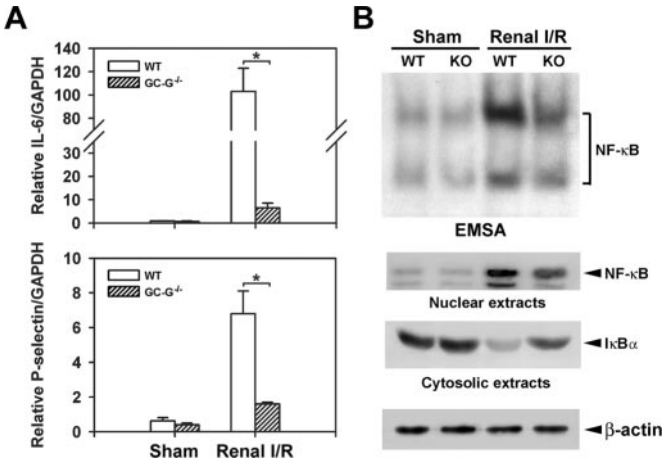

Figure 6. Effect of I/R on the expression of IL-6 and P-selectin or NF-κB DNA-binding activity in WT and GC-G$^{-/-}$ renal tissue. (A) Quantitative RT-PCR analysis of IL-6 and P-selectin level from renal cDNA derived from mice subjected to sham surgery or renal I/R injury. Expression levels were normalized to that of GAPDH. Experiments were performed twice in duplicate with similar results (n = 5 mice in each group). (B) NF-κB nuclear translocation and activation and IκBα degradation. (Top) EMSA results of NF-κB in renal nuclear extracts of WT and GC-G$^{-/-}$ mice subjected to sham operation or I/R injury. (Bottom) Nuclear or cytosolic extracts were probed with NF-κB or IκBα antibody, respectively, to quantify protein levels in these subcellular compartments.

functional NF-κB–binding sites essential for their induction in response to inflammatory stimuli,[26,27] we assessed NF-κB activation by electrophoretic mobility shift assay (EMSA). Renal I/R injury caused increased NF-κB DNA binding activity in the nuclear extracts from WT kidneys, which was markedly suppressed in GC-G$^{-/-}$ mice (Figure 6B, top). The identity of the gel shift band was verified by competition and super-shift analysis with a specific antibody against the NF-κB p65 subunit (data not shown). Furthermore, the increased NF-κB activation in WT renal tissue was accompanied by a concomitant increase in the total amount of NF-κB protein present in the nuclei as well as a simultaneous decrease in cytosolic IκBα, an inhibitory protein that prevents translocation of NF-κB dimers into the nucleus (Figure 6B, middle and bottom). Together, these results suggest that GC-G might play a distinctive role in modulating renal I/R injury through activation of NF-κB.

Validation of the Pathologic Role of GC-G by the Gain- and Loss-of-Functions Methods

As a complementary approach to our knockout mouse model, we used the gain- and loss-of-function methods by overexpressing the WT or dominant negative (DN) mutant of GC-G in cultured renal tubular cells to address further the proapoptotic/proinflammatory role of GC-G. We first generated a DN mutant of GC-G by introducing an alanine (D950A) at a well-conserved Asp-950 critical for the guanylyl cyclase activity (Figure 7A). The GC-G-D950A mutation resulted in an inac-

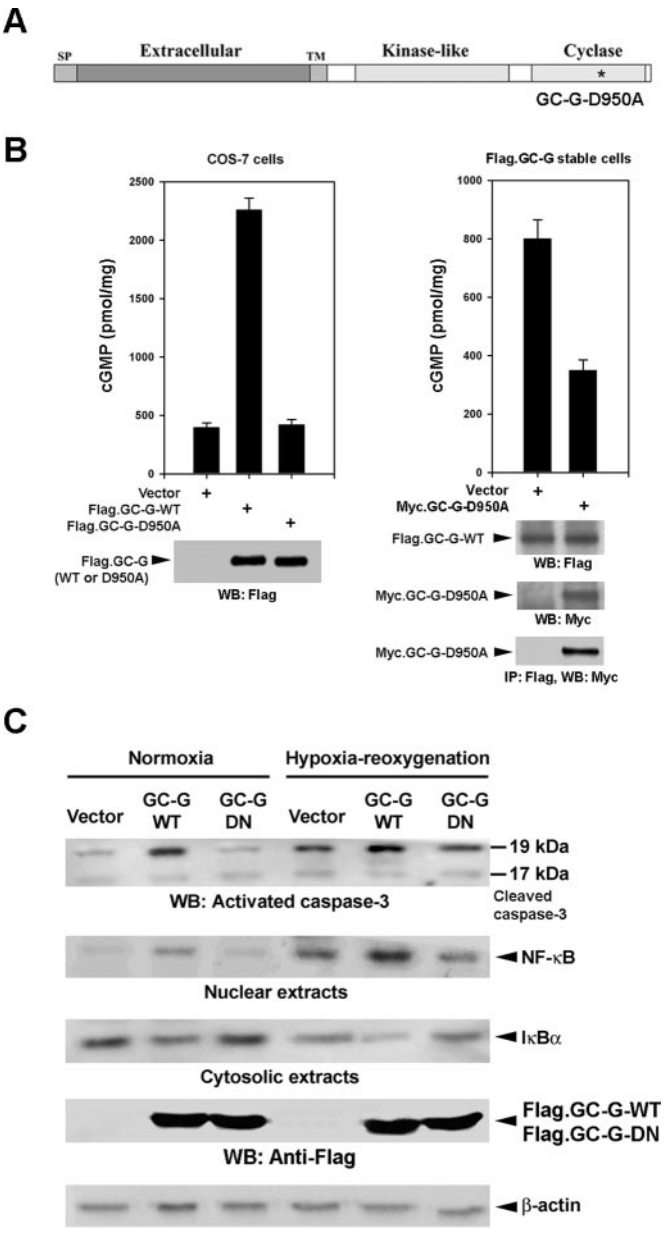

Figure 7. Generation of DN mutant and its effect on the apoptotic/inflammatory responses of renal proximal tubule epithelial cells by hypoxia-reoxygenation. (A) Schematic illustration of a point mutation (D950A) resided within the cyclase domain of GC-G. (B) DN effect of the GC-G-D950A mutant protein. No additional cGMP production could be detected in COS-7 cells expressing the GC-G-D950A construct (left). Importantly, coexpression of GC-G-D950A and GC-G-WT proteins resulted in a formation of dimers detected by immunoprecipitation and a >60% decrease in overall cGMP production (right). (C) Effect of GC-G WT and DN mutant on the apoptotic/inflammatory signaling. The renal proximal tubule epithelial (NRK-52E) cells were transfected with the expression plasmid encoding GC-G-WT or GC-G-DN mutant protein for 24 h before being subjected to normoxia or hypoxia for 24 h and reoxygenation for 18 h. Cells lysates were probes with specific antibody against the cleaved, active form of caspase-3, NF-κB (nuclear extracts), and IκBα (cytosolic extracts). The Flag-tagged GC-G protein expression of WT

tive cyclase that forms dimers with GC-G-WT. Most important, transfection of GC-G-D950A into cells stably expressing the GC-G-WT protein led to a >60% decrease in overall cGMP production, thus acting as a DN protein (Figure 7B). We then performed a series of experiments in which cultured proximal tubule cells (NRK-52E) were transfected with GC-G-WT or GC-G-DN mutant construct, respectively. Under normoxia or hypoxia-reoxygenation, the apoptotic (caspase-3 activation) and inflammatory responses (NF-κB nuclear translocation and cytosolic IκB degradation) were markedly enhanced in cells overexpressing GC-G-WT protein but were attenuated by GC-G-DN mutant overexpression (Figure 7C). Together, these findings strongly support a proapoptotic/proinflammatory role of GC-G.

To address further the pathologic involvement of GC-G/cGMP during renal I/R injury, we directly transferred the GC-G expression plasmid into the kidney by an ultrasound-liposome–mediated system. After I/R injury, the survival, total renal cGMP level, and GC-G protein expression from groups of mice that were transfected with the green fluorescence protein (GFP) or the GC-G expression plasmid were determined. As shown in Figure 8, GC-G gene transfer resulted in a significant increase in GC-G protein expression, total renal cGMP, and blood urea nitrogen concentrations, and this was associated with a dramatic reduction in survival after renal I/R injury, compared with the GFP control mice. Together, these results suggest that GC-G/cGMP may play a pathologic role during renal I/R injury.

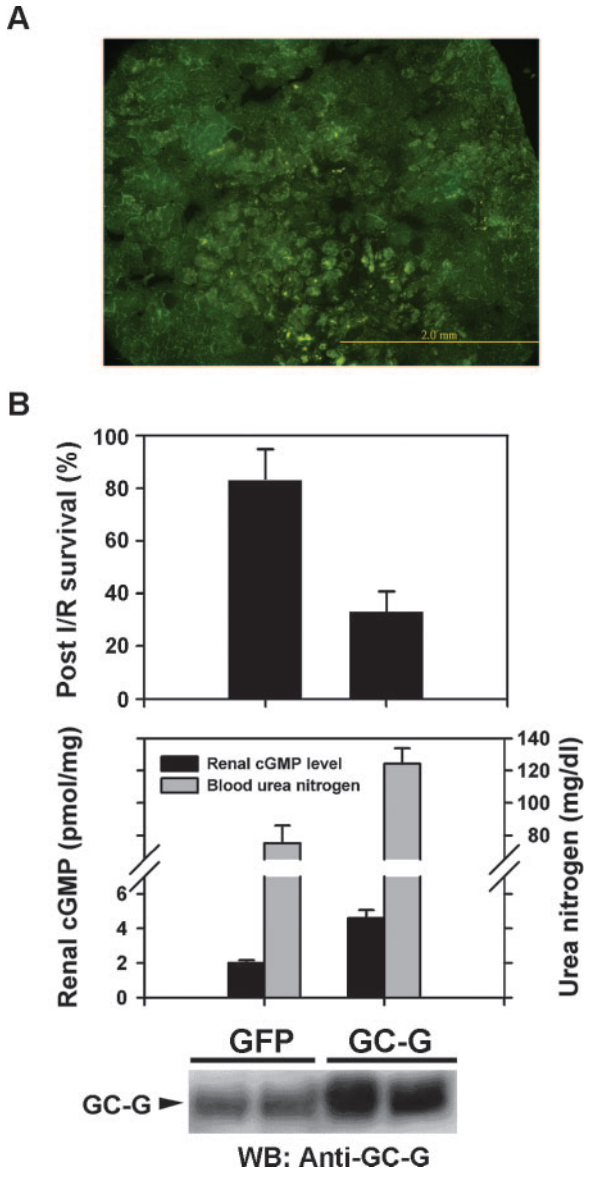

Figure 8. Effect of ultrasound-mediated gene transfer of GC-G on renal I/R injury. Groups of male WT mice were subjected to ultrasound-mediated gene transfer of GFP or GC-G, then followed by renal I/R injury. (A) Kidney sections were examined under fluorescence microscope for GFP expression. Ultrasound-liposome–mediated system strongly directed the GFP expression in the cortex and medullary tissues. (B) GC-G transgene expression enhances I/R injury. GC-G gene transfer resulted in a decreased survival by 3 d after surgery (top, with a concomitant increase in GC-G protein expression, overall renal cGMP. and blood urea nitrogen levels, compared with the GFP control (middle and bottom).

DISCUSSION

GC-G is the most recent and probably the last member of the receptor GC family.[11,28] Although the full-length cDNA for mouse GC-G (*Gucy2g*) was originally isolated from the testis, GC-G mRNA was also found in the kidney by a more sensitive RT-PCR method (Figure 1), as compared with findings from Northern blot analysis.[11] This finding is further supported by immunohistochemistry, validating GC-G protein expression in renal tubular epithelial cells (Figure 2, E and G, and Supplemental Figure 1).

We investigated for the first time the pathologic role of GC-G during renal I/R injury by using a new GC-G gene-ablation mouse. GC-G-null mice not only lacked the expression of functional GC-G (Figure 2) but also showed a marked reduction in the total renal cGMP concentration by approximately 50% as compared with WT mice at baseline conditions (Figure 3A); however, we found there is no difference in urinary cGMP excretion between WT and GC-G$^{-/-}$ male mice (Supplemental Figure 2). Furthermore, deletion of GC-G was not compensated for by an upregulation of other GC isoforms, including GC-A, -B, and -C or soluble α1, α2, and β1 subunits (data not shown). Thus, cGMP formed in the GC-G-null kidney represents the residual nondeleted GC isoforms. It is interesting that renal cGMP levels measured at 24 h after ischemic injury were not significantly increased in WT mice (Figure

and DN mutant was confirmed by anti-Flag Western blot analysis, and overall cGMP production was in agreement with those seen in Supplemental Figure 3 (vector, GC-G-WT, and GC-G-DN: 75 ± 6, 311 ± 19, and 79 ± 7 pmol/mg).

3A), which suggests that a (patho)physiologic effective cGMP increase in level is below the detection limit, as was proposed previously.[29,30] In addition, the cGMP level may dynamically change during the activation process, and local subcellular effects caused by compartmentalization of GC-G may not be detected by measurement of whole-organ content. Alternatively, the disconnection between renal cGMP level and renal I/R injury seen in Figures 3 and 8 suggests that a cGMP-independent function of GC-G may be important for renal I/R pathophysiology. This possibility is supported by recent emerging roles of the kinase-like domain in regulating a variety of signaling/cellular processes[31]; however, further studies are important to clarify whether the cGMP-independent signaling through the kinase-like domain of GC-G is required during renal I/R injury.

In this study, we showed that renal dysfunction after 24 h of reperfusion, as reflected by elevated serum creatinine and urea levels, was markedly prevented in GC-G-null mice as compared with WT mice in response to I/R injury (Figure 3B). This protection was accompanied by a corresponding reduction in renal tubular cell apoptosis and inflammatory responses, including decreased PMN infiltration, renal expression of IL-6 and P-selectin, and activation of NF-κB. Our findings are in line with those from recent studies showing that ANP induced apoptosis in cultured cardiac myocytes and endothelial cells *via* GC-A–mediated elevation of cGMP,[32,33] and inhibition/blockade of GC-A by GC-A$^{-/-}$ mice or a nonpeptide GC-A antagonist alleviated myocardial I/R injury through suppression of NF-κB–mediated P-selectin induction.[34]

Proinflammatory cytokine IL-6 was previously found to be produced by macrophages infiltrating adjacent to ischemic vascular bundles of the outer medulla.[22,23] A local increase in IL-6 was shown to promote PMN accumulation within the ischemic renal tissue by directly amplifying a substantial production of the proinflammatory cytokines IL-1β and TNF-α and/or an upregulation of the adhesion molecules intercellular adhesion molecule-1 and P-selectin on the endothelium.[22,23] Because we failed to localize GC-G expression in any of the infiltrating PMN or in the endothelial layer of peritubular capillaries and interlobular arteries (Figure 2, E and F), the effects of GC-G on I/R-induced inflammation might be mediated through an indirect mechanism in these cell types. On the contrary, double immunostaining revealed that with I/R injury, GC-G and activated caspase-3 were co-localized to the same tubular cells (data not shown), which suggests that GC-G–mediated programmed cell death occurs primarily in renal tubular epithelial cells.

We also found that the protection against renal apoptosis and inflammation in GC-G$^{-/-}$ mice could be seen as early as 6 h after reperfusion (data not shown). Indeed, a recent report demonstrated that renal apoptosis starting as early as 2 h after reperfusion is a critical event that could initiate renal I/R-induced inflammation and subsequent renal tissue injury.[15] Thus, GC-G activation might represent an early injury signal event, probably upstream of caspase-3 activation, after reperfusion. Because two major pathways of death receptor–dependent and mitochondria-mediated apoptosis have been implicated in renal I/R-induced injury and these two pathways culminate in the activation of caspase-3, whether GC-G participates in either or both apoptotic signal pathways remains to be investigated.

Because inhibition of apoptosis, IL-6, or P-selectin has been demonstrated to protect against I/R-induced organ failure in the kidney,[15,22–24] heart,[35,36] brain,[37,38] intestine,[39] and liver,[40] GC-G may also be involved in I/R-induced abnormalities in other organs, such as acute myocardial infarction in the heart or stroke in the brain, which warrants further investigation.

Although the exact mechanism by which GC-G is regulated under renal I/R remains undefined, a simple regulatory mechanism could be that renal I/R induces the release of endogenous ligand(s) and modulates GC-G activity to transmit the injury signal leading to apoptosis and inflammation. This paradigm has been previously demonstrated for GC-A signaling *via* a rapid activation/release of cardiac natriuretic peptides (ANP and B-type natriuretic peptide) after acute myocardial infarction.[41,42] Regardless, the molecular mechanisms underlying the regulation of GC-G under I/R require further elucidation.

In summary, our results point to the important role of GC-G as an early signaling molecule of injury in kidney I/R. Moreover, the GC-G-knockout mouse serves as an excellent model to define further the molecular mechanisms by which GC-G signals apoptosis and inflammation in response to I/R injury. Pharmacologic modulation of renal cGMP production or its signal transduction may provide a viable strategy to prevent acute cell damage and improve the outcome of ischemic injury to the kidney.

CONCISE METHODS

Generation of GC-G$^{-/-}$ Mice

The GC-G gene (*Gucy2g*) from the mouse strain 129/SvJ was used to construct a targeting vector. A neomycin-resistance gene cassette replaced exon 6, which codes for a portion of the extracellular domain, leaving a coding sequence that could yield only a truncated protein (see Figure 2). Standard procedures were used to produce the GC-G$^{-/-}$ embryonic stem (ES) cells. Two independent targeted ES cell clones (197 and 385) were injected into C57BL/6 blastocysts. ES cell–derived progeny were screened for the presence of the targeted allele by Southern blot (Figure 2) or PCR analysis. The targeted allele was backcrossed with C57BL/6 mice for at least five generations before I/R experiment, and comparisons were made among WT littermates. Because the two knockout lines displayed similar phenotypes, we present only the data derived from the 197 line. Primers used for the genotyping analysis were as follows: WT allele (521 bp) F4, 5′-CAG GGA TGC TTA GAG GTG AGT AG-3′, and R4, 5′-ATA ATG AAG AAA GGG AAG GAA GAG; mutated allele (738 bp) F5, 5′-GTG AGA CGT GCT ACT TCC ATT TG-3′, and R5, 5′-GAC TTC AGG CCT CCA TTA CTA TCA-3′.

Table 1. Primers used for RT-PCR and real-time quantitative RT-PCR analyses

Gene	Forward	Reverse	Size (bp)
GC-G	F1: ACCTGGGCTGGGAACATATC	R1: GGAAACCGTCATCTCCAATG	426
GC-G	F2: GGGGAAAAGGCACGTGGATTACTC	R2: TCCATGTGCTGCCGTGCTTGAACT	929
GC-G	F3: TCAGCATGCCGACGAGATTG	R3: CTGCCCCCGAAGGACACC	471
GC-G	Q-F1: TTGCGTATGACATAGTCAATGGC	Q-R1: TCAGGTTGCCGTGAGACCTC	69
GAPDH	F1: ATCATCCCTGCATCCACTGGTGCTG	R1: TGATGGCATTCAAGAGAGTAGGGAG	574
GAPDH	Q-F1: GGCAAATTCAACGGCACAGT	Q-R1: AAGATGGTGATGGGCTTCCC	71

Animal Model for Renal I/R

Our investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996). All surgical procedures were performed according to the protocols approved by the Institutional Animal Care and Utilization Committee, Academia Sinica. Male mice, ages approximately 8 to 10 wk, were subjected to bilateral renal artery occlusion (45 min) and reperfusion (24 h) as described previously.[23] Sham control animals underwent the identical surgery except for pedicle clamping.

Measurement of Biochemical Parameters, RT-PCR, and Quantitative RT-PCR

For monitoring of renal (glomerular) function, levels of serum creatine and blood urea nitrogen were measured by the core laboratory at Academia Sinica. Real-time quantitative PCR analysis was performed twice in duplicate by use of the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City). Normalization involved mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels as controls in parallel reactions. The relative expression ratio of mouse GC-G transcript to GAPDH transcript was calculated as described previously.[43] Primers used are listed in Table 1.

Histologic Evaluation, Immunohistochemistry, TUNEL Assay, and Renal MPO Activity

Mice were killed and perfused with PBS followed by 4% paraformaldehyde. The kidneys were fixed in 10% formalin, then embedded in paraffin. Sections were cut and stained with hematoxylin and eosin. Histologic changes, including tubular necrosis, tubular dilation, cast formations, and loss of brush border, were examined by pathologists in a blinded manner. Micrographs of representative fields were recorded (Figure 4). Immunolocalization for mouse GC-G with a polyclonal specific antibody in renal sections was as described previously.[44] Apoptosis in renal tissues was identified by TUNEL assay with an *in situ* Cell Death Detection kit (Roche Applied Science, Indianapolis, IN) following the manufacturer's instructions. Five fields per section and two sections per kidney were examined in each experimental group ($n = 5$ mice in each group). MPO, indicating neutrophil infiltration into tissue, was measured as described previously.[45]

EMSA and Western Blot Analysis of Nuclear NF-κB and IκBα

DNA probe containing a consensus NF-κB enhancer element (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). EMSA and Western blot analysis of nuclear NF-κB or IκBα was as described previously.[34]

Statistical Analyses

Data are expressed as means ± SEM, and statistical analysis involved *t* test. $P < 0.05$ was considered statistically significant.

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DISCLOSURES

None

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