Recovery from Glycerol-Induced Acute Kidney Injury Is Accelerated by Suramin

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ABSTRACT

Acute kidney injury (AKI) is a common and potentially life-threatening complication after ischemia/reperfusion and exposure to nephrotoxic agents. In this study, we examined the efficacy and mechanism(s) of suramin in promoting recovery from glycerol-induced AKI, a model of rhabdomyolysis-induced AKI. After intramuscular glycerol injection (10 ml of 50% glycerol per kilogram) into male Sprague-Dawley rats, serum creatinine maximally increased at 24 to 72 h and then decreased at 120 h. Creatinine clearance (CrCl) decreased 75% at 24 to 72 h and increased at 120 h. Suramin (1 mg/kg i.v.) administered 24 h after glycerol accelerated recovery of renal function as demonstrated by increased CrCl, decreased renal kidney injury molecule-1, and improved histopathology 72 h after glycerol injection. Suramin treatment decreased interleukin-1 β (IL-1 β) mRNA, transforming growth factor- β_1 (TGF- β_1), phospho-p65

of nuclear factor- κ B (NF- κ B), and cleaved caspase-3 at 48 h compared with glycerol alone. Suramin treatment also decreased glycerol-induced activation of intracellular adhesion molecule-1 (ICAM-1) and leukocyte infiltration at 72 h. Urinary/renal neutrophil gelatinase-associated lipocalin 2 (NGAL) levels, hemeoxygenase-1 expression, and renal cell proliferation were increased by suramin compared with glycerol alone at 72 h. Mechanistically, suramin decreases early glycerol-induced pro-inflammatory (IL-1 β and NF- κ B) and growth inhibitory (TGF- β_1) mediators, resulting in the prevention of late downstream inflammatory effects (ICAM-1 and leukocyte infiltration) and increasing compensatory nephrogenic repair. These results support the hypothesis that delayed administration of suramin is effective in abrogating apoptosis, attenuating inflammation, and enhancing nephrogenic repair after glycerol-induced AKI.

Introduction

Acute kidney injury (AKI) is a critical clinical problem with a high mortality rate and can occur after an acute injury or as a silent event, being identified only after its occurrence by the onset of progressive azotemia (Schrier et al., 2004). Rhabdomyolysis-induced AKI, also termed “crush” kidney injury, develops after skeletal muscle trauma related to physical,

thermal, ischemic, infective, metabolic, or toxic causes, releasing toxic doses of myoglobin and other intracellular proteins into the circulation (Vanholder et al., 2000; Huerta-Alardín et al., 2005; Bagley et al., 2007). Approximately 10 to 50% of patients suffering from rhabdomyolysis develop some degree of AKI (Ward, 1988), and although interventions have improved, the mortality rate may still be as high as 8% (Polderman, 2004; Huerta-Alardín et al., 2005; Bagley et al., 2007). Animal models of glycerol-induced AKI are being used currently to understand the clinical syndrome and study the mechanisms of AKI in general (Nath et al., 1992).

Although the pathogenesis of glycerol-induced AKI is complex and incompletely understood (Vanholder et al., 2000), apoptosis (Homsí et al., 2010; Kim et al., 2010; Wang et al., 2011), inflammation (Homsí et al., 2006), and oxidative stress (Rosenberger et al., 2008; Kim et al., 2010; Wei et al., 2011) have been implicated. Recent studies have demonstrated that timely prophylactic and/or early therapeutic interventions (either pre- or coadministration) ameliorated glycerol-induced AKI (Homsí et al., 2010; Kim et al., 2010; Wang et al., 2011; Wei

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ABBREVIATIONS: AKI, acute kidney injury; CrCl, creatinine clearance; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HO-1, hemeoxygenase-1; IL, interleukin; ICAM-1, intracellular adhesion molecule-1; I/R, ischemia/reperfusion; KIM-1, kidney injury molecule-1; MCP-1, monocyte chemoattractant protein-1; NF- κ B, nuclear factor κ B; NGAL, neutrophil gelatinase-associated lipocalin 2; OSOM, outer stripe of the outer medulla; PCNA, proliferating cell nuclear antigen; TGF- β_1 , transforming growth factor- β_1 ; TNF- α , tumor necrosis factor- α .

et al., 2011). However, no reports have documented the efficacy of the delayed intervention of a therapeutic agent when the renal dysfunction is already well established.

Recently, it was reported that the delayed administration of suramin, a polysulfonated naphthylurea used in humans to treat trypanosomiasis, accelerates recovery from renal dysfunction induced by ischemia/reperfusion (I/R) injury, renal fibrosis, and obstructive nephropathies in mice (Zhuang and Schnellmann, 2005; Zhuang et al., 2009; Liu et al., 2011a,b). The mechanisms by which suramin accelerates recovery from renal dysfunction in these models remain incompletely understood but may be associated with the inhibition of apoptosis, suppression of inflammatory cytokine production, and promotion of epithelial cell proliferation. However, it is unknown whether the delayed administration of suramin is beneficial in other forms of AKI (i.e., glycerol-induced AKI).

In this study, we examined the effects of the delayed administration of suramin on renal function and pathology in a rat model of glycerol-induced AKI. We examined the more clinically relevant question of whether suramin promotes recovery from glycerol-induced renal dysfunction by administering suramin 24 h after AKI, when serum creatinine levels were maximal. We also uncovered some novel mechanisms underlying glycerol-induced AKI and the effects of suramin on predictive biomarkers of AKI, signaling involved in leukocyte infiltration and inflammation, oxidative stress, and tubular cell proliferation.

Materials and Methods

Animals and Treatment. Male Sprague-Dawley rats (Harlan, Indianapolis, IN), 8 weeks of age (180–200 g), were housed in temperature-controlled conditions under a light/dark photocycle with food and water supplied ad libitum. Rats were dehydrated for 16 h before glycerol injection. Rats were divided randomly into three groups. The first group (untreated, $n = 5$) was not injected with any treatment; the second and third groups of rats ($n = 20$) were given intramuscular injections of 50% glycerol (10 ml/kg) in their hind limbs. The first and second groups received sterile water, and the third group received suramin intravenously (1 mg/kg, dissolved in sterile water) 24 h after initial glycerol injection. Blood, urine, and kidney samples were collected at 24, 48, 72, and 120 h. All of the animal and treatment protocols were in compliance with the *Guide for the Care and Use of Laboratory Animals* as adopted and promulgated by the U.S. National Institutes of Health and were approved by our Institutional Animal Care and Use Committee (Institute of Laboratory Animal Resources, 1996).

Chemicals. Unless stated otherwise, all of the chemicals and biochemicals were purchased from Sigma-Aldrich (St. Louis, MO). Goat polyclonal anti-kidney injury molecule-1 (KIM-1) antibody was obtained from R&D Systems (Minneapolis, MN). Rabbit polyclonal anti-neutrophil gelatinase-associated lipocalin 2 (NGAL) and mouse monoclonal anti-transforming growth factor- β_1 (TGF- β_1) antibodies were purchased from Abcam Inc. (Cambridge, MA), rabbit polyclonal anti-caspase-3 was obtained from Enzo Life Sciences, Inc. (Farmingdale, NY), rabbit polyclonal anti-intracellular adhesion molecule-1 (ICAM-1) and rabbit polyclonal anti-proliferating cell nuclear antigen (PCNA) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), rabbit monoclonal anti-phospho-p65 and rabbit monoclonal anti-hemeoxygenase-1 (HO-1) were obtained from Cell Signaling Technology (Danvers, MA), and the loading control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was obtained from Fitzgerald International, Inc. (Acton, MA). Anti-goat secondary antibody conjugated with horseradish peroxidase was purchased

from Millipore Corporation (Billerica, MA), and anti-rabbit and anti-mouse secondary antibodies conjugated with horseradish peroxidase were obtained from Thermo Fisher Scientific (Waltham, MA).

Assessment of Renal Function. Rats were placed in metabolic cages (Tecniplast, Philadelphia, PA) for 24-h urine collections. Renal function was monitored by measuring 24-h urine volume, serum, and urine creatinine using a creatinine assay kit (BioAssay Systems, Hayward, CA) as per the manufacturer's instructions, and creatinine clearance (CrCl) was calculated. Urinary NGAL was measured using an enzyme-linked immunosorbent assay (ALPCO Immunoassays, Salem, NH) as per the manufacturer's protocol.

Histology and Immunohistochemistry. Renal tissues were fixed in 4.5% buffered formalin, dehydrated, and embedded in paraffin. For general histology, sections were stained with hematoxylin and eosin. For the immunohistochemistry of KIM-1, NGAL, ICAM-1, and PCNA, the manufacturer's protocols were followed.

Assessment of Renal Inflammation. Renal inflammation was assessed by measuring leukocyte (neutrophils and monocytes) infiltration using the naphthol AS-D chloroacetate esterase kit (Sigma-Aldrich), and immunohistochemistry was carried out as per the manufacturer's protocol. To quantify leukocyte infiltration, a total of 25 fields (original magnification, 20 $\times$) in the outer stripe of the outer medulla (OSOM) were examined and expressed as the total number of leukocytes in all of the fields.

Quantitative Polymerase Chain Reaction. Rat kidney cortex samples were homogenized with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The mRNA was subjected to reverse transcription to cDNA in the presence of oligo(dT) primers using the RevertAid first strand cDNA synthesis kit from Fermentas Life Sciences (Glen Burnie, MD). Polymerase chain reaction was performed using commercially available primers (Integrated DNA Technologies, Inc., Coralville, IA) for tumor necrosis factor- α (TNF- α), interleukin (IL)-6, IL-1 β , monocyte chemoattractant protein-1 (MCP-1), and tubulin. Tubulin was used to normalize mRNA expression.

Immunoblot Analysis. Rat kidney cortex tissue was homogenized in five volumes of protein lysis buffer [1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 2 mM sodium orthovanadate, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM HEPES (pH 7.6), 1 μ g/ml leupeptin, and 1 μ g/ml aprotinin] using a Polytron homogenizer. The homogenate was stored on ice for 10 min and then centrifuged at 7500g for 5 min at 4°C. The supernatant was collected, and the protein concentration was determined using a bicinchoninic acid kit (Sigma-Aldrich) with bovine serum albumin as the standard. Proteins (50–75 μ g) were separated on 4 to 20% gradient SDS polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked either in 5% dried milk or bovine serum albumin in 0.1% Tween 20 in 1 $\times$ Tris-buffered saline and incubated with 1:1000 dilutions of anti-KIM-1, anti-NGAL, anti-TGF- β_1 , anti-caspase-3, anti-phospho-p65, anti-HO-1, and anti-GAPDH and 1:100 dilutions of anti-ICAM-1 and anti-PCNA overnight at 4°C. After the incubations for 2 h at room temperature with secondary antibodies (1:2000) conjugated with horseradish peroxidase, membranes were detected by chemiluminescence.

Data and Statistical Analysis. Data are expressed as means $\pm$ S.E.M. ($n = 4$ –5) for all of the experiments. Multiple comparisons of normally distributed data were analyzed by one-way analysis of variance, as appropriate, and group means were compared using the Student-Newman-Keuls post hoc test. Single comparisons were analyzed by Student's t test where appropriate. The criterion for statistical differences was $p \leq 0.05$ for all of the comparisons.

Results

Suramin Improves Glycerol-Induced Renal Dysfunction and Damage. Serum creatinine was maximal and sustained from 24 to 72 h and decreased to slightly higher than

Fig. 1. Effect of delayed administration of suramin on renal dysfunction in rats subjected to glycerol-induced AKI. A, serum creatinine in groups of male Sprague-Dawley rats ($n = 5$) that were given intramuscular injections of 10 ml of 50% glycerol per kilogram, with or without 1 mg of suramin per kilogram administered intravenously at 24 h after glycerol treatment. One more group did not receive any treatment. Data are expressed as mean $\pm$ S.E. ($n = 5$). *, statistically significant from respective 0 h controls. #, statistically significant from rats receiving glycerol treatment alone at the corresponding time point ($p \leq 0.05$). B, urine creatinine in groups of male Sprague-Dawley rats ($n = 5$) that were injected intramuscularly with 10 ml of 50% glycerol per kilogram, with or without 1 mg of suramin per kilogram administered intravenously at 24 h after glycerol treatment. One more group did not receive any treatment. Data are expressed as mean $\pm$ S.E. ($n = 5$). *, statistically significant from respective 0 h controls ($p \leq 0.05$). C, urine volume in groups of male Sprague-Dawley rats ($n = 5$) that were given intramuscular injections of 10 ml of 50% glycerol per kilogram, with or without 1 mg of suramin per kilogram administered intravenously at 24 h after glycerol treatment. One more group did not receive any treatment. Data are expressed as mean $\pm$ S.E. ($n = 5$). *, statistically significant from respective 0 h controls. #, statistically significant from rats receiving glycerol treatment alone at the corresponding time point ($p \leq 0.05$). D, CrCl in groups of male Sprague-Dawley rats ($n = 5$) that were injected intramuscularly with 10 ml of 50% glycerol per kilogram, with or without 1 mg of suramin per kilogram administered intravenously at 24 h after glycerol treatment. One more group did not receive any treatment. Data are expressed as mean $\pm$ S.E. ($n = 5$). *, statistically significant from respective 0 h controls. #, statistically significant from rats receiving glycerol treatment alone at the corresponding time point ($p \leq 0.05$).

control levels at 120 h after glycerol treatment (Fig. 1A). Urine creatinine decreased similarly after glycerol treatment in the presence and absence of suramin for 24 to 120 h compared with those of untreated controls (Fig. 1B). In addition, urine volume increased after glycerol treatment in the presence and absence of suramin for 48 to 120 h compared with those of untreated controls (Fig. 1C). CrCl decreased 75% and remained inhibited from 24 to 72 h and returned to control levels at 120 h after glycerol treatment alone (Fig. 1D). However, although CrCl was similar to that of glycerol treatment alone at 48 h, suramin treatment increased CrCl to control levels at 72 and 120 h (Fig. 1D).

Associated with the renal dysfunction, glycerol-treated rats exhibited extensive proximal tubular necrosis throughout the corticomedullary region characterized by eosinophilic tubules with the remnants of karyolytic nuclei (Fig. 2B) compared with the renal architecture in control animals (Fig. 2A). There was minimal to no histological appearance of repair defined by a thin layer of cuboidal re-epithelialization in the tubular lamina at 72 h. Administration of suramin at 24 h after glycerol treatment resulted in decreased pathology

and clear evidence of renal tubular repair characterized by marked renal tubule basophilia and squamous to low cuboidal re-epithelialization inside the residual tubular basal lamina (Fig. 2C).

Suramin Intervention Decreases KIM-1 Expression after Glycerol-Induced AKI. KIM-1 protein expression in the kidney was elevated 24 h after glycerol treatment alone and was sustained until 72 h (Fig. 3, A and B). With suramin treatment, renal KIM-1 levels were lower at 72 h after glycerol administration. KIM-1 expression was not detected in untreated rat kidneys by immunohistochemical staining (Fig. 3C). In glycerol-treated rat kidneys, KIM-1 staining increased and was localized mainly to the apical membranes of proximal tubular epithelial cells (arrows) along with some damaged tubules at 72 h (Fig. 3D). In contrast, glycerol + suramin rats exhibited less KIM-1 staining distributed in tubular epithelial cells, and there were fewer KIM-1-positive tubules compared with glycerol-treated rat kidneys at 72 h (Fig. 3E).

Suramin Increases Urinary and Renal NGAL Levels after Glycerol-Induced AKI. Urinary NGAL in glycerol-

A UNTREATED **B** 72 h AFTER GLYCEROL **C** 72 h AFTER GLYCEROL + SURAMIN

Fig. 2. Effect of delayed administration of suramin on renal injury in rats subjected to glycerol-induced AKI. A–C, representative photomicrographs of hematoxylin and eosin-stained kidney sections from rats that were untreated (A), treated with glycerol alone at 72 h (B), and subjected to suramin intervention after glycerol treatment at 72 h (C). *, denotes necrotic tubules. #, denotes tubules that are lined with regenerating cells. All of the fields were chosen from the OSOM. Original magnification, 200 $\times$.

treated rats was elevated compared with that in control rats, with maximal levels at 48 h after glycerol treatment, and NGAL remained elevated through 120 h (Fig. 4A). Renal protein and tissue NGAL did not increase until 48 h after glycerol treatment (Fig. 4, B–E). After suramin treatment, urinary NGAL was higher at 48 and 72 h (Fig. 4A), and renal protein and tissue NGAL staining was higher at 48 h compared with that of rats receiving glycerol treatment alone (Fig. 4, B–F).

Suramin Decreases Apoptosis after Glycerol-Induced AKI. Caspase-3-mediated apoptosis has been implicated in tubular damage after glycerol-induced AKI (Padanilam, 2003; Homsí et al., 2010; Wang et al., 2011). Suramin decreased renal cleaved caspase-3 at 48 h after glycerol treatment compared with glycerol treatment alone at the same time point (Fig. 5, A and B). However, there was no difference in cleaved caspase-3 among control and glycerol-treated rats $\pm$ suramin at 72 h (densitometric units of renal cleaved caspase-3 normalized to GAPDH for control, 0.45 ± 0.01 ; glycerol alone, 0.94 ± 0.16 ; glycerol + suramin, 0.67 ± 0.14).

Suramin Decreases Leukocyte Infiltration and Inflammation after Glycerol-Induced AKI. Leukocyte-mediated infiltration and inflammation have been suggested to be important events in AKI (Bonventre and Zuk, 2004; Homsí et al., 2006; de Jesus Soares et al., 2007). We analyzed leukocyte infiltration after glycerol-induced AKI in the corticomedullary region of kidneys of rats treated with or without suramin by staining neutrophils and monocytes with naphthol AS-D chloroacetate esterase as described previously (Zhuang et al., 2009). Increased leukocytes were observed at

72 h after glycerol treatment alone (Fig. 6, B and D) and were decreased with suramin intervention at the same time point (Fig. 6, C and D).

Suramin Attenuates ICAM-1 Expression after Glycerol-Induced AKI. Activation of renal endothelial cell ICAM-1 expression by leukocytes and their contributions to the pathophysiology of AKI, in general, and glycerol-induced tubular inflammation and necrosis have been documented previously (Liu et al., 2002; Bonventre, 2010). We did not find any differences in ICAM-1 expression among untreated and glycerol-treated rats $\pm$ suramin at 0 to 48 h (densitometric units of renal ICAM-1 normalized to GAPDH for control, 0.6 ± 0.2 ; 24 h glycerol alone, 0.8 ± 0.3 ; 48 h glycerol alone, 0.6 ± 0.2 ; 48 h glycerol + suramin, 0.4 ± 0.1). However, suramin inhibited ICAM-1 expression at 72 h after glycerol treatment compared to glycerol treatment alone (Fig. 7, A and B). ICAM-1 localization at the basement membranes of proximal convoluted tubules was blocked by suramin treatment compared to glycerol treatment alone (Fig. 7, D and E).

Suramin Decreases Proinflammatory Signaling after Glycerol-Induced AKI. The roles of innate immunity and proinflammatory mediators in the progression of renal inflammation in AKI have been established (Homsí et al., 2009; Gonçalves et al., 2010). There were no differences in the mRNA levels of renal TNF- α and MCP-1 after glycerol treatment with or without suramin over the time course (Fig. 8, A and D). However, renal IL-6 mRNA at 48 h in glycerol-treated rats with or without suramin was higher compared with that in untreated rats (Fig. 8B). Not that renal IL-1 β increased numerically, but not statistically, at 24

Fig. 3. Effect of delayed administration of suramin on KIM-1 in rats subjected to glycerol-induced AKI. A, representative Western blot showing KIM-1 protein expression in kidneys of untreated and glycerol-treated rats ($n = 4$) with or without suramin over a time course. B, densitometric analysis of renal KIM-1 protein expression in untreated, glycerol-treated, and suramin intervention after glycerol treatment groups over the time course. Data were normalized to GAPDH, which served as an internal control. Data are expressed as mean $\pm$ S.E. ($n = 4$). *, statistically significant from respective 0 h controls. #, statistically significant from rats receiving glycerol treatment alone at the corresponding time point ($p \leq 0.05$). U, untreated; G, glycerol treatment alone group; GS, glycerol + suramin treatment group. C–E, representative photomicrographs of KIM-1-stained kidney sections from rats that were untreated (C), treated with glycerol alone at 72 h (D), and subjected to suramin intervention after glycerol treatment at 72 h (E). KIM-1-positive staining was observed on the proximal tubular epithelial cells and damaged tubules (arrows). All of the fields were chosen from the OSOM. Original magnification, 200 $\times$.

and 48 h after glycerol administration, and suramin decreased IL-1 β to below control levels at 48 h (Fig. 8C).

Suramin Decreases Nuclear Factor- κ B Activation after Glycerol-Induced AKI. Phosphorylation of the p65 subunit of NF- κ B leads to the nuclear translocation of nuclear factor- κ B (NF- κ B) and transcription of several genes involved in renal inflammation (Hu et al., 2012). ICAM-1 is one of those genes that is positively regulated by NF- κ B (Zheng et al., 2006; Yang et al., 2010; Chen et al., 2011; Li et al., 2011). Phosphorylated p65 was increased at 24 and 48 h in glycerol-treated rats compared with that in untreated rats (Fig. 9, A and B), and suramin treatment after glycerol treatment decreased the phosphorylation of p65 at 48 h compared to glycerol treatment alone (Fig. 9, A and B).

Suramin Increases HO-1 and Decreases TGF- β ₁ after Glycerol-Induced AKI. HO-1 was shown to be indispensable in protecting against glycerol-induced AKI (Ishizuka et al., 1997; Nath et al., 2000; Rosenberger et al., 2008; Wei et al., 2011). Renal HO-1 protein was decreased after glycerol treatment from 24 to 72 h. However, with suramin treatment, HO-1 increased at 72 h compared to glycerol treatment alone (Fig. 10, A and B). Suramin was reported previously to inhibit fibrotic and growth inhibitory actions of TGF- β ₁ (Liu and Zhuang, 2011; Liu et al., 2011a,b). In this study, we found that suramin decreased TGF- β ₁ protein at 48 and 72 h

after glycerol treatment compared to glycerol treatment alone (Fig. 10, C and D).

Suramin Increases Tubular Cell Proliferation after Glycerol-Induced AKI. Kidneys from rats treated with glycerol and glycerol + suramin exhibited similar increases in PCNA protein expression at 24 and 48 h (Fig. 11, A and B). However, at 72 h after glycerol treatment, PCNA protein expression remained elevated in the glycerol + suramin group, whereas it decreased in the glycerol group (Fig. 11, A and B). Immunohistochemical staining revealed there was an increase in the number of PCNA-positive proximal tubular epithelial cells, and this increase was sustained until 72 h with suramin treatment compared to glycerol treatment alone in rats (Fig. 11, C–E).

Discussion

Previous studies have determined that therapeutic interventional strategies before, at the time of I/R, drug, or toxicant exposure, or within a few hours after the exposure attenuate AKI (Kelly et al., 1994; Chiao et al., 1997; Bates and Lin, 2005; Johnson et al., 2006). Recently, it was demonstrated that the delayed administration of suramin after renal dysfunction was established (24 h) accelerated the recovery of renal function (Zhuang et al., 2009; Liu et al.,

Fig. 4. Effect of delayed administration of suramin on NGAL in rats subjected to glycerol-induced AKI. A, enzyme-linked immunosorbent assay of urinary NGAL levels of untreated and glycerol-treated rats ($n = 5$) with or without suramin over a time course. B, representative Western blot showing NGAL protein expression in kidneys of untreated and glycerol-treated rats ($n = 4$) with or without suramin over a time course. C, densitometric analysis of renal NGAL protein expression in untreated, glycerol-treated, and suramin intervention after glycerol treatment groups over the time course. Data were normalized to GAPDH, which served as an internal control. Data are expressed as mean $\pm$ S.E. ($n = 4$). *, statistically significant from respective 0 h controls. #, statistically significant from rats receiving glycerol treatment alone at the corresponding time point ($p \leq 0.05$). D–F, representative photomicrographs of NGAL-stained kidney sections from rats that were untreated (D), treated with glycerol alone at 48 h (E), and subjected to suramin intervention after glycerol treatment at 48 h (F). NGAL-positive staining was observed on the proximal tubular epithelial cells (arrows). All of the fields were chosen from the OSOM. Original magnification, 200 $\times$.

Fig. 5. Effect of delayed administration of suramin on renal cleaved caspase-3 in rats subjected to glycerol-induced AKI. A, representative Western blot showing cleaved caspase-3 protein expression in kidneys of untreated and glycerol-treated rats ($n = 4$) with or without suramin over a time course. B, densitometric analysis of renal cleaved caspase-3 protein expression in untreated, glycerol-treated, and suramin intervention after glycerol treatment groups over the time course. Data were normalized to GAPDH, which served as an internal control. Data are expressed as mean $\pm$ S.E. ($n = 4$). *, statistically significant from respective 0 h controls. #, statistically significant from glycerol- and suramin-treated rats at the corresponding time point ($p \leq 0.05$).

2011a,b). This is of therapeutic importance, because most cases of AKI in the clinical setting are not identified until sometime after the insult has already occurred. Here, we

demonstrate the beneficial effects of the delayed administration (24 h) of suramin and identified novel protective mechanisms in another clinically relevant model of AKI (rhab-

Fig. 6. Effect of delayed administration of suramin on renal leukocyte infiltration in rats subjected to glycerol-induced AKI. A–C, representative photomicrographs of neutrophil and monocyte staining assessed by the formation of a stable pinkish-red complex (arrows) of free naphthol and diazonium salts after the incubation of kidney sections from rats that were untreated (A), treated with glycerol alone at 72 h (B), and subjected to suramin intervention after glycerol treatment at 72 h (C) in naphthol AS-D chloroacetate reagent. All of the fields were chosen from the OSOM. Original magnification, 200 $\times$. D, quantitative analysis of renal leukocyte infiltration assessed by the number of pink dots in a total of 25 fields in the OSOM region of kidney sections. Data are expressed as mean $\pm$ S.E. ($n = 4$). *, statistically significant from respective 0 h controls. #, statistically significant from rats receiving glycerol treatment alone at the corresponding time point ($p \leq 0.05$).

Fig. 7. Effect of delayed administration of suramin on renal ICAM-1 in rats subjected to glycerol-induced AKI. A, representative Western blot showing ICAM-1 protein expression in kidneys of untreated and glycerol-treated rats ($n = 4$) with or without suramin over a time course. B, densitometric analysis of renal ICAM-1 protein expression in untreated, glycerol-treated, and suramin intervention after glycerol treatment groups over the time course. Data were normalized to GAPDH, which served as an internal control. Data are expressed as mean $\pm$ S.E. ($n = 4$). *, statistically significant from respective 0 h controls. #, statistically significant from glycerol- and suramin-treated rats at the corresponding time point ($p \leq 0.05$). C–E, representative photomicrographs of ICAM-1-stained kidney sections from rats that were untreated (C), treated with glycerol alone at 72 h (D), and subjected to suramin intervention after glycerol treatment at 72 h (E). ICAM-1-positive staining was observed on the basement membranes of proximal tubules (arrows). All of the fields were chosen from the OSOM. Original magnification, 200 $\times$.

domyolysis-induced AKI). Furthermore, we demonstrate that suramin accelerates the recovery of renal damage and function by restoring renal architecture after tubular necrosis. Suramin exerts this beneficial effect through the inhibition of apoptosis, decreased activation of endothelial cells, decreased inflammatory signaling molecules and infiltration of inflammatory cells, decreased oxidative stress, and increased tubular cell proliferation. These results are consistent with previous studies of glycerol-induced AKI in which oxidative

stress, apoptosis, and inflammation are key mediators of renal dysfunction (Homsí et al., 2006; Rosenberger et al., 2008; Homsí et al., 2010; Kim et al., 2010; Wang et al., 2011; Wei et al., 2011).

A proinflammatory response and leukocyte infiltration are important mechanisms in the initiation and maintenance of glycerol-induced AKI (Bonventre and Zuk, 2004; Homsí et al., 2006; de Jesus Soares et al., 2007). We did not observe a difference in the mRNA levels of TNF- α and MCP-1 after

Fig. 8. Effect of delayed administration of suramin on renal proinflammatory cytokines in rats subjected to glycerol-induced AKI. A–D, the mRNA expression profiles in kidneys of untreated and glycerol-treated rats ($n = 4$) with or without suramin over a time course, and quantitative polymerase reactions were performed using primers for TNF- α (A), IL-6 (B), IL-1 β (C), and MCP-1 (D). Tubulin was used as the internal control. Data are expressed as mean $\pm$ S.E. ($n = 4$). *, statistically significant from respective 0 h controls. #, statistically significant from rats receiving glycerol treatment alone at the corresponding time point ($p \leq 0.05$).

Fig. 9. Effect of delayed administration of suramin on renal phospho-p65 in rats subjected to glycerol-induced AKI. A, representative Western blot showing phospho-p65 protein expression in the kidneys of untreated and glycerol-treated rats ($n = 4$) with or without suramin over a time course. B, densitometric analysis of renal phospho-p65 protein expression in untreated, glycerol-treated, and suramin intervention after glycerol treatment groups over the time course. Data were normalized to GAPDH, which served as an internal control. Data are expressed as mean $\pm$ S.E. ($n = 4$). *, statistically significant from respective 0 h controls. #, statistically significant from rats receiving glycerol treatment alone at the corresponding time point ($p \leq 0.05$).

glycerol treatment, which was shown previously in glycerol-induced AKI (Homsy et al., 2009). However, IL-1 β mRNA increased at 24 h and IL-6 mRNA increased at 48 h after glycerol treatment, and suramin treatment blocked the increase in IL-1 β . Suramin did not statistically block the increase in IL-6. Although IL-6 has been reported to play a role

in AKI by increasing the cell-mediated immune response that promotes renal injury and a protective response to tubular cells that maintains renal function (Nechemia-Arbely et al., 2008), the exact role of IL-6 in these experiments remains unclear.

IL-1 β processing and activation have been shown to increase the nuclear translocation of NF- κ B (phosphorylation of the p65 subunit) in endothelial cells, thus resulting in the transcriptional activation of adhesion molecules such as ICAM-1 (Yang et al., 2010). Circulating leukocytes use endothelial cell transmembrane ICAM-1 as an anchor to transigrate and cause inflammation (Lai et al., 2003; Nayak, 2005; Iwai et al., 2006; Yang et al., 2010). We found that IL-1 β mRNA levels increased at 24 to 48 h and phospho-p65 (activated NF- κ B) increased at 24 to 48 h after glycerol treatment alone, that suramin treatment at 24 h after glycerol treatment decreased IL-1 β mRNA and phospho-p65 at 48 h, and that ICAM-1 and leukocyte infiltration were decreased at 72 h. These sequential events may result from the suppressive effect of suramin on IL-1 β mRNA or a direct inhibitory effect on NF- κ B activation as reported previously (Goto et al., 2006). Thus, the sequence of events aligns, and we suggest that the activation of upstream and early events such as IL-1 β (24–48 h) and NF- κ B (24–48 h) may be responsible for the up-regulation of ICAM-1 (72 h) after glycerol treatment alone. The finding that ICAM-1 blockade leads to decreased leukocyte adhesion and renal inflammation is consistent with a previous report that showed an antibody directed against ICAM-1 protected the kidney against I/R injury in rats (Kelly et al., 1994). It is also possible that suramin may block Toll-like receptor 4, which is known to trigger an in-

Fig. 10. Effect of delayed administration of suramin on renal HO-1 and TGF- β_1 in rats subjected to glycerol-induced AKI. A and C, representative Western blot showing HO-1 (A) and TGF- β_1 (C) protein expression in the kidneys of untreated and glycerol-treated rats ($n = 4$) with or without suramin over a time course. B and D, densitometric analysis of renal HO-1 (B) and TGF- β_1 (D) protein expression in untreated, glycerol-treated, and suramin intervention after glycerol treatment groups over the time course. Data were normalized to GAPDH, which served as an internal control. Data are expressed as mean $\pm$ S.E. ($n = 4$). *, statistically significant from respective 0 h controls. @, statistically significant from glycerol- and suramin-treated rats at the corresponding time point. #, statistically significant from rats receiving glycerol treatment alone at the corresponding time point ($p \leq 0.05$).

Fig. 11. Effect of delayed administration of suramin on renal proximal convoluted tubular epithelial cell PCNA in rats subjected to glycerol-induced AKI. A, representative Western blot showing PCNA protein expression in the kidneys of untreated and glycerol-treated rats ($n = 4$) with or without suramin over a time course. B, densitometric analysis of renal PCNA protein expression in untreated, glycerol-treated, and suramin intervention after glycerol treatment groups over the time course. Data were normalized to GAPDH, which served as an internal control. Data are expressed as mean $\pm$ S.E. ($n = 4$). *, statistically significant from respective 0 h controls. #, statistically significant from rats receiving glycerol treatment alone at the corresponding time point ($p \leq 0.05$). C–E, representative photomicrographs of PCNA-stained kidney sections from rats that were untreated (C), treated with glycerol alone at 72 h (D), and subjected to suramin intervention after glycerol treatment at 72 h (E). PCNA-positive staining (intense brown) was observed in cells that are lining the proximal tubules (arrows). All of the fields were chosen from the OSOM. Original magnification, 200 $\times$.

flammatory response by activating IL-1 β and increasing nuclear translocation of NF- κ B (Goncalves et al., 2010).

HO-1 protects against oxidative stress and inflammation during AKI (Ishizuka et al., 1997; Nath et al., 2000; Rosenberger et al., 2008; Bolisetty et al., 2010; Wei et al., 2011; Zarjou and Agarwal, 2011). We found that HO-1 decreased 24 h after glycerol treatment and remained decreased

through 72 h and that suramin up-regulated renal HO-1 at 72 h after glycerol treatment. One possible explanation for this observation could involve NGAL, because NGAL was shown previously to confer a cytoprotective and survival benefit in response to diverse forms of cellular stress by inducing HO-1 (Mori et al., 2005; Schmidt-Ott et al., 2007; Bahmani et al., 2010; Johnson et al., 2010). Consistent with this idea, we

found that suramin markedly increased renal NGAL levels at 48 h after glycerol treatment, before the suramin-induced increase in HO-1 at 72 h.

We also observed that urinary NGAL increased within 24 h after glycerol treatment and increased further at 48 h, whereas renal NGAL did not change at 24 or 48 h. Suramin intervention increased NGAL in urine and renal cortex at 48 h after glycerol treatment. NGAL is thought currently to maintain the balance in iron levels in cells (Johnson et al., 2010). Thus, it seems possible that when proximal tubular cells are exposed to iron-mediated oxidative stress (resulting from glycerol-mediated rhabdomyolysis and release of heme from circulating myoglobin in the tubular lumen) increased NGAL may be produced. It also is documented that NGAL is up-regulated during the time of kidney damage and participates in nephrogenic repair and regeneration (Mishra et al., 2003, 2004; Mori et al., 2005; Schmidt-Ott et al., 2007; Sola et al., 2011). However, the mechanism(s) by which suramin increased proximal tubular NGAL expression currently remains unknown. One hypothesis is that suramin may increase the expression of receptors for NGAL uptake in the proximal tubules. Megalin/24p3R receptor-mediated proximal tubular uptake of NGAL has been suggested (Schmidt-Ott et al., 2007).

It has been documented previously that an inflammatory reaction after muscle injury releases TGF- β_1 , resulting in fibrosis via the activation of fibroblasts and production of extracellular matrix, which halts muscle regeneration and recovery. Suramin administration was shown to significantly decrease the stimulating effect of TGF- β_1 on the growth of muscle-derived fibroblasts and accelerated muscle recovery (Chan et al., 2003; Nozaki et al., 2008). In our study, TGF- β_1 increased at 48 and 72 h after glycerol treatment, and suramin blocked the increase in TGF- β_1 . This effect of suramin on TGF- β_1 was associated with decreased apoptosis 48 h after glycerol treatment and increased proximal tubular cellular proliferation. This finding is consistent with our previous data showing that TGF- β_1 induces apoptosis in renal proximal tubular cells and that suramin stimulates the proliferation of renal proximal tubular cells in primary cultures by activating the Src/Phosphoinositide 3-kinase/Protein kinase B pathway (Zhuang and Schnellmann, 2005).

In addition to being a sensitive biomarker of kidney injury (Bonventre, 2008), KIM-1 may play a role in repair (Ichimura et al., 1998; Bailly et al., 2002; Huo et al., 2010) after AKI. We observed increased and sustained expression of KIM-1 in the group treated with glycerol alone at 24 to 72 h, and suramin treatment decreased KIM-1 at 72 h. Thus, it is unlikely that KIM-1 plays a role in regeneration in this model, because KIM-1 decreased when suramin-induced stimulation of regeneration remains high.

In summary, we found that suramin exerts positive effects on three different end points to promote recovery from glycerol-induced AKI. Suramin inhibited the delayed apoptosis, decreased inflammation, and promoted proliferation of the renal tubular epithelial cells. Further exploration of the signaling pathways revealed that suramin decreased proinflammatory IL-1 β and NF- κ B and growth inhibitory TGF- β_1 at 48 h after glycerol treatment, thereby attenuating subsequent inflammation as demonstrated by a decrease in leukocyte infiltration and ICAM-1 expression at 72 h. In addition, suramin increased the anti-inflammatory protein HO-1 and

increased cell proliferation at 72 h after glycerol treatment. Therefore, delayed administration of suramin, when serum creatinine levels were maximal, markedly promoted recovery after glycerol-induced AKI. These broad actions of a single, low-dose of suramin might prove to have therapeutic potential against rhabdomyolysis-induced and other forms of AKI in clinical settings.

Authorship Contributions

Participated in research design: Korrapati and Schnellmann.

Conducted experiments: Korrapati and Shaner.

Performed data analysis: Korrapati, Shaner, and Schnellmann.

Wrote or contributed to the writing of the manuscript: Korrapati and Schnellmann.

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