Magnesium Supplementation Combined with N-Acetylcysteine Protects against Postischemic Acute Renal Failure

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Magnesium is a potent vasodilator whose effects have not been evaluated in renal ischemia. The antioxidant properties of N-acetylcysteine (NAC) partially protect animals from ischemic/reperfusion injury. This study was designed to evaluate magnesium supplementation, alone or combined with NAC, on ischemic acute renal failure. Rats were maintained on normal diets, supplemented or not with MgCl₂•6H₂O (1% in drinking water) for 23 d, and some rats received NAC (440 mg/kg body wt) on days 20 to 23. On day 21, ischemia was induced by clamping both renal arteries for 30 min. Five groups were studied: Normal, ischemia, ischemia+magnesium, ischemia+NAC, and ischemia+magnesium+NAC. GFR (inulin clearance), renal blood flow (RBF), FEH₂O, and FENa were determined. Serum magnesium was decreased in ischemia-only rats. Magnesium prevented postischemia GFR and RBF decreases but did not protect against tubular damage. However, NAC completely restored the tubular damage induced by ischemia/reperfusion. Semiquantitative immunoblotting showed that NAC prevented the decreased expression of Na-K-2Cl co-transporter and aquaporin 2 after renal ischemia/reperfusion. Untreated rats with acute renal failure demonstrated markedly decreased endothelial nitric oxide synthase expression. Significantly, treatment with NAC, magnesium, or both completely inhibited downregulation of endothelial nitric oxide synthase. The tubular necrosis scores were lower in rats that were treated with NAC alone or with the magnesium-NAC combination. Magnesium prevented postischemia GFR and RBF decreases but did not protect against tubular damage. The NAC protected tubules from ischemia, decreased infiltrating macrophages/lymphocytes, and had a mild protective effect on GFR. In ischemic/reperfusion injury, renal function benefits more from the magnesium-NAC combination than from magnesium alone.

Mortality rates among patients with acute renal failure (ARF) remain unacceptably high (1). Renal ischemia/reperfusion (I/R) injury is still a major cause of ARF. Several experimental ameliorative strategies have been tested. In rats, I/R-induced ARF is known to cause characteristic structural alterations in renal tubule epithelia and decreases in GFR (2). The pathophysiologic changes that are responsible for the postischemic renal injury and profoundly depressed renal function seen in ARF remain incompletely understood. It is well known that the ARF process is multifactorial. It has been proposed that prevention and early treatment of ischemic ARF should include the combination of treatments that act on the mechanisms that are responsible for ARF initiation and simultaneously on those that are responsible for its perpetuation (3).

Magnesium is the fourth most abundant cation in the body. It is a predominantly intracellular cation and is involved in maintaining ionic cellular balance and enzyme activities, as well as in ionic channel modulation. It also has important endocrine functions and is required for protein synthesis (4). Magnesium is also a calcium channel blocker and diminishes the intracellular calcium concentrations, resulting in relaxation of smooth muscle cells (5). In mesangial cells in culture, magnesium inhibits the contraction induced by cyclosporine and angiotensin II (6). In models of nephrotoxic ARF, it has been shown that magnesium supplementation is beneficial when combined with vasoconstrictors such as cyclosporine (7,8) or, more recently, indinavir (9). In previous studies, we demonstrated that hypomagnesemia induced by magnesium depletion in the diet potentiates postischemic renal failure in rats (10). In another study, we showed that hypomagnesemia induced a decrease in GFR and in renal blood flow (RBF) in zidovudine-treated rats (11). However, to date, the effects of magnesium on renal ischemia have not been well studied.

Recent studies suggest that N-acetylcysteine (NAC) ameliorates renal I/R injury (12) and cyclosporine nephrotoxicity (13). It was reported recently that NAC prevents radiocontrast nephropathy in high-risk patients (14,15). The mechanism of NAC-related organ protection is primarily attributed to scavenging oxygen free radicals, either directly or through increas-
ing intracellular glutathione concentrations (16, 17). Decreased F2-isoprostane production indicates attenuation of membrane damage from oxygen-free radicals (15), but other protective mechanisms may also play roles (18).

Because magnesium supplementation has been associated with protection against most forms of organ ischemia (19, 20), we hypothesized that magnesium supplementation alone or in combination with NAC supplementation in rats with ischemia-induced ARF may reduce I/R injury. Therefore, in this study, we examined whether (1) magnesium supplementation, (2) NAC supplementation, or (3) magnesium and NAC supplementation combined reduces renal functional defects, down-regulation of the Na-K-2Cl co-transporter (NKCC2), down-regulation of aquaporin 2 (AQP2), and inflammatory cell infiltration in a rat model of I/R-induced ARF. We also evaluated the effects of magnesium supplementation, NAC supplementation, or magnesium and NAC supplementation combined on endothelial nitric oxide synthase (eNOS) protein in this rat model of I/R-induced ARF.

Materials and Methods
Experimental Animals
For this study, male Wistar rats that weighed 200 to 260 g were provided by the University of São Paulo School of Medicine animal facility. The rats were maintained on a normal diet for 23 d and received, during the same period, MgCl2·6H2O (1%) dissolved in drinking water. From day 20 to day 23, NAC (440 mg/kg body wt) was also added to the drinking water. Throughout the intervention period, water consumption was monitored daily to determine the intake per animal. The Ethics Committee of the University of São Paulo School of Medicine approved the experimental protocol.

Induction of Ischemia/Reperfusion-Induced ARF in Rats
On day 21, the rats were anesthetized with sodium pentobarbital (50 mg/kg body wt), and a suprapubic incision was made. Ischemic renal failure was induced by clamping both renal arteries (with a nontraumatic clamp) for 30 min, followed by reperfusion. Inulin clearance studies were performed 48 h after renal ischemia.

Five groups of animals were studied:
1. Control (normal): Normal diet for 23 d (n = 6)
2. Ischemic (Isc): Normal diet for 23 d, renal ischemia on day 21, and clearance studies 48 h after ischemia (n = 8)
3. Ischemic, magnesium-supplemented (Isc+Mg): Normal diet and magnesium supplementation for 23 d, renal ischemia on day 21, and clearance studies 48 h after ischemia (n = 8)
4. Ischemic, NAC-supplemented (Isc+NAC): Normal diet for 23 d, NAC administration from days 20 to 23, renal ischemia on day 21, and clearance studies 48 h after ischemia (n = 7)
5. Ischemic, magnesium- and NAC-supplemented (Isc+Mg+NAC): Normal diet and magnesium supplementation for 23 d, NAC administration from days 20 to 23, renal ischemia on day 21, and clearance studies 48 h after ischemia (n = 6)

We also created a group (n = 5) composed of normal animals that received normal diet and magnesium supplementation for 23 d.

Clearance Studies
On day 23, the animals were anesthetized with sodium thiopental (50 mg/kg body wt). The trachea was cannulated with a PE-240 catheter, and spontaneous breathing was maintained. The jugular veins were cannulated with PE-60 catheters for infusion of inulin and fluids. For controlling mean arterial pressure and for allowing blood sampling, the right femoral artery was catheterized with a PE-50 catheter. For collecting urine samples, the urinary bladder was cannulated with a PE-240 catheter by suprapubic incision. A median incision was made to measure RBF; the left renal pedicle was dissected carefully, and the renal artery was isolated with care to avoid disturbing the renal nerves. An electromagnetic flow probe (Transonic Systems, Bethesda, MD) was placed around the exposed renal artery, and RBF was measured with an electromagnetic flow meter (T 106 XM; Transonic Systems). After completion of the surgical procedure, a loading dose of inulin (100 mg/kg body wt diluted in 1 ml of 0.9% saline) was administered through the jugular vein. Subsequently, a constant infusion of inulin (10 mg/kg body wt in 0.9% saline) was started and continued at 0.04 ml/min throughout the experiment. Three urine samples were collected at 30-min intervals. Blood samples were obtained at the beginning and at the end of the experiment.

Inulin clearance values represent the mean of the three periods. Blood and urine inulin were determined by the anthrone method, and sodium and potassium concentrations were measured with a flame photometer (model 143; Instrumentation Laboratory, Lexington, MA). Values of GFR (inulin clearance), fractional excretion of sodium (FENa), fractional excretion of potassium (FEK), and fractional excretion of water (FEH2O) were calculated using standard formulas. Renal vascular resistance (RVR) was calculated by dividing BP by RBF and is expressed as mmHg/ml per min.

Blood and urine inulin were determined by the anthrone method, and sodium and potassium concentrations were measured by flame photometry (model 143; Instrumentation Laboratory). Plasma magnesium was determined using a diagnostic kit (Labtest, São Paulo, Brazil).

Primary Antibodies
The peptide-derived polyclonal antibody specific to the NKCC2 was supplied by Dr. Mark Knepper (National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD). The peptide-derived polyclonal antibody specific to the AQP2 water channel was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). MAb to eNOS was from Transduction Lab (Lexington, KY).

Preparation of Membrane Fractions for Na Transporter and AQP2 Protein Expression
Medulla samples were homogenized in ice-cold isolation solution (200 mM mannitol, 80 mM HEPES, 41 mM KOH [pH 7.5]) that contained protease inhibitors (cocktail protease inhibitor; Sigma Chemical Company, St. Louis, MO) using a Teflon pestle glass homogenizer (Schmidt and Co., Frankfurt am Main, Germany). The homogenates were centrifuged at low speed (2000 × g) for 15 min at 4°C to remove nuclei and cell debris. Subsequently, the supernatants were spun at 100,000 × g for 1 h at 4°C using a Ti70r rotor (Beckman Coulter, Fullerton, CA) to produce a pellet that contained membrane fractions enriched for both plasma membranes and intracellular vesicles. The pellets were suspended in isolation solution with protease inhibitors.

Preparation of Renal Samples for eNOS Protein Expression
Kidney samples were homogenized in ice-cold isolation solution (200 mM mannitol, 80 mM HEPES, 41 mM KOH [pH 7.5]) that contained protease inhibitors (cocktail protease inhibitor; Sigma) using a Teflon pestle glass homogenizer (Schmidt and Co.). The homogenates were centrifuged at low speed (3000 × g) for 15 min at 4°C to remove nuclei and cell debris. The pellets were suspended in isolation solution with
Electrophoresis and Immunoblotting

Samples of membrane fractions were run on either 12.5% polyacrylamide minigels (for AQP2) or 10% polyacrylamide minigels (for Na-K-2Cl). After transfer by electroelution to nitrocellulose membranes (Poly-SCREEN, PVDF Transfer; Amersham Bioscience, Little Chalfont, UK), blots were blocked with 5% milk and 0.1% Tween 20 in PBS (87.6 g/L NaCl, 7.2 mM dibasic phosphate, and 2.8 mM monobasic phosphate) for 1 h. Blots then were incubated with one of the following: (1:2000) or NKCC2 antibody (0.12 μg/ml). The labeling was visualized with horseradish peroxidase–conjugated secondary antibody (anti-rabbit IgG, diluted 1:2000, or anti-goat IgG, diluted 1:5000; Sigma) by using an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, NJ).

For evaluation of the eNOS isoform, 100 μg of total protein from each sample was separated on an 8% polyacrylamide gel and transferred to a PVDF membrane. Immunoblotting was performed with antibody to eNOS diluted 1:2000 in Tris-buffered saline (TBS) and Tween 0.1%. Immunodetection was accomplished using the appropriate anti-mouse horseradish peroxidase–linked secondary antibody (dilution 1:2000 in TBS and 0.1% Tween) and the enhanced chemiluminescence kit (Amersham). For controlling for loading, blots were incubated with actin antibody (1:3000; Santa Cruz Biotechnology). The labeling was visualized with horseradish peroxidase–conjugated secondary antibody anti-goat (1:5000; Sigma).

Quantification of Kidney Levels of Proteins

The enhanced chemiluminescence films with bands within the linear range were scanned using the ImageMaster VDS (Pharmacia Biotech, Uppsala, Sweden). These bands were normalized through evaluation of densitometric actin protein abundance.

Light Microscopy

Histologic sections, 3 μm thick, were stained with Masson’s trichrome and examined under light microscopy. Tubular necrosis, tubular lumen dilation, and cellular edema in the renal cortex and outer medulla were evaluated. Injury was graded on a scale of 0 to 4 as follows: 0, no damage; 0.5, small focal areas of damage; 1, <10% involvement of the tubular area; 2, 10 to 25% involvement of the tubular area; 3, 25 to 75% involvement of the tubular area; and 4, extensive damage involving >75% of the tubular area.

Preparation of Tissue for Immunocytochemistry

Identical groups of samples were prepared simultaneously for renal morphology and immunohistochemistry studies.

Antibodies

The following antibodies were used: (1) Monoclonal IgG, antibody to a cytoplasmic antigen present in monocytes, macrophages, and dendritic cells (ED1, Bioproducts for Science, Indianapolis, IN) and (2) monoclonal IgG, antibody to rat T lymphocytes (CD3; clone W3/13HLK; Harlan Sera-Lab, Loughborough, UK).

Immunohistochemistry

The ED1 immunostaining was processed in 4-μm paraffin sections. After deparaffinization, endogenous peroxidase activity was blocked with 0.3% H2O2 in water for 10 min at room temperature. Sections then were subjected to microwave irradiation in citrate buffer to enhance antigen retrieval (12) and incubated with a 1:2500 anti-ED1 antibody for 60 min at room temperature, followed by incubation with a biotinylated mouse anti-rat IgG for 30 min at room temperature. The reaction product was detected with avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA). The color reaction was developed with 3,3-diaminobenzidine (Sigma), and sections were counterstained with methyl green.

The CD3 immunostaining was performed in 4-μm-thick frozen acetone-fixed cryostat sections and in 4-μm-thick deparaffinized sections. Sections were preadsorbed with avidin-biotin blocking reagents (Blocking Kit; Vector). Nonspecific binding sites were blocked through 60-min incubation with 10% horse serum. Sections then were incubated overnight at 4°C with a 1:50 anti-CD3 antibody, then incubated with biotinylated mouse anti-rat IgG for 30 min at room temperature. The reaction product was detected with a streptavidin-alkaline phosphatase complex (Vector). The color reaction was developed with a fast-red dye solution that contained levamisole, an inhibitor of endogenous alkaline phosphatase activity, and counterstained with haemotoxylin.

For all sections, negative controls consisted of substitution of the primary antibody with equivalent concentrations of an irrelevant murine mAb of normal rabbit or goat IgG. Evaluation of all slides was performed by an observer who was unaware of the origin of the slides. The sections were examined using light microscopy at a magnification of ×400.

The number of ED-1– and CD-3–positive cells in each section was calculated by counting the number of positive cells in 30 sequential grid fields of renal cortex measuring 0.245 mm² each and 20 sequential grid fields of outer medulla. Data are expressed as mean ± SEM of cells per grid field.

Statistical Analyses

All quantitative data are expressed as mean ± SEM. Differences among the means of multiple parameters were analyzed by one-way ANOVA followed by Student-Newman-Keuls test or Tukey test. Differences between two parameters were analyzed by either unpaired t test or by nonparametric methods (Mann-Whitney test). Values of P < 0.05 were considered significant.

Results

Ischemia-Induced ARF Is Associated with Severe Acute Renal Insufficiency and Impairment of Renal Water, Sodium, and Potassium Handling

The results of clearance studies and renal hemodynamic analyses are presented in Table 1 and Figure 1. Rats with ARF induced by temporary bilateral renal ischemia for 30 min presented significantly decreased inulin clearance and RBF, indicating acute renal insufficiency. As shown in Table 1 and Figure 2, urinary FENa and FEH2O increased markedly 48 h after renal ischemia.

Ischemia-Induced ARF Is Associated with Decreased Serum Magnesium Levels

Regardless of the cause, hypomagnesemia begins to occur after a relatively small magnesium deficit because there is little rapid exchange of extracellular magnesium with the much larger bone and cell stores. Measurements of total serum magnesium in the five groups of experiments are shown in Table 1 and Figure 3. Ischemic, untreated rats with ARF presented significantly lower serum magnesium levels. An association between renal I/R and decreased serum magnesium has never before been shown. In magnesium-supplemented rats, normal serum magnesium levels were restored.
Comparison with the other groups was NS (Isc). Magnesium supplementation from day 20 to day 23 (Isc + Mg) presented significantly higher inulin clearance versus untreated ischemic rats (Isc). In addition, magnesium and NAC administration supplementation from day 20 to day 23; Isc + Mg + NAC, ischemic rats that received 23 days of a magnesium-supplemented diet combined with NAC administration supplementation from day 20 to day 23; InCl, inulin clearance; RBF, renal blood flow; RVR, renal vascular resistance; FENa and FEH₂O, fractional excretion of sodium and water, respectively.

Supplementation with Magnesium but not with NAC Prevents Deterioration of Renal Function in I/R Injury

Ischemic rats that were treated with magnesium supplementation (Isc + Mg) presented significantly higher inulin clearance (ml/min per 100 g body wt) than did ischemic rats (Isc + Mg versus Isc 0.7 ± 0.06 versus 0.38 ± 0.12; P < 0.05). There was no difference compared with the control rats (normal 0.81 ± 0.07). Also, the administration of magnesium and NAC to ischemic rats resulted in higher values of inulin clearance than in untreated ischemic rats (Isc + Mg + NAC versus Isc 0.9 ± 0.06 versus 0.38 ± 0.12; P < 0.01; Table 1, Figure 1). The NAC supplementation increased inulin clearance, although the difference in comparison with the other groups was NS (Isc + NAC 0.59 ± 0.03). Thus, magnesium supplementation resulted in a complete reversal of the decreased GFR in response to renal I/R. Normal RBF (ml/min) was completely restored in ischemic animals that were treated with magnesium supplementation compared with untreated ischemic rats (Isc + Mg versus Isc 7.4 ± 0.1 versus 5.9 ± 0.2; P < 0.001). Magnesium supplementation prevented the hemodynamic abnormalities seen in untreated ischemic animals. In addition, magnesium and NAC supplementation completely restored normal RBF (Isc + Mg + NAC versus Isc 7.4 ± 0.1 versus 5.9 ± 0.2; P < 0.001). The RBF was significantly lower in NAC-supplemented-only ischemic rats than in magnesium-supplemented or in magnesium/NAC-supplemented ischemic rats (Isc + NAC versus Isc + Mg versus Isc + Mg + NAC 6.7 ± 0.2 versus 7.4 ± 0.1 versus 7.4 ± 0.1; P < 0.05). It is known that ischemia increases RVR. Ischemic rats

Table 1. Plasma magnesium, renal function, and hemodynamic values in normal rats, in Isc, in Isc + Mg, in Isc + NAC, and Isc + Mg + NAC

<table>
<thead>
<tr>
<th></th>
<th>Mg</th>
<th>InCl</th>
<th>RBF (ml/min)</th>
<th>RVR (mmHg/ml per min)</th>
<th>FENa (%)</th>
<th>FEH₂O (%)</th>
</tr>
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<tbody>
<tr>
<td>Normal</td>
<td>1.8 ± 0.1</td>
<td>0.81 ± 0.07</td>
<td>7.4 ± 0.2</td>
<td>16.1 ± 0.9</td>
<td>0.77 ± 0.15</td>
<td>0.85 ± 0.19</td>
</tr>
<tr>
<td>Isc</td>
<td>1.5 ± 0.1</td>
<td>0.38 ± 0.12</td>
<td>5.9 ± 0.2</td>
<td>19.5 ± 0.7</td>
<td>1.60 ± 0.14</td>
<td>1.91 ± 0.33</td>
</tr>
<tr>
<td>Isc + Mg</td>
<td>1.9 ± 0.1</td>
<td>0.70 ± 0.06</td>
<td>7.4 ± 0.1</td>
<td>15.1 ± 0.2</td>
<td>1.70 ± 0.33</td>
<td>2.80 ± 0.61</td>
</tr>
<tr>
<td>Isc + NAC</td>
<td>1.6 ± 0.1</td>
<td>0.59 ± 0.03</td>
<td>6.7 ± 0.2</td>
<td>17.2 ± 0.5</td>
<td>0.63 ± 0.16</td>
<td>0.61 ± 0.14</td>
</tr>
<tr>
<td>Isc + Mg + NAC</td>
<td>2.0 ± 0.1</td>
<td>0.90 ± 0.06</td>
<td>7.4 ± 0.1</td>
<td>15.3 ± 0.3</td>
<td>0.66 ± 0.06</td>
<td>0.67 ± 0.08</td>
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*Isc, untreated ischemic rats; Isc + Mg, ischemic rats that received 23 d of a Mg-supplemented diet; Isc + NAC, ischemic rats that received N-acetylcysteine (NAC) supplementation from day 20 to day 23; Isc + Mg + NAC, ischemic rats that received 23 days of a Mg-supplemented diet combined with NAC administration supplementation from day 20 to day 23; InCl, inulin clearance; RBF, renal blood flow; RVR, renal vascular resistance; FENa and FEH₂O, fractional excretion of sodium and water, respectively.

*P < 0.01 versus normal.
*P < 0.05 versus Isc.
*P < 0.001 versus Isc.
*P < 0.05 versus Isc + Mg and Isc + Mg + NAC.
*P < 0.001 versus normal, versus Isc + Mg, and versus Isc + Mg + NAC.
*P < 0.01 versus Isc + NAC.
*P < 0.05 versus Isc + Mg.
*P < 0.01 versus Isc + Mg + NAC.
*P < 0.05 versus normal.

Figure 1. Inulin clearance in normal rats, in untreated ischemic rats (Isc), in ischemic rats that received 23 d of a magnesium-supplemented diet (Isc + Mg), in ischemic rats that received N-acetylcysteine (NAC) supplementation from day 20 to day 23 (Isc + NAC), and in ischemic rats that received 23 d of a magnesium-supplemented diet combined with NAC administration supplementation from day 20 to day 23 (Isc + Mg + NAC). ●, P < 0.01 versus normal; *, P < 0.05 versus Isc; #, P < 0.001 versus Isc. BW, body weight.
that were treated with magnesium or NAC, alone or in combination, exhibited significantly decreased in RVR compared with untreated ischemic rats (Isc versus Isc/Mg versus Isc/Mg+NAC 19.5 ± 0.7 versus 15.1 ± 0.2 versus 15.3 ± 0.3, P < 0.001; versus Isc+NAC 17.2 ± 0.5, P < 0.01).

Magnesium supplementation did not alter renal function (0.77 ± 0.08 ml/min per 100 g body wt), RBF (7.8 ± 0.13 ml/min), or RVR (15.6 ± 0.7 mmHg/ml per min) in normal rats. In addition, there were no changes in tubular function (FENa 0.41 ± 0.17%; FEK 40.54 ± 9.99%; FEH2O 0.57 ± 0.08%).

Supplementation with NAC but not with Magnesium Completely Restores Normal Tubular Function in Ischemic Rats

Ischemic rats that were treated with NAC supplementation or in combination with magnesium presented significantly lower FENa (Isc+NAC 0.63 ± 0.16%, P < 0.05; Isc+Mg+NAC 0.66 ± 0.06%, P < 0.01) compared with untreated ischemic rats (Isc 1.6 ± 0.14%; Table 1, Figure 2). In contrast, FENa in animals that were treated with magnesium supplementation did not differ significantly (Isc+Mg 1.7 ± 0.33%) from that of untreated ischemic rats (Table 1, Figure 2). Furthermore, NAC supplementation, alone or in combination with magnesium supplementation, reduced FEH2O to a greater degree than did magnesium supplementation alone (Table 1). The data suggest that NAC supplementation reduces the ischemia-induced defects in urinary concentration and normalizes the altered renal sodium handling in response to renal I/R. Thus, ischemic rats that were supplemented with NAC and magnesium presented complete recovery of normal renal function, RBF, and tubular function.

Supplementation with NAC but not with Magnesium Prevents Ischemia-Induced Downregulation of NKCC2 Protein Abundance

It is well known that expression of NKCC2 decreases in postischemic ARF. As shown in Figure 4, semiquantitative immunoblotting, using membrane fractions prepared from the entire medulla, show that NKCC2 protein expression was markedly decreased in untreated ischemic rats (Isc 22.3 ± 6.3% [P < 0.01] versus normal 99.3 ± 1.3%). In contrast, NAC supplementation, alone or in combination with magnesium supplementation, significantly prevented the decrease in the expression of NKCC2 after I/R (Isc+NAC 60.8 ± 3.1% [P < 0.01] versus Isc and Isc+Mg; Isc+Mg+NAC 66.7 ± 8.6% [P < 0.001] versus Isc and Isc+Mg). In rats that had ARF and were treated with NAC, levels of NKCC2 expression were nearly three-fold higher than in untreated rats with ARF. Rats that had ARF and received magnesium supplementation presented markedly decreased NKCC2 expression (27.5 ± 6.5%; P < 0.01 versus normal). These data are in agreement with FENa results obtained in the clearance studies, in which FENa recovery was observed only in the NAC-supplemented group. Magnesium supple-
mentation did not prevent the ischemia-induced decrease in NKCC2 expression. Therefore, NAC supplementation significantly prevents the decrease in the expression of the NKCC2 after renal I/R. This is consistent with the functional improvement in renal sodium handling in response to NAC, alone or combined with magnesium supplementation, compared with untreated ischemic rats.

**Supplementation with NAC but not with Magnesium Prevents Ischemia-Induced Downregulation of AQP2 Protein Abundance**

It is also well known that AQP2 protein expression decreases in postischemic ARF. As shown in Figure 5, semiquantitative immunoblotting, using membrane fractions prepared from the entire medulla, revealed that AQP2 protein expression was lower in untreated ischemic rats (Isc 68.32 ± 4.36% [P < 0.01] versus normal 100 ± 6.4%). In contrast, NAC supplementation, alone or in combination with magnesium supplementation, completely prevented the post-I/R decrease in AQP2 expression (Isc+NAC 95.6 ± 3.34%, P < 0.01 versus Isc and P < 0.05 versus Isc+Mg; Isc+NAC+Mg 88.87 ± 5.23%, P < 0.05 versus Isc). In rats that had ARF are were treated with NAC, levels of AQP2 expression were completely restored. Rats that had ARF and received magnesium supplementation presented decreased AQP2 expression (75.62 ± 5.0%; P < 0.05 versus normal; *, P < 0.01; **, P < 0.001 versus Isc and Isc+Mg.

![Figure 4](image_url)

**Figure 4.** Semiquantitative immunoblotting of membrane fractions of kidney medulla. (A) Densitometric analysis of all samples from normal rats, Isc, Isc+Mg, in Isc+NAC, and in Isc+Mg+NAC. Untreated ischemic rats and magnesium supplemented-rats present markedly decreased Na-K-2Cl co-transporter (NKCC2) expression. Levels of NKCC2 expression are almost three times higher in response to NAC, alone or combined with magnesium supplementation, compared with untreated ischemic rats. (B) Immunoblots that reacted with anti-NKCC2 revealed a band of approximately 146 to 176 kD (centered at 161 kD). #, P < 0.01 versus normal; *, P < 0.01; **, P < 0.001 versus Isc and Isc+Mg.

![Figure 5](image_url)

**Figure 5.** Semiquantitative immunoblotting of membrane fractions of kidney medulla. (A) Densitometric analysis of all samples from normal rats, Isc, Isc+Mg, Isc+NAC, and Isc+Mg+NAC. Magnesium-supplemented rats and untreated ischemic rats presented decreased aquaporin-2 (AQP2) expression. Levels of AQP2 expression were completely restored in rats that were treated with NAC, alone or combined with magnesium supplementation, compared with untreated ischemic rats. (B) Immunoblots that reacted with anti-AQP2 revealed 29- and 35- to 50-kD AQP2 bands, representing nonglycosylated and glycosylated forms of AQP2, respectively. ●, P < 0.01 versus normal and Isc+NAC; #, P < 0.05 versus Isc+Mg+NAC; *, P < 0.05 versus normal and Isc+NAC.
Supplementation with NAC, either alone or in combination with magnesium supplementation, reduced FEH O₂ to a greater degree than did magnesium supplementation alone. Taken together, these data suggest that NAC supplementation reduces the ischemia-induced defects in urinary concentration. Magnesium supplementation did not prevent the ischemia-induced decrease in AQP2 expression (Figure 5).

**Supplementation with NAC and with Magnesium Reduces Ischemic-Induced Downregulation of eNOS**

Untreated rats with ARF demonstrated markedly decreased eNOS expression (55.32 ± 6.0% of normal levels; \( P < 0.001 \)) compared with normal rats (102.1 ± 2.45%). Importantly, treatments with NAC alone, magnesium alone, or both in combination inhibit completely the downregulation of eNOS, compared with untreated rats (Isc+NAC 104.3 ± 3.16, \( P < 0.001 \) versus Isc; Isc+Mg 102.3 ± 5.0, \( P < 0.001 \) versus Isc; Isc+Mg+NAC 106.9 ± 5.4, \( P < 0.001 \) versus Isc). Our results clearly indicate that the beneficial effects of magnesium or NAC supplementation are mediated, in part, by the higher production of NO, as evidenced by the higher levels of eNOS protein (Figure 6).

**Histologic Data**

Light microscopy studies showed tubular necrosis, tubular dilation, inflammatory cell infiltration, and cellular edema in the tubulointerstitium of the renal cortex and outer medulla from animals that were killed 48 h after renal ischemia. These lesions were less intense in rats that were treated with supplementation of NAC alone or magnesium alone or in combination compared with untreated animals. The tubular necrosis scores can be seen in Figure 7.

**Immunohistochemical Analysis**

Because the predominant infiltrating cells during I/R injury are ED1 macrophages, the efficacy of NAC and magnesium supplementation was studied through immunohistochemical ED1 counts. The number of interstitial cells in the cortex and outer medulla that demonstrated ED1 staining for macrophages/monocytes was significantly greater in untreated ischemic rats than in controls. There was no statistical difference in the number of ED1 cells between magnesium-supplemented rats and untreated ischemic rats (Table 2, Figure 8). In rats that received supplementation with NAC, alone or combined with magnesium supplementation, the number of ED1 cells tended to be lower in the outer medulla—and significantly lower in the cortex—than in untreated ischemic rats (Table 2, Figure 8). Representative immunostaining for CD3 in the cortex and outer medulla is presented in Figure 9. We observed that CD3 was upregulated in I/R injury. Supplementation with NAC, alone or combined with magnesium supplementation, prevented the marked expression of CD3 (Table 2, Figure 9C). There was no statistically significant difference between magnesium-supplemented rats and untreated ischemic rats in the number of CD3 cells (Table 3).

**Discussion**

Because it is widely known that ARF is a multifactorial process, the purpose of our study was to assess the therapeutic potential of exogenous administration of a combination of two compounds (NAC and magnesium) in I/R injury in rats. We found that 30 min of renal ischemia induced a significant drop in GFR and RBF, resulting in tubular injury. In addition, there was a significant reduction in NKCC2 and AQP2 expression in the renal medulla of the animals, as well as morphologic damage characterized by leukocyte infiltration involving ED1+ monocytes, ED1+ macrophages, and CD3 cells.

Our results clearly show that the animals that received magnesium supplementation presented significantly higher GFR in comparison with the untreated animals that were subjected to renal ischemia, reaching a value similar to that of the control group.

The renal vasodilation produced by magnesium can be attributed to two mechanisms already well described in other

**Figure 6.** Semiquantitative immunoblotting of fractions of kidney. (A) Densitometric analysis of all samples from normal rats, Isc, Isc+Mg, Isc+NAC, and Isc+Mg+NAC. Untreated ischemic rats present decreased endothelial nitric oxide synthase (eNOS) expression. Levels of eNOS expression were completely restored in response to NAC alone, magnesium alone, or NAC combined with magnesium supplementation, compared with untreated ischemic rats. (B) Immunoblots that reacted with anti-eNOS revealed a 140-kD band. \( * \), \( P < 0.001 \) versus normal, Isc+Mg, Isc+NAC, and Isc+NAC+Mg.
vascular beds, in which the magnesium acts via release of NO, a powerful endogenous vasodilator (4). The RBF of the ischemic rats that received magnesium supplementation was comparable to that of the normal animals. We also demonstrated that magnesium supplementation could completely restore eNOS protein expression in ischemic rats. In addition to the vasodilator effect, magnesium supplementation can increase the ultrafiltration coefficient. It is important to point out that FENa and FEH2O remained high in the animals that received magnesium supplementation, indicating that the tubular alterations might be related to other mechanisms of cellular injury after reperfusion.

Herein, we have demonstrated, for the first time, that renal I/R is associated with hypomagnesemia. It was shown previously that hypomagnesemia leads to hypertension, atherogenesis, and stroke, although the mechanisms remain ill-defined (21). It has also been shown that patients with hypertension, ischemic heart disease, or stroke exhibit significant depletion of serum magnesium (22). Such low levels of ionized magnesium, when mimicked in vitro, result in contraction and spasms of peripheral, coronary, and cerebrovascular muscle cells (23). Recent in vitro studies of aortic and cerebrovascular smooth muscle have demonstrated that reduction in magnesium below normal human, rat, or dog serum values induces the expression of at least two proto-oncogenes (c-fos and c-jun) and the transcription factor NF-κB (24). It is known that there is an increase in c-fos, c-jun, and NF-κB mRNA after renal I/R, and these factors may also be involved in the renal proinflammatory and oxidative processes (25,26). Renal ischemia-induced hypomagnesemia may be associated with proto-oncogene and NF-κB upregulation. Magnesium is involved in myriad reactions and functions in various cells, including blood mononuclear cells. These include enzymatic reactions and channel, receptor, and intracellular signaling molecule operation, as well as nucleic acid and protein conformation (4). Surprisingly, in our study, magnesium supplementation did not decrease monocyte/macrophage infiltration 48 h after renal I/R. It is possible that a latent effect of this magnesium supplementation could have been seen if the observation period had been of a longer duration. Finally, magnesium-supplementation-independent mechanisms are also likely to play a role in inflammatory cell infiltration in I/R injury.

The oxidative stress generated by the imbalance between reactive oxygen species (ROS) and the endogenous antioxidant forces has come to be closely related to renal I/R injury. Some studies have demonstrated the beneficial effect of antioxidant substances such as NAC in the pathogenesis of some illnesses, including ischemic and nephrotoxic ARF (12–15). In addition to being the precursor of the L-cysteine and glutathione reductase, NAC acts as a superoxide scavenger, capable of tripling endothelial NOS expression as well as increasing NO bioavailability (27,28). It has also been shown to inhibit the activation of c-Jun N-terminal kinase, p38 mitogen-activated protein kinase, and the transcriptional factor NF-κB, as well as preventing apoptosis and limiting the activity of some proteins (29,30).

Table 2. Number of ED1+ cells/0.245 mm2 in the renal cortex and outer medulla of control rats and of rats that were subjected to ischemia and treated and untreated with magnesium, NAC, or both

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Ischemia</th>
<th>Isc+Mg</th>
<th>Isc+NAC</th>
<th>Isc+Mg+NAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortex</td>
<td>3.44 ± 0.41</td>
<td>9.72 ± 0.41</td>
<td>10.48 ± 0.85a</td>
<td>7.27 ± 0.59ab</td>
<td>6.27 ± 0.28abc</td>
</tr>
<tr>
<td>Outer medulla</td>
<td>3.56 ± 0.59</td>
<td>10.54 ± 0.34a</td>
<td>12.65 ± 1.29a</td>
<td>9.64 ± 1.25a</td>
<td>7.86 ± 0.61d</td>
</tr>
</tbody>
</table>

aP < 0.001 versus controls, Tukey test.
bP < 0.05 versus untreated ischemia, Tukey test.
cP < 0.01 versus ischemia, Tukey test.
dP < 0.05 versus controls, Tukey test.
Our results show that isolated NAC administration partially protected against the decreased GFR and RBF induced by renal ischemia. We have demonstrated that levels of NKCC2 in the thick ascending limb decreased significantly in response to ischemia. Levels of AQP2 in the renal medulla also decreased significantly in response to ischemia. In addition, the decreased levels of NKCC2 were associated with increased urinary excretion of sodium and potassium in rats with ARF. The reduced expression of both NKCC2 and AQP2 were significantly prevented by co-treatment with NAC. These results are in agreement with the studies of Heyman et al. (31), who also showed a partial improvement in GFR followed by improved reabsorption of sodium in rats that had ARF and were treated with NAC. The authors showed that in normal rats that received NAC supplementation, there were no changes in renal function or RBF. In a previous study conducted in our laboratory, we also demonstrated that NAC supplementation did not alter renal function or tubular function (32). In a previous study, Wang et al. (33) showed a reduction of approximately 88% in mRNA expression of NKCC2 in the first 12 h after 30 min of renal ischemia, which remained suppressed after 48 h. These results are similar to those obtained in our study regarding expression of the NKCC2 protein. Magnesium supplementation did not modify the expression of these proteins (NKCC2 and AQP2) when compared with the animals that were subjected to renal ischemia only. The animals that received NAC.

Figure 8. Immunostaining for ED1 (macrophages/monocytes) in juxtamedullary region from control rats (A) and from rats that were subjected to ischemia untreated (B) or treated with Mg+NAC (C). Note that the number of ED1-positive cells is higher in B. Magnification, ×280.

Figure 9. Immunostaining for CD43 (T lymphocytes) in juxtamedullary region from control rats (A) and from rats that were subjected to ischemia untreated (B) or treated with Mg+NAC (C). Note that the number of CD43-positive cells is higher in B. Magnification, ×280.
presented higher NKCC2 and AQP2 expression when compared with the untreated ischemic animals.

A potent anti-inflammatory agent, NAC inhibits neutrophil migration and production of neutrophil chemokines (34). In this study, we demonstrated that NAC treatment of rats with bilateral ischemia-induced ARF significantly reduced down-regulation of NKCC2 and AQP2 levels and that this was paralleled by functional changes. The exact roles and signaling pathways of NAC, endothelin, and chemokines in altering NKCC2 and AQP2 expression have yet to be identified. In addition, trials aimed at modulating the expression of sodium transporters may potentially provide relevant therapeutic modalities for treating ischemia-induced ARF in human patients. Therefore, we could infer that NAC, probably through its antioxidant action, hinders the lipid peroxidation of the tubular cell membrane, thereby preventing the loss of the transport proteins (e.g., NKCC2 and AQP2) and protecting the renal medulla from I/R injury.

Whether NAC increases NKCC2 and AQP2 expression owing to its antioxidant properties or by other mechanisms is unknown. We have shown that NAC completely reverses the decreased eNOS protein expression induced by ischemia.

The mechanisms that have been proposed to explain the I/R renal injury include anoxia followed by release of oxygen-derived free radicals during reperfusion, leading to endothelial cell dysfunction with decreased NO release and lower leukocyte-endothelial adhesion and activation (35). Superoxide anion, one of these free radicals, can interact with NO to generate peroxynitrite, a potent and cytotoxic oxidant that could cause renal vasoconstriction and medullary ischemia, thus contributing to the persistent reduction in medullary perfusion associated with ARF (36).

Animals that were treated with the combination of magnesium and NAC were completely protected against postischemic ARF. This treatment might restore eNOS protein expression completely. These animals presented normal renal function and higher NKCC2 and AQP2 expression in comparison with animals in the other ischemic groups. Recent studies have shown that the combination of NAC, sodium nitroprusside, and phosphoramidon partially attenuates I/R injury in rats (37).

Our results are in agreement with the proposition that more efficient therapies for the prevention and the treatment of ischemic ARF should involve a combination of procedures in the early stages of ARF.

In conclusion, supplementation with magnesium alone and with NAC alone partially protected the postischemic rats with ARF, the former improving the hemodynamic function and the latter the tubular function. The magnesium-NAC combination completely protected the animals from postischemic ARF, as well as increasing eNOS protein expression.

Table 3. Number of CD3+ cells/0.245 mm² in the renal cortex and outer medulla from control rats and from rats that were subjected to ischemia and treated and untreated with magnesium, NAC, or both

<table>
<thead>
<tr>
<th></th>
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<th>Isc+Mg</th>
<th>Isc+NAC</th>
<th>Isc+Mg+NAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortex</td>
<td>1.77 ± 0.22</td>
<td>7.68 ± 0.68(^a)</td>
<td>6.61 ± 0.11(^b)</td>
<td>4.92 ± 0.34(^b,c)</td>
<td>4.97 ± 0.56(^b,c)</td>
</tr>
<tr>
<td>Outer medulla</td>
<td>2.53 ± 0.47</td>
<td>9.10 ± 0.57(^a)</td>
<td>8.68 ± 1.52(^a)</td>
<td>7.05 ± 0.35(^b)</td>
<td>6.13 ± 0.34(^d)</td>
</tr>
</tbody>
</table>

\(^{a,b,c,d}\)P < 0.001 versus controls, Tukey test.

References

Magnesium and N-Acetylcycteine in Acute Renal Failure

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