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Guanosine supplementation reduces apoptosis and protects renal function in the setting of ischemic injury

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Methods

Renal ischemia. All animal experimentation was conducted in conformity with the Guiding Principles for the Journal of Clinical Investigation | November 2001 | Volume 108 | Number 9

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See related Commentary on pages 1279–1281.
Research Involving Animals and Human Beings. Guanosine (Sigma Chemical Co., St. Louis, Missouri, USA), 30 mg/kg in 0.9% NaCl, or an equal volume of 0.9% NaCl was administered via intraperitoneal injection. The dose of guanosine was based on the in vivo use of nucleosides from cancer literature (13). Male C57 black mice weighing 15–25 g and Sprague-Dawley rats weighing 180–220 g (Harlan, Indianapolis, Indiana, USA) were anesthetized with intraperitoneal sodium pentobarbital (50–70 mg/kg) and placed on a homeothermic table to maintain core body temperature at 37°C. Both renal pedicles were occluded via a midline incision for 32 minutes (14). Sham surgery consisted of an identical procedure with the exception of application of the microaneurysm clamps. Creatinine was determined by standard picric acid reaction in serum obtained from the tail vein or by cardiac puncture.

**Light microscopy.** Twenty-four hours after surgery, kidneys were perfusion fixed in situ with 4% paraformaldehyde. Kidneys were paraffin embedded, sectioned at 4 µm, and stained using hematoxylin and eosin (H&E). The percentage of tubules in the cortex and outer medulla that showed epithelial necrosis with luminal necrotic debris and tubular dilation were each quantified as follows: 0%, <10%, 10–25%, 26–75%, and >75%. The estimates were performed by a blinded observer on coded sections (three to four sections per kidney and 10 to 12 fields per section) (15).

**Fluorescence microscopy.** A Zeiss confocal microscope (LSM 510; Carl Zeiss Inc., Thornwood, New York, USA), equipped with ultraviolet, argon, and helium lasers, was used. Pieces from the in situ fixed kidneys were preserved in 30% sucrose before 10-µm frozen sections were obtained. Some sections were stained with FITC-phalloidin (for evaluation of actin cytoskeleton architecture and as a marker for proximal tubules) and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), a nuclear stain (Molecular Probes Inc., Eugene, Oregon, USA). Other sections were also costained with a rabbit polyclonal anti-human Tamm-Horsfall protein Ab (THP; Biogenesis Inc., Kingston, New Hampshire, USA) and a goat anti-rabbit Cy5-conjugated secondary Ab (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA). This was used as a marker for Henle’s loop.

Finally, separate sections were stained with TUNEL reagent (Promega Corp., Madison, Wisconsin, USA) and DAPI for in situ apoptosis detection. In brief, 10-µm frozen sections were treated with 20 µg/ml proteinase K and then incubated in a nucleotide mixture containing fluorescein-12-dUTP and terminal transferase (TdT). Positive controls were pretreated with 1 U/ml DNase, and negative controls were incubated without TdT. TUNEL-positive nuclei were expressed as a percentage of total nuclei (DAPI positive) per field. Six to eight fields per section and two to three sections per kidney were examined in each experiment.

In some experiments, TUNEL-positive nuclei were expressed as a percentage of total nuclei of a particular tubular segment. This was done by labeling 5-µm sections with TUNEL reagent and a directly contiguous 5-µm section from the same tissue with phalloidin and anti-THP Ab. Overlay of the two sections allowed the localization of TUNEL-positive nuclei to various tubular segments.

**Nucleotide extraction.** Kidney tissue obtained 1 hour after sham surgery or ischemia with cutting forceps precooled in liquid nitrogen was crushed under liquid nitrogen and resuspended in 30% ice-cold acetonitrile and centrifuged at 16,000 g for 10 minutes at –20°C. The supernatant fraction, kept on ice, was gassed with N2 for 30 minutes to evaporate acetonitrile, and the nucleotides were assayed by HPLC as described in detail previously (12). The pellet’s protein content was analyzed with Coomassie blue assay (Pierce Chemical Co., Rockford, Illinois, USA).

**Plasma guanosine levels.** Blood was collected into a chilled stopping solution: 26 µM erythро-9-(2-hydroxy-3-nonyl) adenine hydrochloride, 100 µM dipyridamole, 4 mM EDTA, 2 µM indomethacin, and 100 U/ml heparin. The plasma was separated by centrifugation and the proteins precipitated with 10% trichloroacetic acid. The supernatant was washed with...
tert-butyl-ether and the organic layer discarded. The sample was filtered through a cyano-bonded solid-phase extraction cartridge and assayed by HPLC using established protocols (16).

Studies with LLC-PK1 cells. A4.8 clones of LLC-PK1 porcine proximal tubule cells were grown in 5% CO₂ at 37°C in DMEM with 10% FBS (Sigma Chemical Co.) and 5 mM glucose (referred to as regular medium). For chemical anoxia, 0.1 µM antimycin A was used in depleted media (DMEM without amino acids, glucose, or serum). Nucleotide extraction and measurements were done as described previously (12). Cell death was determined using a Zeiss confocal microscope. Both adherent and floating cells were visualized after staining with Hoechst 33342 and propidium iodide, as described previously (12). Cell viability was based on the criteria of trypan blue exclusion and cell adherence as described by others (10).

Statistics. Creatinine, guanosine, and nucleotide levels are expressed as means plus or minus SE. ANOVA was used to determine if differences among mean values reached statistical significance. Student t test was used for comparisons between groups. Bonferonni adjustment was used for multiple comparisons. Fisher’s exact test was used to determine if differences in histological parameters were significantly different.

Results

Effect of intraperitoneal guanosine on plasma guanosine levels. We first examined the time course of changes in plasma guanosine levels following an intraperitoneal guanosine administration in mice subjected to bilateral ischemia/reperfusion. As shown in Figure 1, there was a significant increase in plasma guanosine that peaked 2 hours after guanosine administration. Three to 7 hours after the intraperitoneal injection, levels were significantly lower than those at 2 hours but still slightly higher than baseline. Thus, guanosine was administered 2 hours before surgery in all subsequent experiments except when stated otherwise.

Effects of intraperitoneal guanosine on renal nucleotide levels during reperfusion. We have shown previously that renal GTP and ATP levels fall to less than 10% of control during renal artery clamp exceeding 20 minutes (12). After 6 to 8 hours of reperfusion, GTP and ATP normalized to preischemia levels. Furthermore, the decline in GTP levels during ischemia could not be prevented by guanosine supplementation (data not shown). Therefore, we investigated the effects of guanosine on GTP at an early time point during reperfusion. In the control kidneys, ATP was 18 ± 3 pmol/µg protein and GTP was 6 ± 2 pmol/µg protein. As shown in Figure 2, renal GTP levels were above 92% of control at 1 hour of reperfusion in the guanosine/ischemia mice (G/I) compared with 56% in normal saline/ischemia mice (NS/I). Furthermore, guanosine supplementation did not affect ATP levels significantly (44% in G/I versus 36% in NS/I). Thus, guanosine selectively increased GTP levels to near baseline values after only 1 hour of reperfusion. Guanosine had no significant effects on GTP or ATP in the sham kidneys. Similar results were obtained in rats: in control sham kidneys, ATP was 28 ± 4 pmol/µg protein and GTP 8 ± 2 pmol/µg protein. Guanosine increased GTP after 1 hour reperfusion to 107% of control compared with 65% in the NS/I (P < 0.01). ATP levels were 49% in the G/I group versus 58% in the NS/I (P = NS).

Effects of guanosine and enhanced GTP recovery on renal histology at 24 hours. Histological sections of kidneys from NS-treated mice (NS/I group) removed 24 hours after ischemia showed extensive tubular necrosis, tubular dilation, and cast formation in the outer medulla (Figure 3). Sections from the guanosine-treated mice (G/I group) had similar histological features. Quantitation of these changes showed no significant differ-
ences between these groups (Table 1). In addition, staining with phalloidin demonstrated comparable disruption and thinning of apical actin rim between the two groups. The sham surgery group showed a well-formed, thick, and continuous actin rim (Figure 3). Thus, guanosine and enhanced GTP recovery after ischemia did not have a significant effect on the morphologic consequences of renal ischemia in this mouse model. The histology and actin architecture of the sham group that received guanosine were normal and indistinguishable from the NS-treated sham group. Similar changes were seen in rat kidneys. The majority of sections of outer medulla from both the NS/I and G/I rats showed evidence of tubular necrosis in >75% of tubules examined. The apical actin rim also showed comparable thinning and disruption in both groups (data not shown).

**Effects of guanosine and enhanced GTP recovery on renal cell apoptosis at 24 hours.** At 24 hours after ischemia, guanosine had a remarkable effect on the occurrence of apoptosis in renal tissues. As shown in Figure 4, kidneys from mice in the NS/I group showed extensive TUNEL-positive staining (Figure 4c), predominantly in the outer medulla. In contrast, kidneys from mice in the G/I group had very little TUNEL-positive staining (Figure 4d). Furthermore, because the TUNEL stain can occasionally be nonspecific, we examined nuclear morphology with DAPI staining at higher magnification (×60). In Figure 4e, nuclei from the NS/I group showed heavy staining and condensation of chromatin. The inset in Figure 4e shows ×120 magnification of a single nucleus. Fragmentation of chromatin into several smaller bodies is seen, a hallmark of apoptosis. Figure 4f is a ×60 magnification of nuclei from G/I mice. They do not show condensation and stain very faintly with DAPI.

In addition, we confirmed the presence of apoptotic nuclei in the NS/I group by high-magnification (×100) light microscopy of H&E-stained tissues. As shown in Figure 5, a and b, very few if any apoptotic nuclei were observed in the G/I group. In contrast, multiple apoptotic nuclei with classic condensation and fragmentation can be seen in the NS/I group (Figure 5, c–f). Elec-
Electron microscopic examination of tissues from the NS/I group clearly identified two types of nuclei in addition to normal ones: apoptotic nuclei (Figure 5g) and necrotic ones (Figure 5h). Thus, the apoptosis found by TUNEL was confirmed by fluorescence, light, and electron microscopic criteria.

The distribution of TUNEL-positive staining between outer medulla and cortex in the four groups is shown in Figure 6. The highest TUNEL-positive staining was in the medulla of the NS/I group and represented 28% of all nuclei stained in this region. In the G/I group, the number of TUNEL-positive nuclei was reduced significantly to 3%. There was less TUNEL positivity in the cortex of the NS/I group (8%), but this was still reduced in the G/I group to less than 2%. Similar results were observed in rats. The NS/I group showed 33% TUNEL-positive nuclei in the medulla. This was reduced in the G/I group to less than 5%. There was minimal cortical TUNEL positivity (<2%) in all groups.

We further examined the window of efficacy of guanosine. We found that guanosine was most protec-
tive when given 2 hours before ischemia. Mean creatinine was no different than in the control postischemic group when guanosine was given 4 or 8 hours before ischemia or 4 or 8 hours after ischemia. When guanosine was given at the time of ischemia or 2 hours after ischemia, the improvement in mean creatinine did not reach statistical significance. Peak guanosine levels occur 2 to 3 hours after an intraperitoneal injection (Figure 1), coinciding well with the enhanced GTP levels observed 1 hour after ischemia (3 hours after guanosine administration).

Effects of guanosine on nucleotide content, cell viability, and apoptosis after chemical anoxia in LLC-PK1 cells. Because of the cellular complexity of kidney tissues, we investigated if the reported changes in nucleotides and cell death can be reproduced in tubular cells in culture. For this purpose, we used the well-characterized LLC-PK1 proximal tubular cell line to model the effects of guanosine. Resting ATP and GTP levels were 36 ± 4 pmol/µg and 8 ± 2 pmol/µg protein, respectively (n = 20). The addition of up to 200 µM guanosine for 2 hours increased ATP to 40 ± 3 pmol/µg and GTP to 9 ± 2 pmol/µg protein (n = 5). Both changes were not statistically significant.

Next we investigated the effects of guanosine on nucleotide recovery after antimycin A–induced chemical anoxia. As shown in Figure 9a, 45 minutes’ exposure to 0.1 µM antimycin A in depleted media reduced ATP to 5% ± 2% and GTP to 8% ± 2% of control levels. After 2 hours’ recovery in regular media, ATP was 33% ± 3% and GTP was 49% ± 4% of control values. When 200 µM guanosine was included in the recovery medium for 2 hours, ATP was 35% ± 2% and GTP was 88% ± 5% of control (Figure 9b). Thus, guanosine enhanced early selective GTP recovery after chemical anoxia.

We then quantitated cell viability after chemical anoxia and recovery for 24 hours. The presence of guanosine significantly increased cell viability at 24 hours: viability was 70% ± 8% in the guanosine-treated group as compared with 44% ± 6% in the control group (P < 0.02). As shown in Figure 9, c and d, the increase in viability in the guanosine group primarily was due to a reduction in apoptotic cell death. Indeed, the guanosine-treated group showed less than 10% apoptosis at 8, 12, and 24 hours as compared with 40% in the control group (the representative fields shown in Figure 9 are from a 24-hour time point). Neither group showed any significant necrosis at these three time points as evidenced by exclusion of propidium iodide. Thus, guanosine supplementation reduces apoptosis and increases viability after in vitro chemical anoxia in LLC-PK1 proximal tubular cells.

Discussion

In this article, we extend our findings relating apoptosis and GTP depletion in cell culture to the in vivo ischemia setting. Indeed, our results in both mice and rats show that guanosine selectively repletes renal GTP levels after 1 hour of reperfusion. This early increase in GTP is accompanied by a significant reduction in medullary apoptosis and a remarkable protective effect on GFR. Therefore, these results introduce two important concepts to be discussed. First is the relationship of GTP levels to apoptosis and second is the relationship of apoptosis to renal function.

The depletion of guanine nucleotides has been implicated in the induction of apoptosis in anoxic cells in

![Figure 7](https://example.com/guanosine.png)

Figure 7 Distribution of TUNEL-positive nuclei among various nephronal segments. (a) A 5-µm thick section from NS/I mice stained with TUNEL and DAPI. The TUNEL-positive nuclei were pseudocolored white instead of green to allow clear distinction from the green FITC-phalloidin. (b) A 5-µm section immediately contiguous to the one shown in a is stained with the actin marker FITC-phalloidin (bright green) to identify proximal tubules (P). Distal tubules (D) show minimal or no staining. The section was costained with anti-THP Ab and a Cy5-conjugated secondary Ab (red). This is predominantly a marker for Henle’s loop segments (LH). (c) An overlay of a and b allows the localization of TUNEL-positive nuclei to various tubular segments. (d) A quantitative estimate of the distribution of TUNEL-positive nuclei normalized to total number of DAPI-positive nuclei for each particular tubular segment or lumen. Other refers to interstitial or vascular TUNEL-positive nuclei.

![Figure 8](https://example.com/guanosine.png)

Figure 8 Effects of guanosine and enhanced renal GTP levels on renal function at 24 hours. Values are means ± SE. Creatinine levels at 24 hours are shown after administration of normal saline (NS) or guanosine (G) 2 hours before sham surgery or bilateral renal ischemia. Mean creatinine was significantly higher in the NS/I mice than in the other groups. (*P < 0.01, n = 6 for NS/I and G/I groups).
Cells were treated for 45 minutes with 0.1 μM antimycin A in depleted media followed by recovery for 2 hours. Nucleotides were measured at 45 minutes of depletion (n = 7) and at 2 hours of recovery (n = 6). (b) Cells were treated identically except for the addition of 200 μM guanosine to the recovery medium (n = 6). (c and d) Representative fields of confocal microscopic images of LLC-PK1 cells at 24 hours after recovery from chemical anoxia. Cells were costained with Hoechst 33342 and propidium iodide as detailed in Methods. In c, cells recovered in regular media. Apoptotic features such as condensation and fragmentation of chromatin is seen in most cells in the field. In d, cells recovered in the presence of 200 μM guanosine. Most nuclei showed normal morphology, and only few had apoptotic features.

**Figure 9**

Effects of guanosine on nucleotide levels and apoptosis after chemical anoxia recovery in LLC-PK1 cells. Values are means ± SE. (a) LLC-PK1 cells were treated for 45 minutes with 0.1 μM antimycin A in depleted media followed by recovery for 2 hours. Nucleotides were measured at 45 minutes of depletion (n = 7) and at 2 hours of recovery (n = 6). (b) Cells were treated identically except for the addition of 200 μM guanosine to the recovery medium (n = 6). (c and d) Representative fields of confocal microscopic images of LLC-PK1 cells at 24 hours after recovery from chemical anoxia. Cells were costained with Hoechst 33342 and propidium iodide as detailed in Methods. In c, cells recovered in regular media. Apoptotic features such as condensation and fragmentation of chromatin is seen in most cells in the field. In d, cells recovered in the presence of 200 μM guanosine. Most nuclei showed normal morphology, and only few had apoptotic features.

Our results in mice and rats are in remarkable agreement with our in vitro findings and strongly implicate GTP depletion in renal tubular apoptosis. However, the complexity of in vivo renal ischemia imposes significant caution to the proposal of a direct link between GTP and apoptosis. For example, the reported GTP levels are average numbers from both cortex and medulla. Thus, the exact GTP changes in these two regions could not be dissected further. In addition, the presence of multiple cell types in these regions also imposes some restriction on our results. However, tubular cells are far more abundant than other cell types, and this justifies our assumption that the reported levels predominantly represent tubular GTP. Furthermore, we were able to reproduce the changes in GTP (and apoptosis) in LLC-PK1 proximal tubular cells in culture. Thus, the reported changes in nucleotides in renal tissues most likely reflect events in tubular cells. Improvements in the sampling technique for GTP measurements will allow a better evaluation of the role of this nucleotide in cortical versus medullary regions.

Recent studies have reported a protective effect of guanosine against neuronal hypoxic injury in culture (24, 25). This beneficial effect was ascribed to an enhancement in ATP production. Our in vivo and in vitro studies do not support such a role for guanosine in renal tubular cells. After guanosine administration, only GTP levels were increased with minimal or no effect on ATP levels. Selective effects of guanosine on GTP as opposed to a nonselective increase in all nucleotides could be related to the doses of guanosine used or cell-specific differences in purine metabolic pathways.

Another degree of caution is imposed by our limited knowledge of possible direct effects of guanosine unrelated to nucleotide production. These could include hemodynamic, vascular, or anti-inflammatory properties. However, unlike adenosine, guanosine has no known receptors or second messenger cascades. It is taken up by specific transporters into cells and then proceeds via the salvage pathway to generate guanine nucleotides (26). This pathway is catalyzed by the rate-limiting enzyme hypoxanthine phosphoribosyl transferase (HPRT). Studies in HPRT-null mice will uncover possible direct effects of guanosine. These mice cannot synthesize GTP from guanosine and therefore will be useful to probe for direct effects of guanosine (27). Nevertheless, the histological findings in the G/I group revealed morphological damage similar to that seen in the NS/I group. These data and those in LLC-PK1 cells argue against significant beneficial hemodynamic or anti-inflammatory properties of guanosine. They also underscore the difference in apoptosis as the main underlying mechanism for the improved function.

Our confidence in the TUNEL assay as a reporter of apoptosis relies on two facts. First is the confirmation of apoptosis by morphological criteria. Indeed, condensation and fragmentation of nuclear chromatin is seen in most cells in the field. In d, cells recovered in the presence of 200 μM guanosine. Most nuclei showed normal morphology, and only few had apoptotic features.

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turn affects function directly. In the kidneys, the link between inhibition of apoptosis and the improved GFR is less obvious. A recent study proposed that apoptosis is proinflammatory and thus detrimental to function (30). Our histological findings do not support such an interpretation. In fact, a lack of correlation between histological evidence of injury and renal function has been found in both human acute renal failure (34, 35) and in animal models of renal injury (36). It is possible that prevention of apoptotic death in nearly 30% of medullary tubular cells confers a protective effect on overall tubular integrity and nephron function.

In conclusion, we report a novel finding regarding the protective effect of guanosine against renal ischemic injury in vivo. Our results show that guanosine selectively increases GTP and has no significant effects on ATP pools. Thus, this protective effect is likely secondary to enhanced GTP levels leading to reduced medullary programmed cell death. Our study highlights the importance of cellular GTP levels as targets for therapeutic intervention. It also proposes guanosine as a potential tool to manipulate GTP levels in vivo and alter the outcome of ischemic injury to the kidneys.

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