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Increased expression of p38 mitogen-activated protein kinase is related to the acute renal lesions induced by gentamicin

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Abstract

Mitogen-activated protein kinases (MAPK) may be involved in the pathogenesis of acute renal failure. This study investigated the expression of p-p38 MAPK and nuclear factor kappa B (NF-κB) in the renal cortex of rats treated with gentamicin. Twenty rats were injected with gentamicin, 40 mg/kg, *im*, twice a day for 9 days, 20 with gentamicin + pyrrolidine dithiocarbamate (PDTC, an NF-κB inhibitor), 14 with 0.15 M NaCl, *im*, twice a day for 9 days, and 14 with 0.15 M NaCl , *im*, twice a day for 9 days and PDTC, 50 mg kg^{-1} day^{-1}, *ip*, twice a day for 15 days. The animals were killed 5 and 30 days after the last of the injections and the kidneys were removed for histological, immunohistochemical and Western blot analysis and for nitrate determination. The results of the immunohistochemical study were evaluated by counting the p-p38 MAPK-positive cells per area of renal cortex measuring 0.05 mm^2. Creatinine was measured by the Jaffé method in blood samples collected 5 and 30 days after the end of the treatments. Gentamicin-treated rats presented a transitory increase in plasma creatinine levels. In addition, animals killed 5 days after the end of gentamicin treatment presented acute tubular necrosis and increased nitrate levels in the renal cortex. Increased expression of p-p38 MAPK and NF-κB was also observed in the kidneys from these animals. The animals killed 30 days after gentamicin treatment showed residual areas of interstitial fibrosis in the renal cortex, although the expression of p-p38 MAPK in their kidneys did not differ from control. Treatment with PDTC reduced the functional and structural changes induced by gentamicin as well as the expression of p-p38 MAPK and NF-κB. The increased expression of p-p38 MAPK and NF-κB observed in these rats suggests that these signaling molecules may be involved in the pathogenesis of tubulointerstitial nephritis induced by gentamicin.

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- Inflammation
- Renal fibrosis
- Gentamicin-induced acute tubular necrosis
- Nitric oxide

Cell growth, differentiation and apoptosis are regulated by a variety of extracellular signals. The type, duration and magnitude of these signals are efficiently transmitted to the inside of the cells, where signaling complexes are assembled for appropriate integration and processing (1,2). Mitogen-activated protein kinases (MAPK) are important mediators involved in the intracellular network of interaction proteins that transduce extracellular stimuli to intracellular responses (3). Three distinct MAPK pathways have

been described in mammalian cells: extracellular signal-regulated kinase, c-Jun-N-terminal kinase, and p38 MAPK (4).

p38 MAPK is a ubiquitous, highly conserved protein kinase that plays an important role in the inflammatory response and in the apoptosis process (2,5). p38 MAPK is activated by cytokines and cellular stress, and its activation results in increased production of inflammatory cytokine genes including interleukin-1ß and tumor necrosis factor-α (TNF-α) (5,6). There is also evidence that p38 MAPK may be involved in nuclear factor kappa B (NF-κB) activation (7). NF-κB is an important transcription factor which is present in the cytoplasm of every cell type in an inactive form and, upon stimulation, is released from an inhibitory subunit (IκB) and translocates into the nucleus, promoting the transcriptional activation of target genes related to the inflammatory process such as interleukin-1ß and TNF-α (8).

Gentamicin treatment provokes acute tubular necrosis and acute renal failure in about 20% of high-risk patients (9). Animal models of aminoglycoside nephrotoxicity also present residual areas of interstitial fibrosis in the renal cortex and progressive tubular injury (10,11). Therefore, since there is evidence that both the MAPK and NF-κB systems can be activated by oxidative stress in gentamicin-treated animals, we investigated the expression of p-p38 MAPK and NF-κB in the kidney during the evolution of tubulointerstitial nephritis and its relationship with histological features and renal function in gentamicin- or gentamicin + pyrrolidine dithiocarbamate (PDTC, an NF-κB inhibitor)-treated rats.

Sixty-eight female Wistar rats (180-200 g) were used. The animals were divided into four groups: group 1 (N = 20), rats injected with gentamicin (Schering-Plough S/A, Rio de Janeiro, RJ, Brazil), 40 mg/kg, *im*, twice a day for 9 days; group 2 (N = 20) rats injected with gentamicin, *im*, twice a day for 9 days and PDTC, 50 mg kg^{-1} day^{-1}, *ip*, twice a day for 15 days, starting one day before the first gentamicin injection; group 3 (N = 14), control, rats injected with 0.15 M NaCl, *im,* twice a day for 9 days; group 4 (N = 14), control PDTC, rats injected with 0.15 M NaCl, *im*, twice a day for 9 days and PDTC, 50 mg kg^{-1} day^{-1}, *ip*, twice a day for 15 days, starting 1 day before the first gentamicin injection.

Blood samples were collected 5 and 30 days after the end of treatment with gentamicin or NaCl to quantify creatinine (12). The animals were killed on the same days as blood collection, 32 rats at day 5: 8 injected with gentamicin, 10 with gentamicin + PDTC, 7 with NaCl and 7 with NaCl + PDTC and 36 rats at day 30: 12 injected with gentamicin, 10 with gentamicin + PDTC, 7 with NaCl and 7 with NaCl + PDTC. The organs were perfused with PBS (0.15 M NaCl containing 10 mM sodium phosphate buffer, pH 7.4) and the kidneys were removed for histological and immunohistochemical analysis, for nitrate determination, and for Western blot studies.

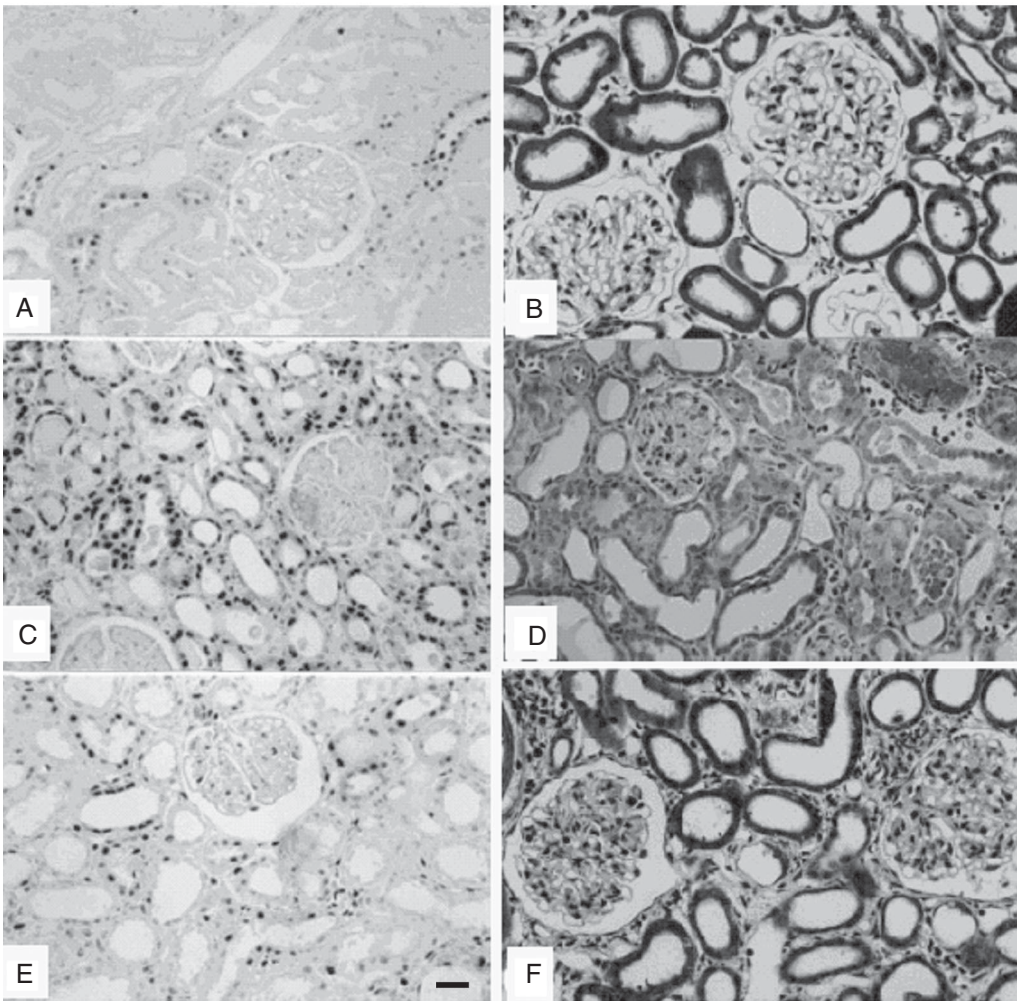
Whole kidney cortical tissue from animals killed 5 days after the end of gentamicin or saline treatment was homogenized in 2 mL Triton X-100 lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 1 µg/mL aprotinin, 1 µg/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride) at 4ºC. After incubation for 5 min, the lysate was centrifuged at 4ºC for 15 min at 10,000 *g* and stored frozen at -70ºC. Lysate samples were thawed and deproteinized with 95% cold ethanol (4ºC) for 30 min and centrifuged. The supernatant solution was used for nitrate determination by the nitric oxide (NO)/ozone chemiluminescence technique as described by Hampl et al. (13), using a Sievers Analyzer (Sievers 280 NOA, Sievers, Boulder, CO, USA).

The whole lysate was also used for Western blot studies. Thirty micrograms of protein was loaded per well and separated on a

10% SDS-PAGE gel. Gels were electroblotted onto a nitrocellulose membrane, incubated for 4 h in 30 mL blocking buffer (PBS, 5% skim milk), washed in buffer (PBS, 0.1% Tween 20, pH 7.6) and incubated with anti-p-p38 MAPK (Sigma-Aldrich, St. Louis, MO, USA; 1/800) or anti-NF-κB p65 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1/200) overnight at 4ºC. To determine the equivalence of protein loading the membranes were also incubated with anti-α_1-tubulin (Sigma; 1/2000) in 5% bovine serum albumin overnight at 4ºC. Blots were washed and incubated with horseradish peroxidase-conjugated goat 1/10000 anti-mouse or 1/5000 anti-rabbit IgG (Dako, Glostrup, Denmark) for 1 h at room temperature. Membranes were washed and the membrane-bound antibody was detected using the Supersignal West Pico Chemiluminescent substrate (Pierce, Rockford, IL, USA) and captured on X-ray film. The intensity of the identified lanes was quantified by densitometry using ImageJ NIH image software and is reported in arbitrary units.

The kidneys from control animals and from animals killed 5 and 30 days after gentamicin or gentamicin + PDTC treatment were fixed in 4% paraformaldehyde, post-fixed in Bouin's solution for 4-6 h, and processed for paraffin embedding. Four-micrometer histological sections were stained with Masson's trichrome and examined under the light microscope. In addition, the kidneys from control animals (N = 7) and from animals killed 5 days or 30 days after gentamicin or gentamicin + PDTC treatment (N = 8 per group) were subjected to immunohistochemical staining (14). The sections were incubated overnight at 4ºC with 1/1,000 p-p38 MAPK monoclonal antibody and the reaction product was detected with an avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA, USA). The color reaction was developed with 3,3'-diaminobenzidine (Sigma) and 8% nickel chloride and the material was then counter-stained with methylgreen, dehydrated and mounted. To obtain the mean number of p-p38MAPK-positive cells in the renal cortical tubulointerstitium, 30 grid fields measuring 0.05 mm^2 each were evaluated, and mean counts per kidney were calculated. All data were submitted to analysis of variance with multiple comparisons by the Tukey test, with the level of significance set at $P < 0.05$.

Gentamicin-treated rats presented a transitory increase in plasma creatinine levels evaluated on day 5 after treatment (1.65 ± 0.34 mg/dL) compared to control (0.48 ± 0.03 mg/dL; $P < 001$) that returned to normal levels at day 30 (0.57 ± 0.04 mg/dL). This change observed on day 5 was less intense in gentamicin + PDTC-treated rats (1.08 ± 08 mg/dL; $P < 0.05$). The histological study showed alterations characteristic of acute tubular necrosis in the renal cortex from the rats treated with gentamicin only and killed 5 days after treatment: tubular cell necrosis, focal areas of denuded basement membrane, intraluminal casts, swelling and flattening of proximal tubular cells with brush border loss, diffuse interstitial edema, and interstitial inflammatory cell infiltrates (Figure 1D,F). Histological features of chronic nephropathy such as interstitial fibrosis, tubular atrophy or dilatation were observed on day 30 after this treatment (data not shown). Glomerular morphology remained unchanged. We observed in previous studies that the percentage of damaged areas in the renal cortical tubulointerstitium from rats killed on day 30 after gentamicin treatment was 23.2% and the percentage of glomeruli with sclerosis was 6.4% despite the recovery of renal function. Thus, we can infer that those animals probably had enough normal glomeruli and tubulointerstitial areas to maintain renal function (15). Houghton et al. (10) also observed that glomerular filtration rate was preserved in rats with chronic gentamicin nephrotoxicity despite the progressive tubular injury. Treatment with PDTC attenuates the increase in plasma creatinine



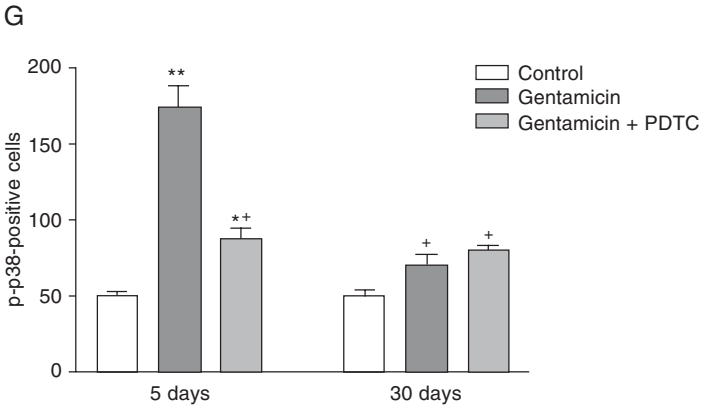


Figure 1. Immunolocalization of p-p38 MAPK in the renal cortex (A, C and E) and in Masson's trichrome-stained histological sections (B, D and F) from rats killed 5 days after treatment with NaCl (A and B), gentamicin (C and D) or gentamicin + PDTC (E and F). Note that the number of p-p38 MAPK-positive cells is higher in C than in A and E and that the acute tubular necrosis, flattening of proximal cells and increase in interstitial area are more intense in D than in F. *G*, Number of p-p38-positive cells per grid field of renal cortex measuring 0.05 mm^2. MAPK = mitogen-activated protein kinases; PDTC = pyrrolidine dithiocarbamate. Original magnification X280 (G). Bar = 24.94 µm for all panels. *P < 0.05 *vs* control; **P < 0.001 *vs* control; +P < 0.001 *vs* gentamicin 5 days (Tukey test).

levels and the histological changes observed in gentamicin-injected animals. PDTC administered alone did not affect the parameters of renal function and structure investigated (data not shown).

Our studies regarding the levels of tissue nitrate, a final product of NO reaction, showed that rats killed 5 days after gentamicin treatment presented significantly ($P < 0.05$) increased renal nitrate levels (0.660 ± 0.220 μM/mg protein) when compared to control animals (0.032 ± 0.006 μM/mg protein). Numerous effects of reactive oxygen species (ROS) in the regulation of cellular functions are mediated by NO (16). Although endothelial NO may have a beneficial role as a vasodilator by inducing an increase in renal blood flow and in glomerular filtration in these animals, excessive NO production can lead to cytotoxic injury. Peroxynitrite anion formation, protein tyrosine nitration, and hydroxyl radical production may contribute to the evolution of the renal lesions induced by gentamicin (17).

The immunohistochemical studies showed a significantly increased number of p-p38 MAPK-positive cells ($P < 0.001$) in the renal cortex from gentamicin-treated animals killed 5 days after treatment (174.0 ± 14.63 per 0.05 mm^2) when compared to control (50.0 ± 2.65 per 0.05 mm^2). Most cells from the proximal tubules of animals treated only with gentamicin expressed p-p38. This alteration was reduced by treatment with PDTC and was not observed in the animals killed 30 days after gentamicin treatment. These data show that p38 MAPK is more activated in the tubulointerstitial cells during the early stages of this disease when the inflammatory process is more intense and diffuse. The p-p38 MAPK immunoreaction was localized in the tubular and interstitial cells, showing a diffuse distribution pattern in the renal cortex from gentamicin-treated animals killed 5 days after treatment (Figure 1C) and a focal distribution primarily in the damaged areas on day 30 (data not shown).

Western blot analysis performed with antibodies against p-p38 MAPK or NF-κB demonstrated the presence of 43-kDa (p-p38 MAPK) and 65-kDa (NF-κB) protein lanes in the renal cortex from all groups studied. The corresponding lanes from gentamicin-treated animals killed 5 days after treatment were more prominent than those obtained for control and PDTC + gentamicin-treated animals (Figure 2). There was no

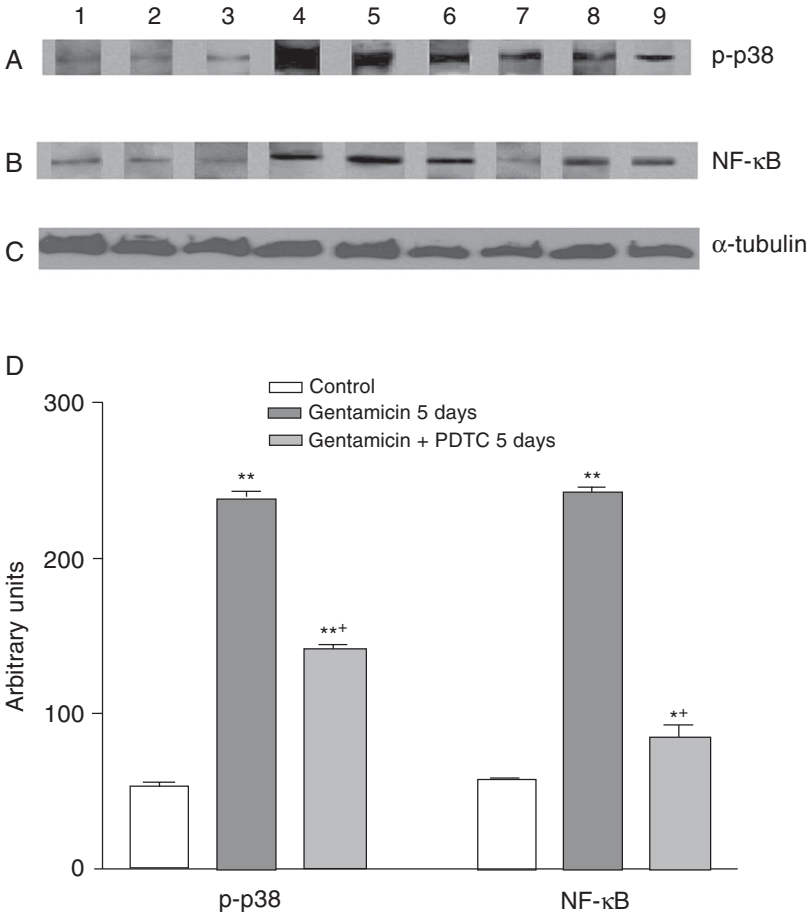


Figure 2. Western blot analysis of p38 MAPK (A), NF-κB (B) and α_1-tubulin (C) in renal cortex from control rats (lanes 1-3), from rats killed 5 days after treatment with gentamicin (lanes 4-6), or gentamicin + PDTC (lanes 7-9). Note that the corresponding lanes from gentamicin-treated rats (4-6) are more prominent than those corresponding to the control or PDTC + gentamicin groups and that there was no difference in the intensity of the signal between the different groups for α_1-tubulin (C). *D*, Densitometry of p-p38 MAPK and NF-κB lanes in the renal cortex from control rats (N = 3) and from rats killed 5 days after treatment with gentamicin (N = 3) or PDTC + gentamicin (N = 3). Data are reported as mean ± SEM. MAPK = mitogen-activated protein kinases; NF-κB = nuclear factor kappa B; PDTC = pyrrolidine dithiocarbamate. *$P < 0.01$ *vs* control; **$P < 0.001$ *vs* control; $^{+}P < 0.001$ *vs* gentamicin 5 days (Tukey test).

difference in the intensity of the lanes for α_1-tubulin between the different groups, showing the equivalence of protein loading (Figure 2C). Several studies have shown that both the MAPK and NF-κB systems can be activated by oxidative stress (18), with consequent changes in gene expression that can progress to cell survival or cell death (19). PDTC as an antioxidant can reduce the ROS present in the gentamicin-induced inflammatory process (18,20). PDTC also has a direct effect on NF-κB activation by blocking IκB degradation (20). It has been reported that specific and selective p38 MAPK inhibitors can block the production of inflammatory molecules, reducing the apoptotic cell death and ameliorating the acute renal injury observed in some animal models of renal disease such as anti-GBM glomerulonephritis and ischemia/reperfusion (2,4). Furthermore, there is evidence that the production of TNF post-renal injury is triggered by the locally produced ROS, which activate NF-κB through p38 MAPK (7). The signaling cascade leading to inflammatory cytokine production includes the phosphorylation of Ras, which in turn initiates the protein kinase cascade which ultimately leads to activation of the MAPK family of protein kinases. Activation of p38 MAPK can also induce NF-κB activation and subsequent transcription of inflammatory cytokines (7). In addition, these inflammatory cytokines and ROS can act as a positive feedback loop, amplifying the inflammatory process observed in acute tubular necrosis.

Our data show that p-p38 MAPK expression is increased during the development of gentamicin-induced interstitial nephritis and that such alteration is associated with enhancement of NF-κB expression and of the inflammatory process in the renal cortex, suggesting that the p38 MAPK pathway may be involved in the renal lesions induced by gentamicin.

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