

# Regulation of JNK/ERK activation, cell apoptosis, and tissue regeneration by monoamine oxidases after renal ischemia-reperfusion<sup>1</sup>

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## SPECIFIC AIM

Reactive oxygen species (ROS) contribute to ischemia-reperfusion (I/R) injury. In the kidney, the intracellular sources of ROS during ischemia-reperfusion are still unclear. We investigated the role of the catecholamine-degrading enzyme monoamine oxidases (MAOs) in hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) generation after reperfusion and their involvement in cell events leading to tissue injury and recovery.

## PRINCIPAL FINDINGS

Sham-operated and ischemic groups of rats were treated with saline or the irreversible MAO inhibitor pargyline (6 mg/kg, i.v.) 15 min prior to 45 min of unilateral ischemia. The kidneys were removed at the end of the ischemic period and after 5 min, 15 min, 30 min, 1 h, 6 h, 24 h, 48 h, 72 h, and 7 days of reperfusion.

### Renal MAO-A activity and MAO-dependent H<sub>2</sub>O<sub>2</sub> production during I/R: effect of pargyline treatment

MAO-A activity was measured using [<sup>14</sup>C]serotonin as substrate and MAO-dependent H<sub>2</sub>O<sub>2</sub> production was determined by luminol-amplified chemiluminescence assay in renal cortex homogenates. Generation of chemiluminescence triggered with a MAO substrate, tyramine, was monitored for 60 min. MAO-A activity and MAO-dependent H<sub>2</sub>O<sub>2</sub> production were inhibited by 65% during ischemia and recovered to control sham-operated values 5 min after reperfusion. Rat treatment with pargyline strongly inhibited MAO activity and MAO-dependent H<sub>2</sub>O<sub>2</sub> production, indicating that pharmacological MAO inhibition prevents H<sub>2</sub>O<sub>2</sub> generation in the early phases of reperfusion.

### MAO inhibition prevents lipid peroxidation induced by I/R

To determine whether H<sub>2</sub>O<sub>2</sub> generation by MAOs participates in oxidative stress during reperfusion, we measured the kidney levels of a marker of lipid peroxi-

dation, malondialdehyde (MDA). After 5 min of reperfusion, MDA transiently increased in saline-treated rats compared with sham-operated animals. Pargyline treatment prevented the MDA increase, suggesting that MAO plays a critical role in oxidative stress.

### Effect of pargyline treatment on tubular necrosis and apoptosis induced by I/R

Cell necrosis and apoptosis, which are highly dependent on ROS production, represent the major postreperfusion events leading to renal injury. In reperfused kidneys of saline-treated rats, extensive necrosis (>75% in 17 of 24 rats), mainly located at the proximal tubules, was evident between 24 and 72 h of reperfusion. Pretreatment with pargyline 15 min before ischemia significantly reduced the degree of tubular necrosis (<25% in 19 of 24 rats). This protective effect was evident only when pargyline was administered before ischemia.

Cell apoptosis was evaluated by TUNEL immunostaining and morphological analysis. The number of tubular epithelial cells containing TUNEL-positive nuclei increased after 6 and 24 h of reperfusion compared with the sham-operated controls (Fig. 1A, B). In contrast, cell staining in pargyline-treated rats (P+I/R) was not different from that observed in sham-operated rats untreated (S) or treated (P) with pargyline. The typical morphological features of apoptosis such as chromatin and cytoplasmic condensation are depicted in Fig. 1c.

### Effect of pargyline treatment on renal cell proliferation after I/R

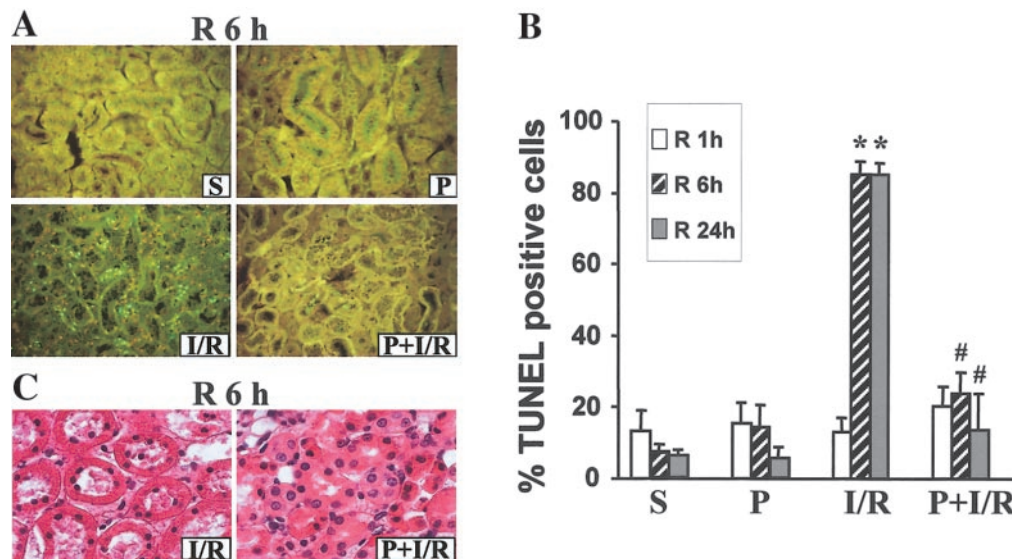
To investigate whether in addition to controlling cell death processes MAO inhibition promotes cell regeneration, we evaluated PCNA expression in kidney sections

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**Figure 1.** Effect of pargyline pretreatment on apoptosis induced by renal ischemia-reperfusion. Apoptosis was evaluated by TUNEL staining (A, B) and morphological examination (C) of sections of kidneys. Sham-operated and ischemic groups of rats were treated with saline or pargyline (6 mg/kg, i.v.) 15 min before 45 min of unilateral ischemia. Rats were killed after 1, 6, and 24 h of reperfusion. A) Light photomicrographs (magnification  $\times 40$ ) of kidney sections 6 h after reperfusion. The bright green dots correspond to a representative TUNEL-positive (fluorescent) nucleus. B) Percentage of TUNEL-positive cells in kidney sections at different times of reperfusion. Data shown are mean  $\pm$  SE of 4 independent experiments quantified in triplicate.  $*P < 0.001$  vs. sham group;  $\#P < 0.001$  vs. ischemic group. C) Representative light micrographs (magnification  $\times 100$ ) of hematoxylin and eosin-stained sections showing morphological features of tubule cells after 6 h of reperfusion. S: sham-operated rats treated with saline; P: sham-operated rats treated with pargyline; I/R: rats subjected to ischemia followed by reperfusion; P+I/R: rats treated with pargyline before ischemia followed by reperfusion.



from the different groups of rats. Renal PCNA expression increased 2 and 3 days after I/R vs. kidneys from sham-operated rats. Pargyline treatment did not modify PCNA staining in sham-operated rats, but significantly increased the number of PCNA-positive nuclei in proximal tubules at 6, 48, and 72 h after I/R compared with untreated rats (Fig. 2). Together, these data indicate that MAO inhibition promotes the tubular cell proliferation involved in the recovery of renal structure after I/R.

### Effect of pargyline treatment on mitogen-activated protein kinase (MAPK) activation after I/R

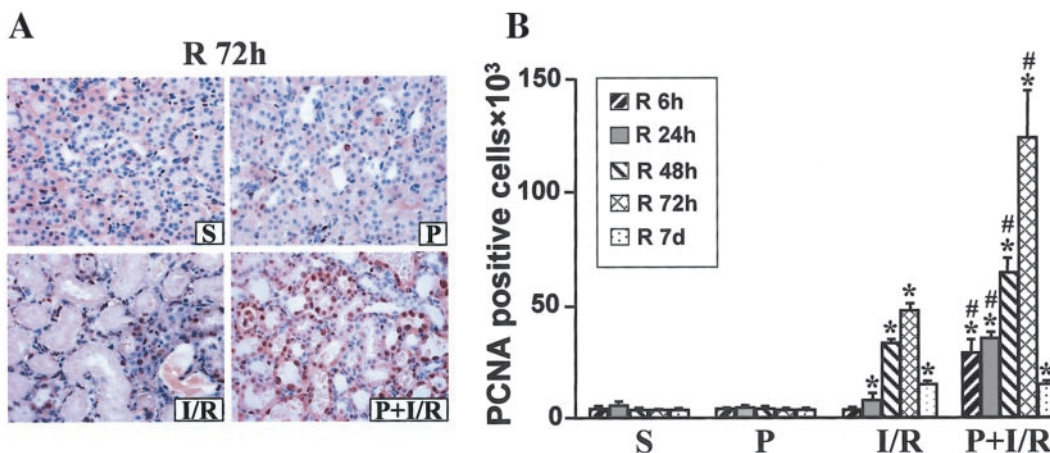
To determine the mechanisms involved in controlling cell death processes and promotion of regeneration by MAO inhibition, we examined extracellular signal-regulated ki-

nases (ERKs) and Jun amino-terminal kinases (JNKs), described in ischemia and reperfusion injury as part of a 'survival' and 'death' pathway, respectively. The Thr/Tyr phosphorylation status of ERKs and JNKs corresponding to the activated forms of these enzymes was faintly detectable in sham-operated rats. ERK1/ERK2 phosphorylation remained weak after 6 and 24 h of reperfusion whereas JNK2 phosphorylation strongly increased. Pargyline treatment reversed JNKs phosphorylation and caused ERK1/ERK2 activation, indicating that MAO inhibition can regulate the balance of ERK/JNK activities.

### CONCLUSIONS AND SIGNIFICANCE

The extent of renal necrosis and the time required for tissue regeneration are major determinants of the

**Figure 2.** PCNA expression in rat kidneys subjected to ischemia-reperfusion after pargyline treatment. Immunohistochemistry for PCNA counterstained with hematoxylin, showing positive staining (brown) for proliferating cells. A) Representative photographs ( $\times 40$ ) of kidney sections. B) Number of PCNA positive nuclei in saline- and pargyline-treated rats after 6 h, 24 h, 48 h, 72 h, and 7 days of reperfusion. Data are means  $\pm$  SE of 4 independent experiments performed in triplicate.  $*P < 0.001$  vs. sham group;  $\#P < 0.001$  vs. ischemic group. S: sham-operated rats treated with saline; P: sham-operated rats treated with pargyline (6 mg/kg, i.v.); I/R: rats subjected to 45 min ischemia followed by reperfusion; P+I/R: rats treated with pargyline 15 min before ischemia followed by reperfusion.



## Renal unilateral ischemia/reperfusion

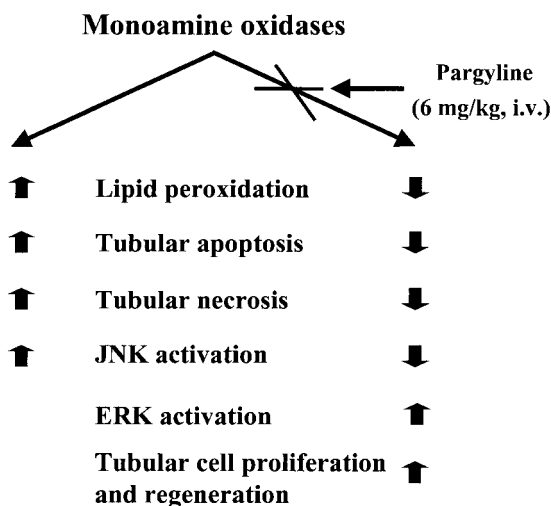


Figure 3.

prognosis of renal failure after I/R. We show that postreperfusion renal injury and regeneration are strongly dependent on MAO activity. In fact, inhibition of renal MAO by pargyline administration before ischemia significantly reduces renal oxidative stress, JNK activation, apoptosis, and necrosis induced by reperfusion and increases ERK activation and tubular cell proliferation. These effects were not observed when pargyline was administered 1 h after the beginning of reperfusion suggesting the critical role of MAO in the initiation of renal cell injury.

These results provide new insights into the function of MAOs in the kidney. To date, renal MAOs have been regarded exclusively as factors regulating the availability of their substrates, particularly dopamine and serotonin and their effects on sodium excretion. The results of the present study suggest that MAO may regulate other renal functions through ROS production. In the kidney, the sources of ROS during reperfusion and their effect on tissue injury and recovery are still ill defined. Our results showing that postreperfusion  $H_2O_2$  production and lipid peroxidation were fully prevented by pargyline treatment indicate that in the kidney 1) MAOs are a major source of  $H_2O_2$  in the early phases of reperfusion and 2) MAO-dependent  $H_2O_2$  production is responsible for the postreperfusion oxidative stress. The large impact of  $H_2O_2$  produced by MAOs on renal apoptosis and necrosis may be related to the regional distribution and subcellular location of these enzymes. MAO expression is particularly high in the proximal tubule cells, which not only produce large amount of ROS during reperfusion but are also the major target of reperfusion injury mediated by ROS. In addition, proximal tubules produce and therefore contain most of the renal dopamine and serotonin, two

MAO substrates. We have recently demonstrated that serotonin and dopamine concentrations significantly increased in the early phase of reperfusion. Acting together, the high MAO expression and elevated substrate concentration may promote the production of large amounts of  $H_2O_2$  in proximal tubules during reperfusion. The subcellular location of MAOs at the outer mitochondrial membrane is another factor that may explain the importance of these enzymes in the induction of cell apoptosis. It has been shown extensively that mitochondria play a key role in apoptosis by the production of ROS and the release of apoptotic factors such as cytochrome *c*, AIF, and calcium. The series of results obtained *in vitro* suggested that mitochondrial dysfunction and cell apoptosis can be induced by MAO-dependent  $H_2O_2$  production. It has been shown that  $H_2O_2$  production by MAO increases the intramitochondrial formation of oxidized glutathione, enhances mitochondrial  $Ca^{2+}$  efflux, and reduces mitochondrial electron transport. It has been reported that MAO inhibitors such as pargyline or clorgyline prevent the apoptosis of human melanoma M14, PC12, and rat renal proximal tubule cells. As observed *in vitro*, production of  $H_2O_2$  by MAOs at the outer mitochondrial membrane may accelerate the mitochondrial processes responsible for apoptosis observed after I/R.

Activation of the JNK cascade, which is dependent in part on  $H_2O_2$  formation, is considered as an important intermediate of cell apoptosis. We show here that postreperfusion tubular cell apoptosis is associated with strong JNK activation. That MAO inhibition prevents JNK phosphorylation suggests that activation of this MAPK participates an intermediate step between  $H_2O_2$  production by MAOs and apoptosis after I/R.

Another member of the MAPK family, ERK, has been implicated in protection against cell injury, and its activation has been considered a sign of renal regeneration and protection. In contrast to JNKs, we found that MAO inhibition by pargyline induced a significant increase in ERK phosphorylation associated with accelerated tubule cell proliferation and recovery of normal renal morphology. Therefore, the beneficial effects of MAO inhibition on post-I/R renal injury may be related to the inversion of the JNK-ERK activation ratio.

Although irreversible MAO inhibitors have been considered potent antidepressive drugs, their use has been limited by the side effects of chronic administration. Our demonstration that a single injection of pargyline is sufficient to decrease post-I/R renal injury and accelerate tubule regeneration opens new perspectives for the therapy of post-I/R renal failure, a major complication of kidney transplantation and angioplasty of renal artery. Clinical studies will be necessary to evaluate the therapeutical properties of irreversible MAO inhibitors in post-I/R syndromes not only in kidney, but also in heart and brain, two organs containing a large amount of MAOs.

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