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Role of reactive oxygen in phospholipase A_2 activation by ischemia/reperfusion of the rat kidney

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Abstract

Purpose. To investigate the role of phospholipase A_2 (PLA_2) in reperfusion injury of the kidney in an in vivo animal model, renal mitochondrial PLA_2 activity was measured under three different conditions.

Methods. Male Wistar rats (n = 72) anesthetized with pentobarbital underwent renal ischemia surgically for 45 min and were reperfused for the indicated time (renal ischemia/reperfusion). Treatments included reperfusion for various predetermined periods (phase 1), exposure to hyperbaric oxygen (phase 2), and administration of reactive oxygen species (ROS) scavenger (phase 3). Thereafter, each kidney was harvested, and mitochondrial PLA_2 activity was measured by a radioisotope technique.

Results. Ischemia/reperfusion resulted in time-related PLA_2 activation in the renal mitochondria up to 48 h of reperfusion after renal ischemia. Renal mitochondrial PLA_2 activity was further augmented by hyperbaric oxygen exposure prior to reperfusion, whereas administration of the ROS scavengers suppressed mitochondrial PLA_2 activity.

Conclusion. These data suggest that ROS may play an important role in the in vivo activation of PLA_2 associated with renal ischemia/reperfusion.

Key words: Phospholipase A_2, Rat kidney, Ischemia/reperfusion, Superoxide dismutase, Dimethylthiourea

Introduction

Phospholipase A_2 (PLA_2) has been reported to play an important role in ischemia/reperfusion injury of some organs, including the kidney [1]. PLA_2 acts on cellular membranes, including mitochondrial membranes, and alters permeability characteristics, thus impairing cellular bioenergetics. Arachidonic acid, an important product of PLA_2 action, is converted to eicosanoids that are vasoactive and chemotactic and contribute to deterioration of the ischemic injury.

Some in vitro studies have shown that a synergistic interaction exists between PLA_2 and reactive oxygen species (ROS) in ischemia/reperfusion injury of the kidney [2]. Membranes exposed to ROS are peroxidized and become more susceptible to PLA_2 action. This synergy occurs at the level of the mitochondria, where PLA_2 acts in concert with ROS to uncouple oxidative phosphorylation and to impair the electron transport chain [2]. However, there is little evidence indicating that this synergistic interaction actually occurs in the living body. The present study was carried out to evaluate in vivo interaction between PLA_2 and ROS in renal ischemia/reperfusion in rats.

Materials and methods

Radioactively labeled phospholipid substrate (1-stearoyl-2-[1-^{14}C] arachidonyl phosphatidylcholine, ^{14}C-PC) was obtained from Amersham Japan, Tokyo, Japan. The ROS scavenger, superoxide dismutase (Cu,Zn-SOD), was from Nippon Kayaku, Tokyo, Japan, and dimethylthiourea (DMTU) was from Sigma Chemical, St. Louis, MO, USA.

Renal ischemia

Male Wistar rats (8–11 weeks of age, weighing 250–350 g, n = 72) were anesthetized with sodium pentobarbital (50 mg·kg^{-1} body weight) administered intraperitoneally and placed on a heated operating table. Following right nephrectomy, the left renal pedicle was exposed and occluded by a microvascular clamp for 45 min, then reperfused for a predetermined

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time. After the indicated period, the left kidney was removed under pentobarbital anesthesia. Sham-operated animals were used as controls, in which the left renal pedicle was exposed but not clamped after right nephrectomy. Following observation for 45 min, the abdomen was surgically closed.

Phase 1 experiment: time course after renal ischemia

To assess reperfusion time-related changes in renal mitochondrial PLA_2 activity after 45 min of renal ischemia, the animals were anesthetized and the kidneys were harvested after 1, 6, 12, 24 and 48 h of reperfusion. Sham-operated animals were allocated into two groups: baseline (rats with immediate sacrifice after the operation) and 48 h control (rats with 48 h observation following the operation) ($n = 7$ in each group).

Phase 2 experiment: effects of hyperbaric oxygen (HBO)

Rats were allocated into one of four groups ($n = 7$ in each). Rats in the control group were nephrectomized and sham-operated without subsequent HBO exposure. In the ischemia-alone group, the kidneys were rendered ischemic without subsequent HBO exposure. In the HBO-alone group, the rats were nephrectomized and sham-operated with no renal ischemia but were exposed to HBO. In the HBO-alone and the ischemia-plus-HBO group, ischemia/reperfusion was performed followed by HBO exposure. In each group, PLA_2 activity was measured after 48 h of reperfusion. In the HBO-alone and the ischemia-plus-HBO group, rats were exposed to HBO for 2 h at 3 absolute atmospheres (ATA) in a hyperbaric chamber starting 2 h after release of the renal clamp. The kidney was excised 44 h after the end of HBO exposure.

Phase 3 experiment: effects of ROS scavengers

Rats were allocated into one of four groups ($n = 7$ in each). The ROS scavengers used were SOD and DMTU. Two minutes before reperfusion, 10 $mg \cdot kg^{-1}$ SOD or 500 $mg \cdot kg^{-1}$ DMTU was administered intravenously. The injection volume was adjusted to 2 $ml \cdot kg^{-1}$ with saline. The same volume of saline was administered to the control and to the saline-treated ischemic group. The kidney was harvested after 24 h of reperfusion.

Mitochondrial fractionation and extraction of PLA_2

In phase 2 and 3 experiments, the excised kidneys were rinsed in ice-cold HEPES buffer (containing 50 mM *N*-hydroxyethyl-piperazine-*N*-ethanesulfonic acid [HEPES, pH 7.5], 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, aprotinin [1000 kallikrein-inactivating units$\cdot ml^{-1}$], leupeptin [20 μM], pepstatin A [20 μM], and PMSF [1 mM]) and immediately minced with iced scissors. The pieces were transferred into homogenizing HEPES buffer, and the kidney was homogenized in 10 ml of buffer for 30 s. The homogenate was then centrifuged at 900 *g* for 10 min to precipitate cell debris and nuclei, followed by centrifugation at 9000 *g* for 20 min to precipitate mitochondria at 4°C. The mitochondrial pellet was washed with HEPES buffer and resuspended in 5 ml of the buffer. This suspension represents the mitochondrial fraction.

PLA_2 assay

PLA_2 activity was measured as previously described, with some modifications [3]. Radioactively labeled phospholipid ^{14}C-PC was used as a substrate. All samples were matched for protein content before the PLA_2 assay.

Two microliters of the substrate (final concentration 15 μM) resuspended in DMSO was pipetted into a microcentrifuge tube. Reactions were initiated by the addition of 1.0 mM Ca^{2+} to each sample (as the final concentration) at pH 7.5. After incubation for 30 min at 37°C, the reaction was terminated by the addition of ethanol containing 2% acetic acid and 100 $\mu g \cdot ml^{-1}$ free arachidonic acid (AA). The release of ^{14}C-AA was analyzed using thin-layer chromatography with development fluid (organic phase of ethylacetate/isooctane/H_2O/acetic acid [55:75:100:8]). The phospholipid and free AA bands were scraped, and radioactivity was counted with a liquid scintillation counter. The specific activity of PLA_2 was expressed as picomoles of AA released from PC per minute per milligram of protein at 37°C.

Statistics

All values are presented as means ± SD. Statistical comparisons were performed using two-way ANOVA with repeated measures for the phase 1 experiment and one-way ANOVA with Fisher's PLSD test for the phase 2 and 3 experiments, with $P < 0.05$ regarded as significant.

Results

Time course of PLA_2 activation

Progressive increases in renal mitochondrial PLA_2 activity were noted after 45 min of renal ischemia and a reperfusion period up to 48 h (Fig. 1). The highest value

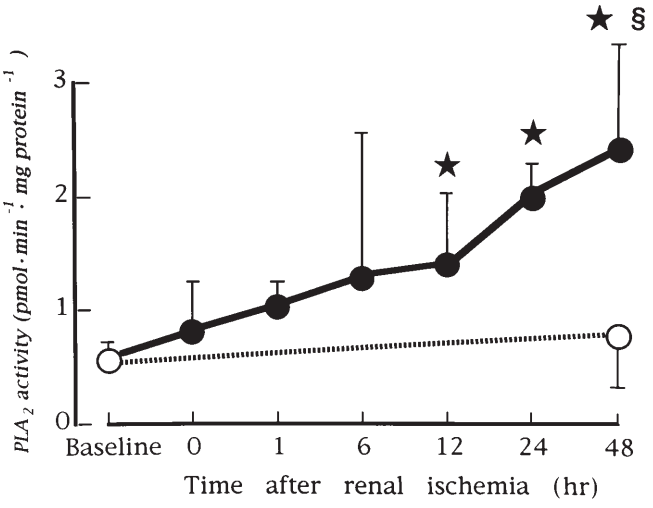

Fig. 1. Time course of changes in renal mitochondrial PLA_2 activity after 45 min of ischemia followed by reperfusion (mean ± SD; $n = 7$ for each value). *Solid circles*, ischemia; *open circles*, control. ★ $P < 0.05$ *vs* baseline, § $P < 0.05$ *vs* 48-h control

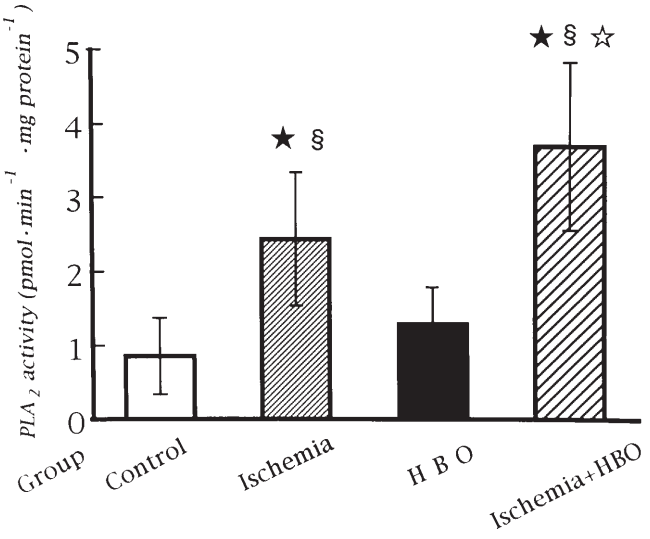

Fig. 2. Effects of ischemia and hyperbaric oxygen on renal mitochondrial PLA_2 activity (mean ± SD; $n = 7$ for each value). Ischemia was applied for 45 min followed by 48 h of reperfusion. Hyperbaric oxygen (HBO) was applied at 3 absolute atmospheres (ATA) for 2 h in a hyperbaric chamber. Kidneys were harvested after 48 h of reperfusion in the ischemia-alone and ischemia + HBO groups, and after corresponding observation in the control and HBO-alone groups. ★ $P < 0.05$ *vs* control group, § $P < 0.05$ *vs* HBO-alone group, ☆ $P < 0.05$ *vs* ischemia-alone group

of PLA_2 activity, 420% of baseline, was observed 48 h after reperfusion. In contrast, PLA_2 activity in sham-operated animals (48 h control) showed no significant changes over time.

Effect of HBO on PLA_2 activation

The renal ischemia group showed enhanced mitochondrial PLA_2 activity to 290% of control group, whereas PLA_2 activity did not change significantly in the HBO-alone group (Fig. 2). However, the addition of HBO to ischemia markedly increased PLA_2 activity up to 430% and 150% of the control and the ischemia-alone groups, respectively.

Effects of ROS scavengers on PLA_2 activation

Renal mitochondrial PLA_2 activities after ischemia/reperfusion in SOD- and DMTU-treated rats were significantly lower than those in saline-treated rats (Fig. 3).

The vehicle did not influence the assay system, i.e., PLA_2 activity was not affected by DMSO added at doses of 0.22, 0.43, 1.06, 2.18, and 4.22 M (data not shown).

Discussion

We previously reported that renal ischemia/reperfusion resulted in an increase in PLA_2 activity of the cytosolic, mitochondrial, and microsomal subcellular fractions of the kidney [4,5]. It has been suggested that mitochondrial PLA_2 activation would be the key process for postischemic cellular injury, since PLA_2 degrades membrane phospholipids, including the cellular membrane and the mitochondrial membrane itself. Thus, in the present study, we focused on the mitochondrial fraction. Renal mitochondria would be exposed to superoxide during postischemic reperfusion [6], and simultaneously it would generate ROS due to abnormalities in intramitochondrial electron transport [7]. Moreover, ROS acted synergistically with calcium to activate isolated kidney mitochondrial PLA_2 [2,8].

In the present study, HBO alone did not induce any significant change in PLA_2 activity in the nonischemic kidney, indicating that this type of oxidative stress alone would not cause PLA_2 activation in the renal mitochondria. However, when HBO was combined with ischemia/reperfusion, renal mitochondrial PLA_2 activity was markedly enhanced, suggesting that the activation of PLA_2 caused by ischemia/reperfusion would be enhanced by oxidative stress-induced toxic oxidant species. The results are in accord with a recent study demonstrating that exposure to 100% oxygen resulted in a significant decrease in SOD activity in the postischemic rat kidney, probably due to consumption by excessive ROS [9]. In the present study, marked renal mitochondrial PLA_2 activation was demonstrated after 45 min of renal ischemia followed by 1 h of reperfusion, and a time-related increase in PLA_2 activation after

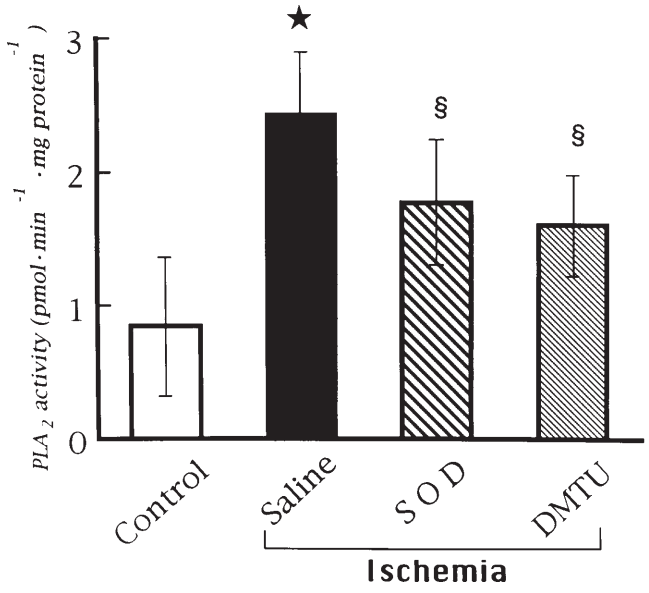

Fig. 3. Effects of ROS scavengers on renal mitochondrial PLA_2 activation after ischemia/reperfusion (mean ± SD; n = 7 for each value). Kidneys were harvested after 24 h of reperfusion in the saline, SOD, and DMTU groups, and after corresponding observation in the control group. ★ $P < 0.05$ *vs* control group, § $P < 0.05$ *vs* saline group

reperfusion was observed up to 48 h after the initiation of reflow (Fig. 1). This result is compatible with the time-related progression of postischemic acute renal failure.

Our data also showed that pretreatment with ROS scavengers could inhibit postischemic PLA_2 activation at renal mitochondria in vivo. The cytoprotective effects of SOD and DMTU against ischemic injury might be due at least in part to prevention of mitochondrial PLA_2 activation. Although SOD (M_r = 31200) is much larger than DMTU (M_r = 104) and may not enter cells with intact membranes, both of these scavengers were effective for suppression of PLA_2 during the period of reperfusion. It is possible that SOD might enter the reversibly injured cells or that SOD might be effective by allowing access to ROS derived from neutrophils in the extracellular space.

In conclusion, oxidative stress enhances the activation of renal mitochondrial PLA_2 by ischemia/reperfusion, whereas scavengers for superoxide and hydroxyl radicals inhibit it. On the other hand, oxidatve stress without ischemia/reperfusion did not cause PLA_2 activation. It is suggested that ROS may play a role in mitochondrial PLA_2 activation during the development of postischemic renal injury.

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