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The cytoprotective effects of verapamil and iloprost (ZK 36374) on ischemia/reperfusion injury of kidneys

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Abstract. We investigated the cytoprotective effects of verapamil, a Ca channel blocker, and of iloprost (ZK 36374), a stable prostacyclin analogue, on ischemia/reperfusion (I/R) injury in Wistar albino rat kidneys that were subjected to 60 min of warm ischemia and reperfusion. The groups included sham, ischemia-untreated (ISCH), verapamil-treated (VER), iloprost-treated (ILO), and verapamil + iloprost (VER + ILO)-treated rats. The 7-day survival of all the treated groups was better than that of the ISCH group. The creatinine concentration on the 3rd day was significantly lower in the VER + ILO group than in the ISCH group. Serum creatinine on day 3 was also low in the VER + ILO groups compared to the ISCH group, although the differences were not significant. The creatinine values on day 7 were significantly lower in the VER and ILO group than in the control, VER, or ILO groups. The malondialdehyde (MDA) concentrations of the kidney cortex tissue after reperfusion in all groups were higher than normal. The tissue-reduced glutathione (GSH) concentrations of the kidneys sampled immediately after reperfusion were significantly lower in the ISCH group than in all of the other treated groups. These results indicate that although verapamil and iloprost have independent cytoprotective effects on 60-min warm ischemia/reperfusion injury of rat kidneys, the protection afforded when both drugs are combined is synergistic. The mechanism of cytoprotection is not limited to the suppression of lipid peroxidation, and a nearly complete protection of reperfusion injury can be obtained by such an intervention.

Key words: Reperfusion, kidney, rat – Rat, kidney reperfusion – Preservation, reperfusion – Verapamil, rat, kidney reperfusion – Iloprost, rat, kidney reperfusion

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Introduction

Warm ischemia of the kidney is often encountered in a number of clinical situations, such as cardiac arrest and resuscitation, hypovolemic shock, and renal vascular interventions. Ischemic renal damage is also one of the leading causes of the loss of transplanted organs [5]. This is important because the supply of transplantable organs is far from that needed to meet the growing demand. There is much interest in research about the prolongation of ischemia time and the conservation of organ function after transplantation.

It has been suggested that reperfusion of ischemic organs produces more damage than ischemia alone [11]. During ischemia, tissue adenosine triphosphate (ATP) levels fall and xanthine and hypoxanthine accumulate in the cell. Also, the cell is unable to preserve its ion gradients – especially calcium (Ca) – across the membranes. The tissue damage that occurs after the reintroduction of oxygen is reported to result from oxygen-free radical (OFR) derivatives that form during reperfusion [9, 15]. An increase in cytosolic Ca during ischemia or reperfusion is reported to activate some proteases and phospholipase A_2 (PLA_2), which, in turn, causes more OFRs and other inflammatory mediators to form and, hence, to produce more tissue destruction [7, 23, 28].

It has been suggested that iloprost (ZK36374), a stable and synthetic prostacyclin analogue, protects the cells from ischemia/reperfusion (I/R) injury by keeping the intracellular ATP levels high and temporarily reducing intracellular Ca levels [2], as well as by exerting its well-known effects of vasodilatation, antiaggregation, and stabilization of the lysosomal membranes [1, 3, 29].

It has been suggested that the accumulation of Ca during ischemia causes activation of proteases, leading to the conversion of xanthine dehydrogenase (XDH) to xanthine oxidase (XO), which is the main source of OFRs [19]. Recent evidence indicates [17] that Ca potentiates the damaging effects of OFR on the mitochondrial electron transport chain due to impairment of NADH-

coenzyme Q-reductase activity through PLA_2 activation, which also causes activation of the arachidonic acid cascade. Verapamil, a slow Ca channel blocker, has been shown to prevent tissue damage in I/R injury by preventing the influx of Ca ions during the period of ischemia [21, 24, 27].

Although a wide variety of drugs have been investigated for cytoprotection of different organs [4, 10, 12, 21, 22, 24, 28], a combination of drugs that exert their actions through different mechanisms has not been fully investigated. In this study, the effects of two different groups of drugs on warm I/R injury of the kidneys were investigated, and the potential mechanisms of interaction betweeen these two drugs are discussed.

Materials and methods

Surgical procedure

Wistar albino rats (200–300 g) were anesthesized with light ether anesthesia, and internal jugular veins were cannulated with a 24 no cannula (Abbott). Blood samples (0.3–0.4 ml) were obtained through this cannula for serum creatinine determination. Following cleaning of the abdomen with povidon-iodine (Batticon) solution, a midline abdominal incision was made, and the left renal vascular pedicle was isolated. A total of 2.5 ml of fluid was infused through the central cannula, 0.5 ml of which included 80 IU heparin, followed by 2.0 ml of isotonic saline, with or without medication (iloprost, verapamil, or both). Next, the pedicle was occluded with atraumatic vascular clamps, except in the sham-operated group. The abdomen was then closed with 3/0 silk sutures, the central cannula was removed, and the rat was returned to its cage. The rat was again anesthesized with light ether anesthesia and the clamp was removed following 60 min of total occlusion. The left kidney was observed for 3–4 min to determine if reperfusion occurred; if not, the rat was not included in the study. Four rats – two in the ISCH group and one each in the VER and ILO groups – were excluded in this manner.

A right nephrectomy was performed and the 2 ml/100 g isotonic saline was left in the peritoneal cavity before the abdomen was closed in two continuous layers with 3/0 silk sutures. In some animals, the left kidney was removed for histologic examination, malondialdehyde (MDA) and reduced glutathione (GSH) determinations, and the animal was sacrificed.

Animals that died before 48 h were excluded from the study because a preliminary study with the same strain of rats demonstrated that bilateral nephrectomy did not result in death within the first 48 h. The surviving rats were anesthesized on the 3rd and 7th days, and blood samples were obtained through jugular catheterization for creatinine determinations using the Technicon RA-1000 autoanalyzer, which utilizes the picric acid reaction. The results are given in mg/dl. All surviving rats were sacrificed on the 7th day, and the left kidney was sampled for histologic examination and MDA and GSH determinations.

Experimental protocols

Five groups of experimental animals were studied. All rats underwent right nephrectomy; thus, all animals in the experimental groups were dependent upon their left kidney for renal function and survival. The groups included:

1. Sham-operated animals (n = 6): These animals underwent jugular vein catheterization and right nephrectomy.
2. Untreated ischemic rats (ISCH, n = 24): These animals underwent the above-mentioned procedure but received only heparin and isotonic saline intravenously.
3. Verapamil-treated ischemic rats (VER, n = 15): These animals were treated the same way as the second group except that before ischemia the 2.0-ml infusion fluid given after heparin contained 0.3 mg/kg verapamil.
4. Iloprost-treated ischemic rats (ILO, n = 12): These animals were treated the same way as the second group except that the 2.0-ml infusion fluid contained 25 μg/kg iloprost.
5. Verapamil + iloprost-treated ischemic rats (VER + ILO, n = 12): These animals were treated the same way as the second group except that the 2.0-ml infusion fluid contained 0.3 mg/kg verapamil and 25 μg/kg iloprost.

Six animals in each group, except for the sham-operated group, were sacrificed 5 min after reperfusion. Their left kidneys were removed, sampled for histologic examination, immersed in liquid nitrogen and stored at −70°C for later determination of MDA and GSH levels.

Histology

The left kidney samples taken at the 5th min of reperfusion or on the 7th day were put into formaldehyde (4%). The tissues were embedded in paraffin, cut into 6-μm-thick slices, and stained with hematoxylin and eosin. The evaluation was made by an observer who had no knowledge of the sampling procedure, according to the criteria outlined by Jablonski et al. [14].

Malondialdehyde (MDA) and glutathione (GSH) determinations

Thiobarbituric acid (TBA) reaction [6] and a modification of the Ellmann procedure [3] were used to determine the MDA and GSH levels, respectively. The molar extinction coefficient for MDA at 532 nm ($1.56 \times 10^5\ M^{-1} \cdot cm^{-1}$) was used and the lipid peroxide levels were expressed as nmol MDA/g dry weight. For GSH, the molar extinction coefficient at 412 nm ($13{,}600\ M^{-1} \cdot cm^{-1}$) was used and the concentration was expressed as μmol GSH/g dry weight).

Statistical analysis

The results are given in the text as mean ± standard error of the mean (SEM). Comparisons of the means were made using Student's t-test. Survival was compared between groups using Fischer's exact test.

Results

Survival

The survival rate of the sham, ILO, and VER + ILO groups was 100%, while it was 89% in the VER group and 61% in the ISCH group (Fig. 1). The difference in survival

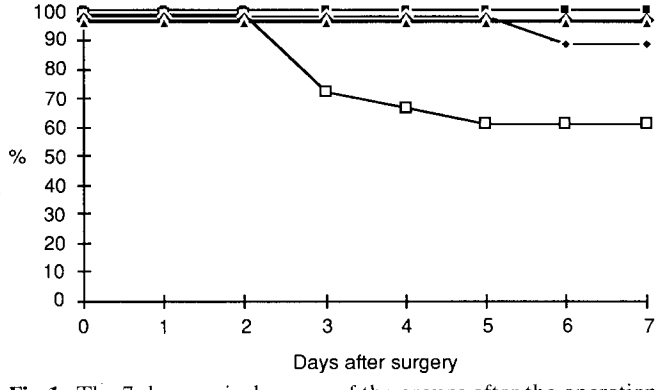

Fig. 1. The 7-day survival curves of the groups after the operation. ■ Sham (n = 6); □ ISCH (n = 18); ◆ VER (n = 9); ◇ ILO (n = 6); ▲ VER + ILO (n = 6)

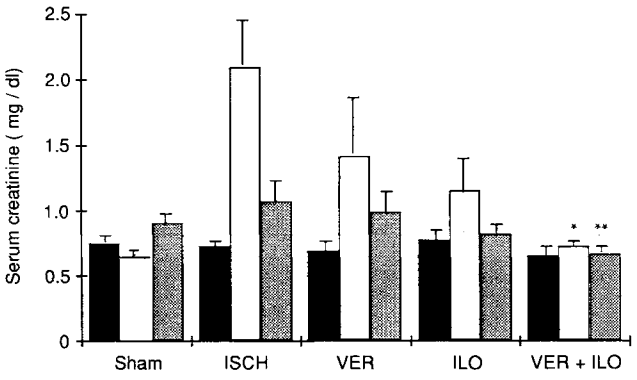

Fig. 2. Serum creatinine levels on days 0 (■), 3 (□), and 7 (▩). Values given represent the mean ± SEM. * $P < 0.01$ when compared to the ISCH group, ** $P < 0.05$ when compared to the ISCH group and $P < 0.01$ when compared to the VER or ILO groups

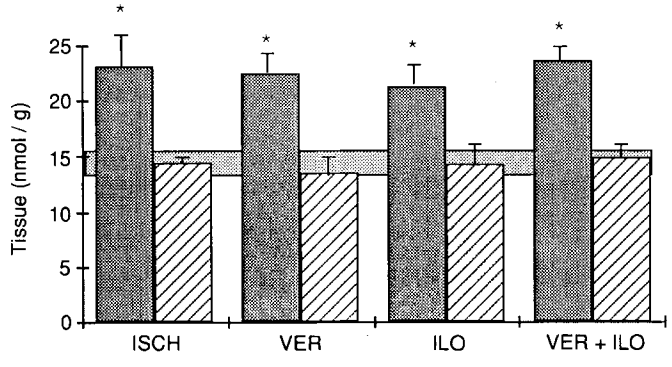

Fig. 3. MDA levels of the tissue samples taken at the 5th min (▩) and on the 7th day (▨). Values given represent the mean ± SEM. The *horizontal bar* indicates the normal level (± SEM). * $P < 0.01$ when compared to the normal level

was not significant between the ISCH group and the other groups ($P < 0.07$). All of the rats that died in the ISCH group died between the 3rd and 5th days following surgery.

Creatinine

The mean creatinine level of the animals before ischemia was found to be 0.72 ± 0.02 mg/dl and did not differ between groups. The mean creatinine levels (± SEM) 3 days after injury were found to be 0.69 ± 0.02 mg/dl in the sham group, 2.06 ± 0.23 mg/dl in the ISCH group, 1.41 ± 0.28 mg/dl in the VER group, 1.35 ± 0.18 mg/dl in the ILO group, and 0.72 ± 0.02 mg/dl in the VER + ILO group (Fig. 2). Significant differences were found between ISCH and VER + ILO groups ($P = 0.007$) and between ISCH and sham groups ($P = 0.005$).

The mean creatinine levels of the animals on the 7th day were found to be 0.9 ± 0.03 mg/dl in the sham group, 1.07 ± 0.09 mg/dl in the ISCH group, 0.98 ± 0.05 mg/dl in the VER group, 0.82 ± 0.03 mg/dl in the ILO group, and 0.66 ± 0.02 mg/dl in the VER + ILO group (Fig. 2). The differences between the ISCH and VER + ILO groups

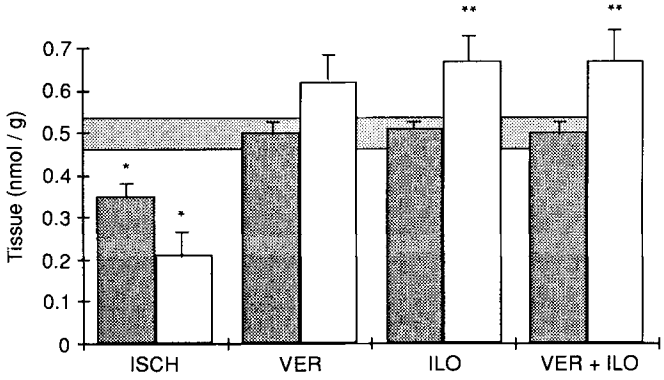

Fig. 4. GSH levels of the tissue samples taken at the 5th min (▩) and on the 7th day (□). Values given represent the mean ± SEM. The *horizontal bar* indicates the normal level (± SEM). * $P < 0.01$ when compared to the normal level or to the other groups, ** $P < 0.05$ when compared to the levels in samples taken at the 5th min of the same group

($P = 0.027$), between the VER and VER + ILO groups ($P = 0.004$), and between the ILO and VER + ILO groups ($P = 0.008$) were statistically significant.

Malondialdehyde (MDA)

The MDA concentration in normal, untreated kidneys was found to be 14.68 ± 0.79 nmol/g. MDA concentrations at the 5th minute of reperfusion were found to be 23.06 ± 1.38 nmol/g in the ISCH, 22.46 ± 0.76 nmol/g in the VER, 21.19 ± 1.08 nmol/g in the ILO, and 23.49 ± 0.65 nmol/g in the VER + ILO group. The concentrations on the 7th day were 14.36 ± 0.36 nmol/g in the ISCH, 13.57 ± 0.34 nmol/g in the VER, 14.19 ± 0.95 nmol/g in the ILO, and 14.87 ± 0.55 nmol/g in the VER + ILO group (Fig. 3). The concentration of MDA taken at the 5th minute of reperfusion in all groups was significantly higher than the normal concentration and than the concentration on the 7th day ($P < 0.01$). On the 7th day, the MDA concentrations in all groups returned to normal levels.

Glutathione (GSH)

The GSH concentration in normal, untreated kidneys was found to be 0.51 ± 0.02 μmol/g. GSH concentrations at the 5th minute of reperfusion were found to be 0.35 ± 0.02 μmol/g in the ISCH, 0.50 ± 0.01 μmol/g in the VER, 0.51 ± 0.01 μmol/g in the ILO, and 0.50 ± 0.01 μmol/g in the VER + ILO group. The concentrations on the 7th day were 0.21 ± 0.02 nmol/g in the ISCH, 0.62 ± 0.4 μmol/g in the VER, 0.68 ± 0.01 μmol/g in the ILO, and 0.67 ± 0.01 μmol/g in the VER + ILO group (Fig. 4). The concentration of GSH taken at the 5th minute of reperfusion in all groups was significantly higher than in the ISCH group ($P < 0.001$), but there was no difference between other groups. On the 7th day, the difference in GSH concentrations between the ISCH and other groups remained ($P < 0.01$), while there was no difference between the

other groups. The GSH concentrations on the 7th day in the ISCH group were significantly lower than the normal level ($P < 0.01$) while they were significantly higher than the normal concentration in the VER ($P < 0.05$), ILO ($P < 0.01$), and VER + ILO ($P < 0.05$) groups. When the concentrations at the 5th min and on the 7th day were compared within groups, a significant reduction was observed in the ISCH group ($P < 0.01$) and a significant increase was observed in the ILO ($P < 0.01$) and VER + ILO ($P < 0.05$) groups; there was no significant increase in the VER group.

Histologic evaluation

We could not find a significant difference in tissue samples taken at the 5th min and on the 7th day. There were no signs of damage in the samples taken on the 7th day. Although ischemic injury findings up to grade 3 were observed in the samples taken early, there was no significant difference between groups.

Discussion

Most studies concerning I/R injury investigate only one aspect of the mechanism. However, multiple mechanisms of injury may be involved, and a synergistic effect may be obtained by combining different groups of drugs to suppress I/R injury [12]. We investigated the effects of two drugs – a slow Ca channel blocker, verapamil, and a synthetic prostacyclin analogue, iloprost (ZK36374) – in a warm I/R model of rat kidneys.

Wait et al. [27] described a chronic, clamp-induced model of ischemic renal failure, occluding the renal artery of rats for 45 min, followed by contralateral nephrectomy, and reported protection by verapamil (66 mg/dl) infusion. Nauta et al. [21] have also demonstrated a beneficial effect of verapamil after 90 min of warm liver ischemia in the in vivo rat liver when given before the ischemic insult but not if given at the end of ischemia or before or during early reperfusion.

It has been suggested that verapamil protects the cells by preventing Ca accumulation during ischemia, which causes activation of the proteases leading to the conversion of XDH to XO [19]. A recent paper by Marubayashi et al. [18] questioned the rate of conversion during ischemia and the significance of this conversion in the formation of OFRs in I/R injury. The authors suggested that the conversion of XDH to XO may not be the mechanism of protection in verapamil. The lack of a protective effect by verapamil given just before reperfusion [21] suggests that it does not effectively prevent the influx of Ca ions through the defects on the cell membrane that are created by the effects of OFRs, as shown by Franceschi et al. [8]. This suggests that if it prevents the influx of Ca, it must be through its effect on voltage-dependent pathways, fast Na channels, or arachidonic acid pathway metabolites. Malis and Bonventre [17] suggested that Ca potentiates the damaging effects of OFR on the mitochondrial electron transport chain due to impairment of NADH-coenzyme Q-reductase activity through PLA_2 activation, which also causes the activation of the arachidonic acid cascade. The inability to increase ATP levels during reperfusion causes a delay in the restoration of cell function. The inability of the ATP-dependent Ca transport systems to move the high intracellular Ca outside of the cell or into the endoplasmic reticulum and mitochondria adds to the problem. The persistance of high Ca levels in a OFR-saturated milieu causes the persistence of the arachidonic acid cascade; thus, a vicious cycle begins. As intracellular Ca levels were not measured in this study, it is not possible to conclude whether the protective effect of verapamil is through Ca channels or other pathways.

The detrimental effects of the arachidonic acid cascade metabolites, such as thromboxane A_2 (TXA_2) and leukotrienes, have been shown to be negated by prostacyclin and its analogues [2, 13, 20, 25]. The protective effects of prostacyclins include vasodilatation, inhibition of thrombocyte aggregation, and stabilization of all membranes, including the lysosomes that contain proteases. Although the mechanism of action of these drugs is not thoroughly understood, maintenance of ATP levels, increasing cAMP levels, and the removal of Ca ions from the cell have all been implicated [25]. It has also been reported that the Ca-dependent formation of blebs by thromboxane can be prevented by prostacyclin infusion [13]. These studies suggest that prostacyclin may antagonize the effects of increased intracellular Ca and may even lower the level of intracellular Ca ions.

The OFRs that form during reperfusion cause peroxidation of membrane lipids [18], and a second massive influx of Ca occurs [8]. The ability of the OFR scavengers to prevent the formation of the metabolites of the arachidonic acid cascade [16] suggests that Ca-dependent PLA_2 activation is mediated by the OFRs. Since verapamil cannot adequately prevent this influx of Ca, the effects of this influx may be prevented by the high levels of iloprost. Iloprost may provide this protection by maintaining high cAMP levels, by its effluxing effect, or by reducing the Ca-dependent PLA_2 activation in the presence of OFRs.

In this study survival did not differ between groups treated with drugs and it was better in these groups than in the nontreated, control group. The creatinine levels on days 3 and 7 were lower in the VER group, but they were not statistically significant when compared with levels in the ISCH group. We obtained similar results with iloprost infusion. However, protection of I/R injury was enhanced when both drugs were used. The creatinine levels of the VER + ILO group and of the sham group were identical. A comparison of the serum creatinine levels between VER, ILO, and VER + ILO groups suggests better preservation of renal function with the simultaneous use of both drugs.

There was no difference between groups in tissue concentrations of MDA; however, concentrations at the 5th min of reperfusion were all higher than the normal and 7-day concentrations, which were normalized in all groups. This suggests that the protective effects of these drugs were not mediated by the prevention of lipid peroxydation of cell membranes. However, MDA concentrations are not always good indicators of lipid peroxidation,

and timing of sampling may also affect the results. GSH concentrations were found to be within the normal range in drug-treated groups at 5 min of reperfusion. GSH concentrations were higher in ILO and VER + ILO groups on the 7th day than they were at the 5th min. This suggests that both drugs have the capacity to lower oxidant levels in I/R. Preventing the reduction of GSH tissue levels makes it possible to preserve ATP levels by breaking the futile cycle that causes increased ATP consumption as a result of the H_2O_2-induced Ca efflux from the mitochondria.

The histologic evaluation did not correlate with the other parameters, but the lack of any microscopic evidence of injury on the 7th day suggests the total healing of the tissues in the living rats.

In this study, the combined use of verapamil and iloprost in this 60-min warm ischemia/reperfusion model of rat kidneys resulted in excellent preservation of renal function. This suggests that this combination may be worth studying in other models of ischemia. Since prostacyclin analogues have been reported to prevent acute rejection after experimental transplantation [26], it is worth performing clinical investigations into the efficacy of these drugs.

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