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Influence of the 21-aminosteroid U74389F on ischemia-reperfusion injury in the rat

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Abstract

We examined the effects of the administration of 21-[4-(2,6-di-1-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl]-pregna-1,4,9(11)-triene-3,20-dione, monomethansulfonate (U74389F), a 21-aminosteroid and so-called lazaroid, that is characterized by an inhibitory activity against iron-dependent lipid peroxidation, on ischemia-reperfusion renal injury in a rat model. After either 60 or 90 min of ischemia, plus 2 or 24 h of reperfusion, kidneys were assayed for glutathione, adenine nucleotides and lipid peroxidation products. 60 min of ischemia produced too little oxidative stress and/or too much spontaneous recovery to allow visualization of the protective effect of the drug. 90 min of ischemia followed by reperfusion induced significant glutathione oxidation, the free oxidized glutathione to total glutathione redox ratio (%) being enhanced from $4.6 \pm 0.7\%$ before kidney clamping to 11 ± 1 and $8.6 \pm 1.4\%$ at 2 and 24 h reperfusion, respectively. Treatment with the lazaroid provided significant protection against this oxidation ($4.9 \pm 1.05\%$ at 24 h reperfusion). Results of lipid peroxidation confirmed the antioxidant effect of the lazaroid. In conclusion this study provides evidence for a protective role of the tested lazaroid against ischemia-reperfusion renal injury in the rat.

Keywords: Glutathione; Lipid peroxidation; Oxidative stress; Ischemia-reperfusion injury; 21-Aminosteroid; Kidney, rat

1. Introduction

Surgical procedures such as renal revascularization and renal transplantation are often associated with acute renal ischemia as a consequence of low perfusion states and shock. Ischemic renal damage is also one of the leading causes of the loss of transplanted organs (Bronphy et al., 1980), and therefore acute renal failure is related to considerable morbidity and death (Stanley et al., 1993). Although ischemia itself produces tissue injury, it is well known that damage is mainly due to reperfusion. Several different mechanisms for this injury have been proposed, but the hypothesis about the role of oxygen-free radicals in generating ischemic-reperfusion injury seems the most reliable (Paller and Hedlund, 1988; Linas et al., 1987; Nath and Paller, 1990). Free radicals affect cell function by oxidizing lipids, proteins and nucleic acids (Halliwell, 1987; Del Maestro, 1980). In particular, lipid peroxidation of unsaturated fatty acids has been shown to disrupt membrane integrity, causing organ impairment and cell death (Weinberg, 1991). During ischemia, degradation of purine nucleotides results in accumulation of hypoxanthine and xanthine, and their oxidation to uric acid by xanthine oxidase after reperfusion causes superoxide anions to be produced (Joannidis et al., 1990; McCord, 1985; Adkison et al., 1986). Moreover, free radical insult results in production of oxidized forms of glutathione (GSH), such as glu-

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tathione disulfide (GSSG) or soluble mixed disulfides with small thiols (GSSX). The ratio of the various forms of cytosolic glutathione (GSSG + GSSX)/(GSH + GSSG + GSSX) can be utilized as a relative index of redox activity (McCoy et al., 1988).

Antioxidant drugs are designed either to prevent free radical formation or to break free radical chain reactions (Halliwell, 1987; Basaga, 1990). In the last years, several new compounds that act as antioxidants have been proposed (Döslüoglu et al., 1993; Catroux et al., 1990; Rabl et al., 1993).

Recently a new class of antioxidant agents, the 21-aminosteroids, named 'lazaroids', has been developed and extensively investigated (Braughler et al., 1987). Among these, the efficacy of 21-[4-(2,6-di-1-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl]-16-methyl-(16α)-pregna-1,4,9(11)-triene-3,20-dione monomethansulfonate (U74006F) in the management of acute central nervous system injuries, cerebral ischemia, hemorrhagic shock, traumatic shock, cardiopulmonary arrest and renal ischemia-reperfusion injury has been demonstrated by using animal models (Bronphy et al., 1980; Hall et al., 1988; Hall and Yonkers, 1988; Natale et al., 1988; Stertz et al., 1991; Stanley et al., 1993; Shackleton et al., 1994). The protective action of the new 21-aminosteroid 21-[4-(2,6-di-1-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl]-pregna-1,4,9(11)-triene-3,20-dione monomethansulfonate (U74389F) has been shown in in vitro (Gadaleta et al., 1994; Kalra et., 1994) and in vivo studies in different animal models (Sinardi and Montanini, 1992; Bernstein et al., 1992; Shin et al., 1994; Shenkar and Abraham, 1995).

We became interested in studying the protective effect of U74389F against kidney damage induced by warm ischemia. Adenine nucleotides were measured to check the ischemic status of the kidney, while glutathione (total and oxidized forms) and lipid peroxidation (diene conjugates and fluorescent products) were measured to check the protective effect of the drug.

2. Materials and methods

2.1. Chemicals

Glutathione, GSSG, *N*-ethylmaleimide, dithiothreitol, Trizma Base (Tris), Phosphate Buffer Saline (PBS) and adenine nucleotides were purchased from Sigma Chemicals Co. (St Louis, MO). All other reagents were obtained from Fluka (Zürich, Switzerland), while organic solvents were from BDH (Poole, UK).

2.2. Protocol

Male Wistar rats (Charles River, Calco, Italy) weighing 200–220 g were used. Animals had free access to food and water until the time of the study. U74389F was dissolved by 2 h mechanical shaking in citrate buffer pH 3.0 (0.5 mg/ml) the day before the study and stored at 4°C until injected i.v. (2 mg/kg; 4 ml/kg) into the tail vein. Rats were anesthetized with 40 mg/kg thiopentone (Farmitalia, Milan, Italy) intraperitoneally and treated with either the lazaroid solution or the vehicle.

The abdomen was opened by a midline incision and the left and right renal arteries were clamped. After the occlusion period, the kidneys were either immediately excised, or unclamped, reperfused with blood for the times indicated below and then removed. After excision, kidneys were quickly divided lengthwise into two parts and frozen in liquid nitrogen. A weighed portion of tissue was dried overnight at 80°C and reweighed for determination of dry weight. During the reperfusion periods rats were allocated to single cages and had free access to food and water.

Rats were randomly assigned to treated or control groups as follows: group 1 ($n = 13$) and 2 ($n = 14$): 60 min ischemia; group 3 ($n = 14$) and 4 ($n = 13$): 60 min ischemia plus 2 h reperfusion; group 5 ($n = 6$) and 6 ($n = 7$): 60 min ischemia plus 24 h reperfusion; group 7 ($n = 6$) and 8 ($n = 5$): 90 min ischemia; group 9 ($n = 11$) and 10 ($n = 15$): 90 min ischemia plus 2 h reperfusion; group 11 ($n = 12$) and 12 ($n = 8$): 90 min ischemia plus 24 h reperfusion. Groups 1, 3, 5, 7, 9, 11 received vehicle alone, while groups 2, 4, 6, 8, 10, 12 were treated with the lazaroid as described above. Control measurements were made with sham-operated animals ($n = 17$).

All data have been expressed on a per gram dry weight basis; glutathione disulfide concentration is expressed as equivalents of glutathione throughout the text.

2.3. Assays

Immediately before analysis frozen kidneys were homogenized in cold 3.5% perchloric acid (1:5, w/v) with an Ultraturrax homogenizer (Janke and Kunkel, GmbH and Co, Staufen, Germany). After centrifugation at $10000 \times g$ for 1 min at 4°C in Microfuge 11 (Beckman, Palo Alto, CA), the supernatant was used for glutathione and nucleotide determinations.

Glutathione was determined after reduction with dithiothreitol, derivatization with *o*-phthalaldehyde and separation by reversed phase high-performance liquid chromatography, as described previously (Paroni et al., 1995). A System Gold Beckman chromatograph equipped with a pump model 126, an autosampler (model 507), an interface (model 406) and a RF-551 fluorescence detector (Shimadzu, Kyoto, Japan) (λ_{exc} 360 nm, λ_{em} 420 nm), was used. Pre-column derivatization and injection were carried out automatically by

mixing equal amounts of the sample and the derivatizing agent and injecting the mixture after exactly 1 min.

Adenine nucleotides and their catabolic products were measured by means of a modified chromatographic method (Stocchi et al., 1985) with UV detection at 254 nm. An aliquot of the acid supernatant from kidney homogenate was neutralized with sodium bicarbonate and injected. Nucleotides were separated by use of a binary gradient system: phase A consisted of 0.1 M KH_2PO_4 pH 6.5, while phase B consisted of buffer A and methanol (90:10, v/v). After 15 min at 100% A, phase B was raised to 100% in 1 min; 5 min later, initial conditions were restored in 2 min; after an additional 7 min the subsequent sample was injected. A Merck LiChroCART RP18 (250 × 4 mm, 5 μm) column eluted at a flow rate of 1 ml/min was used.

Lipid peroxidation was evaluated by measuring diene conjugates and Schiff base formation according to the method of Green et al. (1986). A portion of kidney was homogenized in PBS pH 7.4 (1:10, w/v). For Schiff base determination, homogenates (1 ml) were extracted with 4 ml of chloroform-methanol (2:1, v/v), both before and after incubation in open vessels for 90 min at 37°C with mechanical shaking. The fluorescence of the organic phase was monitored at 430 nm with excitation wavelength at 360 nm in a Perkin Elmer LS-3 spectrofluorimeter (Norwalk, CT). For diene conjugate determination, 200 μl of homogenate was extracted with 2 ml of chloroform-methanol (2:1, v/v), dried under a nitrogen stream, redissolved in heptane and examined for absorbance at 233 nm in a Uvikon spectrophotometer (Kontron Instruments, Zürich, Switzerland).

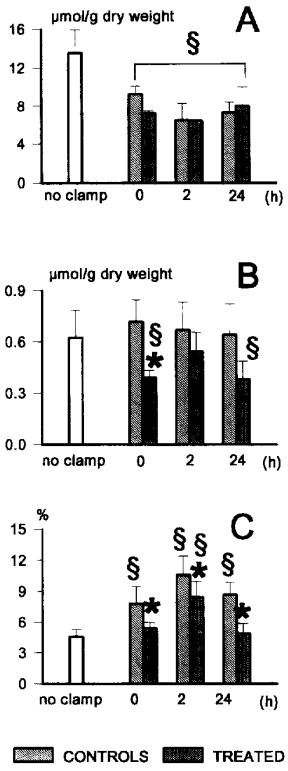

Fig. 1. Kidney glutathione during 90 min of ischemia and reperfusion. (A) Total soluble glutathione (GSH + GSSG + GSSX). (B) Free oxidized glutathione (GSSG + GSSX). (C) Glutathione redox ratio (GSSG + GSSX/GSH + GSSG + GSSX). Kidneys were treated as described under Methods. Values are means ± S.D. * $P < 0.05$ vs. control group, § $P < 0.05$ vs. preischemic values. § (over arrowed line) $P < 0.05$ for both treated and control groups.

2.4. Statistical analysis

All data were expressed as means ± standard deviation (means ± S.D.). Comparison between treatments and preischemic values (sham-operated) was carried out with 2-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test as post-hoc test. Fisher's exact test was used to assess mortality data. Differences were considered significant for a value of $P < 0.05$. All analyses were performed using the Sigma Stat (Statistical Analysis System, version 1.01) statistical package by Jandel Scientific (Germany) on an IBM computer.

3. Results

The studied variables did not show significant differences between treatments in all rats undergoing 60 min of ischemia. Mortality rate was 12% in treated animals vs. 15% in the matched vehicle-treated control group, without significant differences between the groups. After ischemia, total glutathione decreased to about 65% of the initial value in both groups. Although not significant, by 2 h of reperfusion glutathione in the treated group recovered to 87.5% of the sham-operated value, while in controls total glutathione remained depressed. Lipid peroxidation products increased in both groups during reperfusion, but the treated group showed a more pronounced return to preischemic levels.

The positive effect of the investigated drug was observed after a more sustained ischemic insult. Only 2 (7.1%) out of 28 rats receiving the lazaroid and undergoing 90 min of ischemia died in the following 24 h of reperfusion as compared to 10 (34%) out of 29 rats treated with the citrate vehicle alone ($P < 0.05$). After 24 h creatinine levels rose from 0.64 ± 0.17 in the sham-operated to 4.06 ± 0.90 and 3.05 ± 0.95 mg/dl in control and treated rats, respectively, without differences between the two groups.

90 min of ischemia produced a depletion of intracellular glutathione stores, which failed to recover to initial levels in the following 24 h in both groups (Fig.

Table 1
Adenine nucleotides, hypoxanthine and xanthine after 90 min of ischemia and reperfusion

Reperfusion	Number of animals	ATP	ADP	AMP	HYP [a]	XAN [b]
Sham-operated	17	5.0 ± 0.6	4.72 ± 1.1	4.47 ± 0.9	0.39 ± 0.1	n.d. [c]
0 h control	6	0.63 ± 0.12	0.93 ± 0.12	3.04 ± 0.75	2.85 ± 0.29	4.26 ± 0.54
0 h treated	5	0.47 ± 0.03	0.49 ± 0.16	2.68 ± 0.17	2.84 ± 0.43	3.93 ± 0.63
2 h control	9	1.44 ± 0.44	2.29 ± 0.60	2.87 ± 0.74	0.46 ± 0.60	0.67 ± 1.23
2 h treated	15	2.18 ± 0.40 [d]	2.87 ± 0.67	2.07 ± 0.42	0.38 ± 0.52	0.42 ± 0.85
24 h control	4	1.00 ± 0.50	1.60 ± 0.65	4.42 ± 0.33	0.17 ± 0.04	n.d. [c]
24 h treated	6	1.26 ± 0.63	1.64 ± 0.85	4.23 ± 0.73	0.23 ± 0.08	0.08 ± 0.08

Values are expressed as μmol/g dry weight. [a] Hypoxanthine, [b] xanthine, [c] non-detectable, [d] $P < 0.05$ vs. control group.

1A). Immediately after unclamping and during the reperfusion period, the levels of oxidized glutathione (GSSG + GSSX) were found to be lower in the treated group than in the control one ($P < 0.05$ at time 0), thus suggesting protection by the lazaroid against oxidation (Fig. 1B). As a consequence, a significant alteration of the glutathione redox ratio was observed in the control group at each studied time, while, by contrast, treatment with lazaroid significantly reduced this increment, allowing preischemic conditions to be re-established after 24 h of reperfusion (Fig. 1C). Protein-bound glutathione, expressed as % of the total content, did not show any appreciable alteration at any studied time either vs. preischemic value or between treatments.

After 90 min ischemia, renal ATP levels decreased to about 10% of the sham-operated levels (Table 1). In control rats ATP recovered to 29% of normal values by 2 h of reperfusion and fell to 20% after 24 h, as already described after 50 min ischemia (Arnold et al., 1986). By contrast, treated rats showed a better recovery (44%) by 2 h ($P < 0.05$), while this effect was lost after 24 h. Ischemia caused a transient and significant increase in hypoxanthine and xanthine content, which was rapidly neutralized on reperfusion in all studied groups.

Lipid peroxidation was determined by measuring diene conjugates and fluorescent Schiff bases. Diene conjugate formation was significantly enhanced by 90 min of ischemia and remained elevated during the following reperfusion period both in control and in treated rats. A discriminant protective effect of U74389F on lipid peroxidation was observed after 2 h of reperfusion (Fig. 2A). The same was seen for Schiff bases, which were reduced by lazaroid during reperfusion, with significant differences between treated and control groups after 2 h (Fig. 2B).

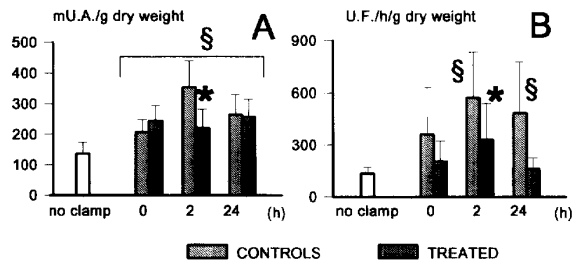

Fig. 2. Kidney lipid peroxidation products during 90 min of ischemia and reperfusion. (A) Diene conjugates. (B) Fluorescent products. Kidneys were treated as described under Methods. Values are means ± S.D. * $P < 0.05$ vs. control group, § $P < 0.05$ vs. preischemic values. § (over arrowed line) $P < 0.05$ for both treated and control groups.

4. Discussion

Readmission of blood after ischemia often results in severe tissue injury, which is in part the consequence of reperfusion rather than the ischemic episode itself. Several mechanisms have been proposed to explain the postischemic renal injury, including depletion of adenine nucleotides, loss of electrolyte homeostasis, alterations in phospholipid metabolism, intracellular acidosis and cell Ca^{2+} influx (Bronphy et al., 1980; Weinberg, 1991; Joannidis et al., 1990). Recently, the role of free radicals in postischemic renal damage has received considerable attention. The presence of oxygen reactive species has been demonstrated by measuring lipid peroxidation or by pointing out the protective role of compounds known to interfere with free radical production (Paller et al., 1984; Paller and Hebbel, 1986; Takenaka et al., 1981; Catroux et al., 1990; Rabl et al., 1992, 1993).

In the last years a new class of antioxidants, named 21-aminosteroids, characterized by their ability to inhibit iron-dependent lipid peroxidation (Braughler et al., 1987; Podrazik et al., 1989; Kalra et al., 1994), apolarity, absence of glucocorticoid activity and potential side effects, has been introduced. Although a large number of studies appeared on the use of these drugs for treatment of central nervous system injuries (Bernstein et al., 1992; Clark, 1994; Sato and Hall, 1992), few investigations have been carried out on the protective action of lazaroids in renal reperfusion injury (Stanley et al., 1993; Podrazik et al., 1989; Shackleton et al., 1994). These authors used U-74006F (tirila-

zad) and reported a protective effect on morphological and functional parameters, while only Shackleton et al. (1994) investigated tissue thiobarbituric acid-reactive products after 15 min of blood flow resumption as a marker of lipid peroxidation.

We focused our attention on the use of the recently developed U74389F, which has a very similar structure to that of the parent drug U74006F, only differing by the absence of a methyl group in position 16 in the new formulation, and on the determination of more sensitive markers of ischemic damage. In our experimental procedure the drug was administered before prolonged periods of ischemia (60 or 90 min) and at a rather low dosage (2 mg/kg).

Our data seem to indicate that 60 min of ischemia produced too little oxidative stress and/or too much spontaneous recovery to allow visualization of the protective effect of the lazaroid, while the efficacy of the treatment was evidenced after a more sustained ischemic insult (90 min).

The effects of the drug on survival during 90 min ischemia and 24h reperfusion seem not to be related to an improvement in renal function when assessed by plasma creatinine. However, although the mortality rate could be affected by a large number of factors not connected with ischemia/reperfusion (anesthesia, surgery, hypothermia), we cannot exclude a positive effect of the drug on other parameters, such as glomerular filtration rate, Na^+ excretion and histologic appearance, as already reported by Stanley et al. (1993) with a 3 mg/kg dosage and Shackleton et al. (1994) with the same dose repeated before and after the ischemic period.

As already observed by others (Scaduto et al., 1988) we found that prolonged renal ischemia caused a loss in glutathione content due to oxidative stress and to depletion of ATP stores necessary for glutathione synthesis, thus resulting in a shift of the glutathione redox ratio to a more oxidized value. Treatment with U74389F, although it did not save glutathione intracellular stores, significantly attenuated the increment in redox ratio, by lowering intracellular oxidized glutathione levels during reperfusion.

Adenine nucleotides were profoundly diminished after 90 min ischemia, and reflow slowly restored them to normal levels (50% of normal at 24 h), as reported by others (Arnold et al., 1986). The only difference in ATP levels between control and treatment groups was seen after 2 h of reperfusion, while after 24 h no benefit was seen anymore, probably because of the low drug levels at this time.

Lipid peroxidation is a well-known consequence of ischemia and reperfusion reported by many authors in different models (Catroux et al., 1990; Rabl et al., 1992, 1993). Diene conjugate formation is a marker of lipid peroxidation and occurs early in the cascade of reactions leading to this structural modification. Diene conjugates are subsequently converted into endoperoxides which are able to react with a variety of compounds including hydrocarbons to form alkyl radicals and lipid hydroperoxides. Alternatively, the oxidation of diene conjugates may lead to the formation of aldehydes; the principal requirement for formation of fluorescent Schiff bases is the formation of a 1,3-iminopropene conjugate derived from the condensation of aldehydes, such as malonyldialdehyde, with primary amine donors such as phospholipids, amino acids, proteins or DNA (Rabl et al., 1992). The tested drug was effective in reducing the early events of lipid peroxidation (diene conjugates formation) and the later phases, leading to enhancement of fluorescent end products. Our results, obtained in vivo in a rat model, confirm those of in vitro studies that evidenced multiple mechanisms underlying U74006F's action, namely an increase in membrane rigidity, scavenging of both lipid peroxyl and oxygen radicals, enhancement of vitamin E activity and reduction of peroxidation-induced arachidonic acid release (Braughler et al., 1987; Hall et al., 1988). Although the low dose used and the short reperfusion periods studied did not have any effect on renal function, our data provide suggestive evidence for the protective action of U74389F on ischemic injury in acute renal failure.

In conclusion, results obtained in this study by using the rat model suggest that pretreatment with the 21-aminosteroid U74389F may be beneficial in protecting against oxygen free radical-mediated damage of the kidney which occurs during the reperfusion of the tissue after a period of warm ischemia.

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