Protection from renal ischemia-reperfusion injury by the 2-methylaminochroman U83836E

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Background. In a prior study the 21-aminosteroid (lazaroid) U74389F provided *in vivo* protection from oxidative stress when used as a preventive therapy in ischemia-reperfusion injury in the kidney. As the cell membrane is the principal site for lipoperoxidation, in the current study the very lipophilic 2-methylaminochroman U83836E, a recently developed lazaroid, was administered to rats at 3 mg/kg before renal ischemia-reperfusion. In addition to the biochemical parameters, the renal function and the histological appearance were carefully evaluated.

Methods. Glutathione, adenine nucleotides and lipid peroxidation products were determined in kidneys reperfused for 2 and 24 hours after 90 minutes of ischemia. Renal function was assessed by plasma creatinine, and renal injury by histological examination.

Results. Reperfusion-induced glutathione oxidation, expressed as an oxidized-to-total glutathione ratio, was significantly attenuated both after 2 and 24 hours of reperfusion by treatment with U83836E. Adenosine triphosphate (ATP) was still significantly depleted after 24 hours in the control group, while at the same time treated animals had already recovered to baseline values. Lipid peroxidation products were significantly lower in lazaroid-groups both after 2 and 24 hours of reperfusion. Renal function after 24 hours of reperfusion was notably better in the treated rats. Histological examination confirmed the protective action of the drug. After 24 hours the control group showed large areas of parenchymal hemorrhage and necrosis with dilated tubules and blood vessel thrombosis, while treated animals showed small necrotic areas with a background of mild interstitial inflammatory cells.

Conclusions. Our results suggest that there is a protective effect of U83836E in ischemia-reperfusion injury, in that tissue damage due to oxidative stress is reduced, thus ameliorating renal function impairment.

The 21-aminosteroids, known as "lazaroids," are drugs developed during the past decade initially for the treatment of central nervous system trauma, ischemia and subarachnoid hemorrhage [1–4]. Later experimental studies showed the efficacy of some of these compounds in the management of cardiopulmonary arrest [5], skeletal muscle [6], liver [7], and renal ischemia-reperfusion injury [8–10]. Several mechanisms have been proposed to explain the protective effects of lazaroids. They can inhibit lipid peroxidation by interacting with ferrous ions, thus blocking iron-mediated reactions, or act like vitamin E by scavenging peroxyl radicals [3, 11]. Moreover, they likely present membrane stabilizing properties and tend to inhibit the release of free arachidonic acid from injured cells [1]. More recently, a second generation of lazaroids has been introduced. These compounds, named 2-methylaminochromans, are based on a non-steroidal structure combining amino functionality of the 21-aminosteroids with the tetramethyl-chroman ring portion of α-tochopherol. The antioxidant potency of these new compounds has been shown to be more than tenfold that of the first generation lazaroids [12]. In particular, U78517F and its enantiomer U83836E have been successfully employed as antioxidants in models of focal and global cerebral ischemia [13], in cerebrovascular spasm [14], acute lung injury [15] and in treatment of diabetes complications [16]. Experimental evidence indicating that U83836E improves survival of embryonic mesencephalic neurones and U78517F inhibits oxidant-stimulated calcium influx have been reported [17, 18].

In 1995 we observed that administration of the 21-aminosteroid U74389F to rats before renal ischemia and reperfusion sensibly reduces oxidative stress and lipid peroxidation [10]. To have a more comprehensive view of the physiological protection offered by lazaroids against ischemic injury in acute renal failure, in this study we tested the effects of the antioxidant 2-methylaminochroman U83836E on renal function (assessed by serum creatinine) and on histological appearance. Moreover, as in our previous

investigation, we carefully evaluated the biochemical status of the cells expressed by the disturbance of prooxidant/antioxidant balance associated to alterations in the reduced-to-oxidized glutathione ratio, the oxidative stress-induced lipid peroxidation, and the high energy nucleotide content.

METHODS

Drug

U83836E ((-)-2-[[4-(2,6-di-1-pyrrolidinyl-4-pyrimidinyl]-1-piperazinyl]methyl]-3,4-dihydro-2,5,7,8-tetramethyl-2H-1-benzopyran-6-ol, dihydrochloride) (Upjohn Company, Kalamazoo, MI, USA) was dissolved in citrate buffer pH 3.0 at a concentration of 0.75 mg/ml the day before the study and stored at 4°C until use. Drug (3 mg/kg) or vehicle (citrate buffer pH 3.0) were administered five minutes before ischemia by injection into the tail vein.

Experimental protocol

Male Wistar rats (Charles River, Calco, Italy), weighing 200 to 220 g, were randomly divided into groups to receive vehicle (groups 1 and 3) or U83836E (groups 2 and 4). Groups 1 ($N = 8$) and 2 ($N = 8$) underwent 90 minutes of ischemia plus two hours of reperfusion, and in groups 3 ($N = 6$) and 4 ($N = 6$) the reperfusion period was extended to 24 hours. The animals were anesthetized with thiopentone (40 mg/kg body wt, i.p.; Farmitalia, Milan, Italy) and kidneys made ischemic for 90 minutes by occluding the left and right renal vessels as described elsewhere [10]. Reperfusion was allowed for 2 or 24 hours before kidney removal. Sham operated controls at 2 and 24 hours were also included in the protocol. During the operation (ischemia-reperfusion), all rats were kept on a surgical table under a heating lamp in order to avoid dramatic changes of body temperature. During the 24 hour reperfusion, rats were allocated to individual cages and had free access to food and water. After excision kidneys were rapidly divided lengthways into two parts: one was immediately frozen in liquid nitrogen for biochemical assays and the other was 10% formalin fixed and paraffin embedded for histological examination. Blood samples were drawn before sacrifice in a heparinized syringe and, after centrifugation in heparinized tubes, plasma was used for creatinine determination.

Assays

Just prior to analysis frozen kidney tissue was rapidly weighed and homogenized in cold 3.5% perchloric acid (2.5 ml), or in PBS pH 7.4 (4.5 ml) with an Ultraturrax® homogenizer (Janke and Kunkel, GmbH and Co., Staufen, Germany). Before homogenization, a small portion of kidney was excised, weighed and lyophilized for the determination of dry weight.

After centrifugation (10,000 × g at 4°C, 1 min) of the acidic homogenate, total and oxidized soluble glutathione

were determined by a reversed-phase high pressure liquid chromatography (HPLC) method, as described by Paroni et al [19]. Adenine nucleotides were measured by HPLC after neutralization of acid supernatant with sodium bicarbonate [10]. Lipid peroxidation was assessed by determination of diene conjugates and Schiff bases formation according to the method of Green et al [20], slightly modified [10]. Glutathione disulphide was always expressed as glutathione equivalents throughout the text. All data are expressed on a per gram dry weight basis. Plasma creatinine was determined by the Jaffè method without deproteinization and blood urea nitrogen (BUN) by the enzymatic-colorimetric method of Berthelot (Boehringer Mannheim Italy, Milan, Italy).

Histological analysis

For conventional histological analysis one section, 5 μm thickness, was cut from each paraffin block and hematoxylin-eosin stained. Histological assessment was semiquantitatively performed according to a previously published guideline [21]. Renal morphological changes were graded on a 0 to 3 scale in relation to the extent of kidney alterations: 0 = none; 1 = up to 20%; 2 = from 20 to 50%; 3 = more than 50%. Seven morphologic changes were assessed: (*1*) neutrophil accumulation in the vasa recta, (*2*) tubular necrosis (that is, the presence of necrotic cells, apparently denuded areas of tubular basement membranes, or ruptured tubular basement membranes), (*3*) tubular regeneration, (*4*) tubular cell mitoses, (*5*) interstitial inflammation, (*6*) tubular casts, and (*7*) loss of PAS-positive brush border. Blind analysis was performed on all samples.

Statistical analysis

All data are expressed as mean ± SE of the mean. Data were analyzed by two-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test as the post-hoc test. Creatinine and BUN were analyzed by the Duncan's multiple test. Concerning histological examination, differences between groups were evaluated using the Mann-Whitney rank sum test. Probabilities ≤ 0.05 were considered to be statistically significant. All analyses were performed using the Sigma Stat (Statistical Analysis System, version 1.01) statistical software package (Jandel Scientific GmbH, Erkrath, Germany) on an IBM computer.

RESULTS

No animal died during this study. Sham-operated animals at 0 and 24 hours gave statistically indistinguishable non-ischemic values, so they were pooled and considered as a single group ($N = 15$). Body temperature monitored in 3 rats at the end of 90 minutes of ischemia did not go below 37°C.
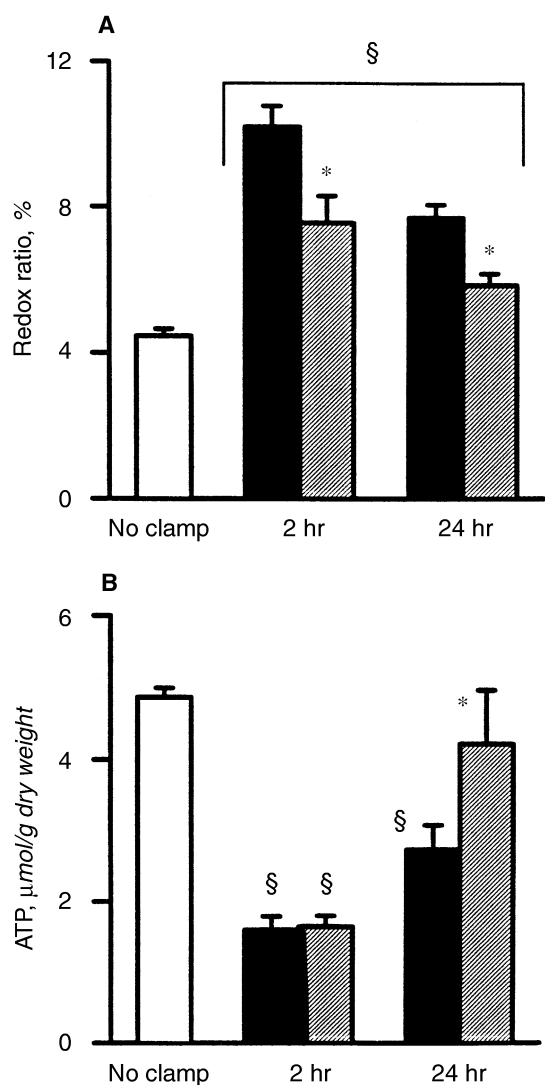
Fig. 1. Effects of U83836E after 90 minutes renal ischemia plus 2 or 24 hours reperfusion. (*A*) Glutathione redox ratio (free oxidized to total free, ratio %). (*B*) ATP. Kidneys were treated as described in the **Methods** section. Values are means ± SE. Symbols are: (□) sham-operated animals; (■) control groups; (▨) treated groups. *P < 0.05 versus control group. §P < 0.05 vs. sham-operated value.

Biochemical parameters

Ischemia and reperfusion resulted in a depletion of total intracellular glutathione stores (from 14.3 ± 1.33 to 8.20 ± 0.56 and 9.01 ± 0.51 μmol/g at 2 hrs of reperfusion for controls and treated rats, respectively), that was associated with a significant increment of glutathione redox balance (oxidized vs. total glutathione %) both in treated and control rats (Fig. 1A). In animals treated with lazaroid, both after 2 and 24 hours of reperfusion, the alteration of glutathione redox ratio was significantly reduced.

After 24 hours of reperfusion, intracellular ATP stores showed a trend to regain baseline values both in treated and control rats. However, while U83836E caused an almost complete recovery to pre-ischemic conditions, these levels were still significantly depleted in control animals, indicating a lower metabolic rate (Fig. 1B). Total adenine nucleotide levels (TAN, which is the sum of ATP, ADP and AMP) followed the ATP behavior, being higher in treated animals both after 2 and 24 hours of reperfusion (7.36 ± 0.9 vs. 6.56 ± 1.0 and 14.18 ± 6.5 vs. 9.74 ± 3.4 μmol/g, 2 and 24 hr, treated vs. control). After 24 hours, the recovery to the pre-ischemic value (15.7 ± 1.9 μmol/g) was complete in treated animals, while it was still in progress in controls ($P < 0.05$ vs. basal value). In a previous set of experiments on U74389F efficacy [9], we observed that the ATP/ADP ratio lowers significantly immediately after a 90-minute ischemic period (0.69 ± 0.18 vs. 1.39 ± 0.27 in the sham operated). In the present experiments a trend to a quicker recovery could be recognized after 24 hours in the treated group (0.83 ± 0.8 vs. 0.78 ± 0.6 μmol/g in the controls), but it was not significant.

Diene conjugates and fluorescent Schiff bases increased notably during reperfusion ($P < 0.05$ vs. baseline), and remained high after 24 hours in both control and treated animals (Fig. 2). U83836E, however, proved quite effective in reducing the extension of lipid peroxidation both at 2 and 24 hours of reperfusion ($P < 0.05$ vs. controls).

Renal function

Plasma creatinine levels and BUN before ischemia and after 2 and 24 hours of reperfusion are shown in Table 1. As expected, no difference vs. basal value or between treatments was observed after two hours of reperfusion. The beneficial effect of U83836E was clearly shown after 24 hours of reperfusion, when the increment in plasma creatinine and BUN was much more evident in the control group ($P < 0.05$ vs. treated group).

Renal morphology

The histologic features assessed in rat kidneys as described in the **Methods** section are reported in Table 2. After two hours of reperfusion, the control group showed kidney necrosis that was not significantly different from the U83836E group, with detectable renal abnormalities limited to mild interstitial edema, with few scattered foci of hemorrhage. Only the presence of tubular casts differed significantly in the control group versus the U83836E treated group ($P < 0.01$ vs. treated). Histological analysis after 24 hours of reperfusion (Fig. 3) showed that necrosis of individual tubular epithelial cells and the presence of tubular casts were the most severe alterations in controls, with large and irregular confluent areas of parenchymal hemorrhage and necrosis, tubular dilation, vacuolization and thrombosis of blood vessels. Conversely, histological examination of the kidneys of treated animals revealed focal and small hyperemic and necrotic areas ($P < 0.03$ treated vs. controls) with dilated tubules and a background of mildly interstitial inflammatory cells. Moreover, with regards to the non-necrotic response, the U83836E showed
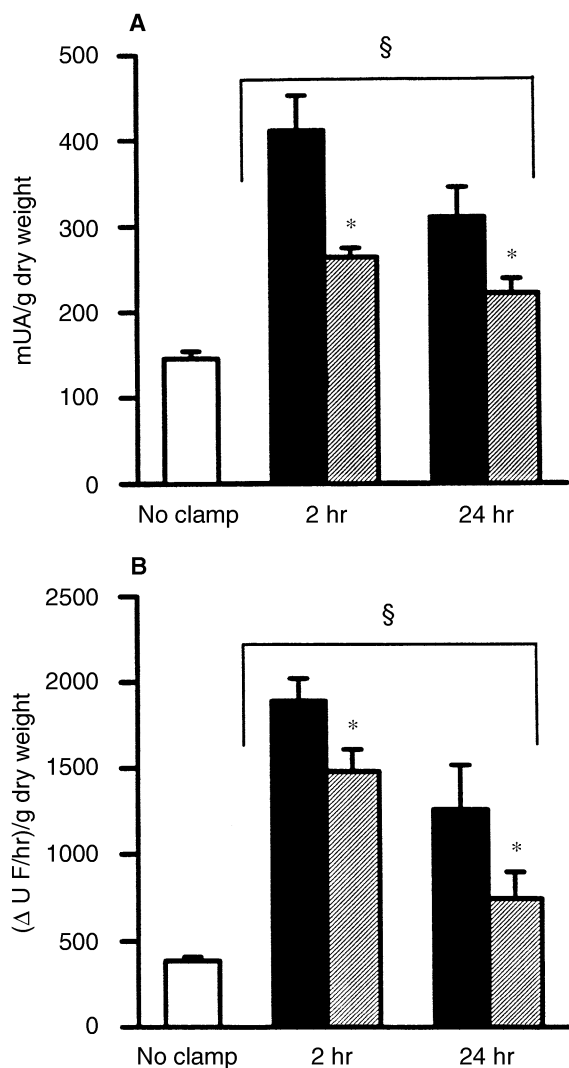
Fig. 2. Effects of U83836E after 90 minutes renal ischemia plus 2 or 24 hours reperfusion. (*A*) Diene conjugates. (*B*) Fluorescent products. Kidneys were treated as described under Methods. Values are means ± SE. Abbreviations are: U.A., units of absorbance; U.F., units of fluorescence. Symbols are: (□) sham-operated animals; (■) controls groups; (▨) treated groups. *$P < 0.05$ vs. control group. §$P < 0.05$ vs. sham-operated value.

a lack of significant change when compared to the sham group.

DISCUSSION

Ischemia-reperfusion injury sustained by kidney during surgical revascularization of the renal artery or after transplantation often results in temporary or permanent alterations of renal function [22, 23]. During ischemia and reperfusion, a massive production of oxygen free radicals associated with an impairment of the antioxidant system triggers lipid peroxidation by removing hydrogen atoms from polyunsaturated fatty acids, thus leading to tissue injury [24–26]. In the last years, several studies have found

that high doses of steroids can inhibit lipid peroxidation [1]. This observation led to the development of the so-called "lazaroids" (21-aminosteroids), which are characterized by a steroid structure and absence of glucocorticoid activity. Among these compounds, U74006F, known as Tirilazad mesylate, has been employed in the treatment of subarachnoid hemorrhage in a multicenter clinical trial [27]. The antioxidant capacity of the 2-methylaminochroman, U78517F, a second generation lazaroid, has been reported to be tenfold that of the 21-aminosteroid U74006F and 100-fold that of vitamin E, probably because the association of the antioxidant properties of vitamin E with the amino portion of U74006F enhances drug antioxidant potency and efficacy [13].

In our previous experiments on U74389F (structurally similar to U74006F) at 2 mg/kg, in a mild model of acute renal failure (60 min ischemia) evidenced some drug efficacy only after two hours reperfusion, while after 24 hours most of the tested biochemical parameters also returned to baseline values in untreated animals. A longer clamp period (90 min) was therefore necessary to establish the drug's effect on the glutathione redox ratio at 24 hours of reperfusion. Nevertheless, U74389F failed to protect against lipid peroxidation and ATP depletion.

Based on these results, the protection of the 2-methylaminochroman U83836E (enantiomer of U78517F) at 3 mg/kg was tested using a 90-minute clamp time. The results are suggestive of a stronger efficacy, and consistent with an additive or synergistic effect of the chromanol moiety together with the amino functionality of U74389F. The 2-methylaminochromans as well as the 21-aminosteroids exert their effects via several mechanisms such as scavenging of peroxyl radicals [11], inactivation of iron-mediated toxic reactions by interaction with ferrous iron [2], and the blocking of calcium channels by inhibition of ion pump ATPase [18]. The long lasting protection from lipid peroxidation and from nucleotide depletion in the kidney by U83836E even after 24 hours of reperfusion can also be ascribed to a longer metabolic half-life in comparison with the relatively short one of the old generation of 21-aminosteroids (U34389F) [11]. In consequence, it could be speculated that there is a different bioavailability because of the higher solubility of U83836E at physiological pH than U74389F. This could lead to a more efficient $O_2^{\cdot}$ and $OH^{\cdot}$ scavenging ability of U83836E to inhibit toxic reactions in the aqueous cell environment.

It is well known that ATP levels fall within a few seconds of ischemia (<1 mmol/g wet wt). Therefore, despite the rapid time course between the removal of the kidney from the animal and subsequent freezing in liquid nitrogen, this degradation is to be kept in mind when analyzing the validity of present study's methods. Nevertheless, our experimental model clearly showed the protective action of the tested lazaroid versus controls, as it well supported the restoration of kidney ATP pool together with the other

Table 1. Baseline and post-ischemic creatinine and blood urea nitrogen (BUN) levels

Reperfusion period	Creatinine *mg/dl*		BUN *mg/dl*	
	Controls	Treated	Controls	Treated
2 hours (*N* = 8)	0.37 ± 0.06	0.43 ± 0.04	57 ± 3.9	52 ± 2.33
24 hours (*N* = 6)	1.95 ± 0.13[a]	1.13 ± 0.27[ab]	71.7 ± 8.2	47.4 ± 3.5[b]

Data are expressed as mean ± SE. The number of animals for each experimental group is given in parentheses. Pre-ischemic values determined in 15 sham-operated rats were 0.44 ± 0.03 mg/dl for creatinine and 48 ± 2.3 mg/dl for BUN.
[a] *P* < 0.05 vs. baseline value
[b] *P* < 0.05 vs. control group

Table 2. Grading of lesions in controls and treated rat kidneys

| Reperfusion time	Neutrophils in vasa recta	Tubular necrosis	Tubular regeneration	Tubular cell mitoses	Interstitial inflammation	Tubular casts	Brush border loss
2 Hours							
C1	0	1	1	0	0	2	2
C2	0	2	1	0	0	2	2
C3	0	1	0	0	1	1	2
C4	0	2	1	0	0	2	2
C5	0	2	1	0	1	2	2
C6	0	2	1	0	1	2	2
C7	0	1	1	0	1	2	2
C8	0	2	1	0	1	2	2
T1	0	1	0	0	0	1	2
T2	0	1	1	0	0	1	2
T3	0	2	1	0	1	2	2
T4	0	1	1	0	0	1	2
T5	0	1	1	0	0	1	2
T6	0	2	1	0	0	1	2
T7	0	1	1	0	1	1	2
T8	0	1	2	0	0	1	2
24 Hours							
C1	0	2	2	0	0	3	2
C2	0	3	1	0	1	2	2
C3	0	2	2	0	1	2	2
C4	0	2	2	0	0	3	2
C5	0	3	3	0	1	3	2
C6	0	3	1	0	1	2	2
T1	0	2	1	0	0	2	2
T2	0	1	1	0	1	1	2
T3	0	1	1	0	0	3	2
T4	0	2	2	0	0	2	2
T5	0	2	1	0	1	1	2
T6	0	1	1	0	1	1	2

Scores are defined as: 0 = negative; 1 = <20%; 2 = 20–50%; 3 = >50% of tissue area morphologic changes. Abbreviations are: C, controls; T, treated.

adenine nucleotides depleted during the ischemic period, with an almost complete recovery to baseline values after 24 hours of reperfusion.

The degree of renal function protection by U83836E was also confirmed by the significant differences found in plasma creatinine after 24 hours of reperfusion, a beneficial effect that was not evidenced with U74389F [10]. A similar result on renal function was obtained by Stanley et al in 1993, who employed a rather high dosage of U74006F (10 mg/kg^{-1}), while at a lower dosage (3 mg/kg) no notable effects were observed [8]. Although the possible influence of U83836E on the glomerular filtration rate of the kidney was not directly assessed, the efficacy of U83836E is clearly evidenced by plasma creatinine, which is also the main

parameter used to evaluate renal function in clinical practice.

In the present study the tissue's histological appearance was also examined. As well as for renal function, histological examination did not reveal marked differences between treated and control rats after two hours of reperfusion, which appears to be too short a period to evidence such alterations. Morphological damage induced by prolonged ischemia was well established after 24 hours reperfusion and clearly counteracted by U83836E administration, as was already reported in a similar animal model of kidney ischemia-reperfusion injury with U74006F [8]. The amelioration of microvascular thromboses documented at two hours and more extensively at 24 hours in control kidneys
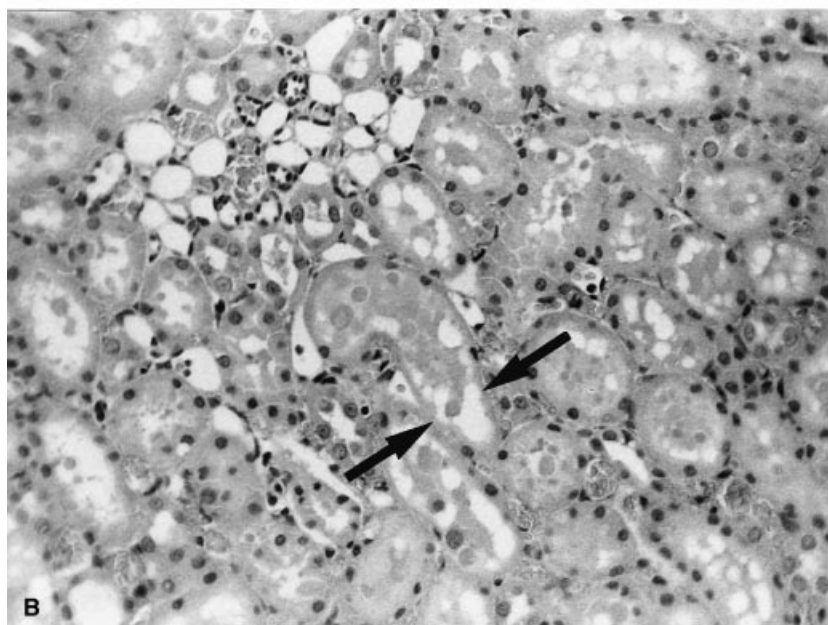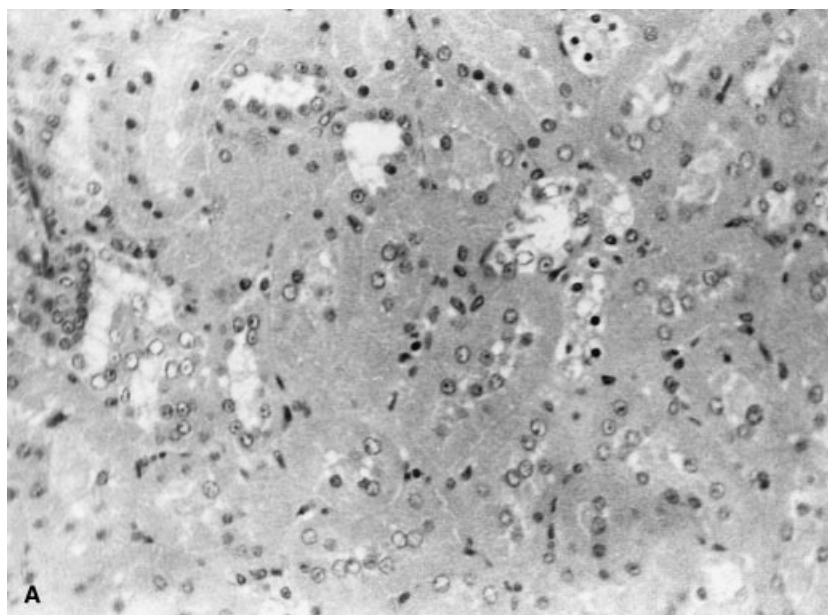
Fig. 3. Histological appearance of a cortical field of a rat kidney sacrificed 24 hours after 90 minutes ischemia. (*A*) Control kidney. (*B*) U83836E treated kidney. In the control group, kidney tubular cell necrosis (presence of necrotic cells or denuded areas of tubular basement membrane; arrows) is less discrete and less focally distributed than in the treated animals (hematoxylin and eosin, ×25).

can be explained by the possible anticoagulant effect of U83836E. Although to our knowledge no data on this issue exist, this possibility cannot be completely excluded.

In conclusion, our data confirm the validity of lazaroid treatment against ischemia-reperfusion injury in a rat model of severe ischemia (90 min). The protection of U83836E against a milder model of acute renal failure (60 min clamp), under strictly regulated normothermic conditions, which is perhaps physiologically more relevant, can also be hypothesized, although it was not experimentally tested in this protocol. Employing 2-methylchromans for the prevention of renal ischemia-reperfusion injury in humans merits further investigation.

APPENDIX

Abbreviations used in this article are: ADP, adenosine 5′-diphosphate; AMP, adenosine monophosphate; ANOVA, analysis of variance; ATP, adenosine triphosphate; BUN, blood urea nitrogen; HPLC, high performance liquid chromatography; PAS, periodic acid Schiff; PBS, phosphate buffered saline; TAN, total adenine nucleotide.

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