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J Mol Cell Cardiol **29**, 2825–2835 (1997)

Beneficial Effects of the 21-Aminosteroid U 74389G on the Ischemia-reperfusion Damage in Pig Hearts

C. Nediani, A. M. Perna,[2] P. Liguori[2], L. Formigli[1], L. Ibba-Manneschi[1], S. Zecchi-Orlandini[1], C. Fiorillo, G. Rizzuti and P. Nassi

Department of Biochemical Sciences, [1]Department of Human Anatomy and Histology, University of Florence; [2]Department of Cardiac Surgery, Careggi Hospital, Florence, Italy

(Received 10 March 1997, accepted in revised from 9 July 1997)

C. Nediani, A. M. Perna, P. Ligvori, L. Formigli, L. Ibba-Manneschi, S. Zecchi-Orlandini, C. Fiorillo, G. Rizzuti and P. Nassi. Beneficial Effects of the 21-aminosteroid U 74389G on the Ischemia-reperfusion Damage in Pig Hearts. *Journal of Molecular and cellular Cardiology* (1997) **29**, 2825–2835. 21-Aminosteroids (Lazaroids), acting as free radical scavengers and as membrane stabilizers, proved to have beneficial effects in various pathological conditions. In the present study we explored the effectiveness of one of these compounds, U 74389 G, in protecting pigs myocardium against the ischemia-reperfusion damage induced by transient coronary occlusion achieved by clampling the left anterior descending coronary artery. Animals were divided into three groups: control, untreated and treated. Control animals were operated but not subjected to ischemia-reperfusion; the untreated group underwent to ischemia-reperfusion without pharmacological treatment; while the treated group received the aminosteroid (4 mg/kg) before coronary occlusion and at the time of reperfusion. Specimens of myocardial tissue and blood samples were taken for morphological and biochemical studies. In the ischemic-reperfused myocardium of the untreated animals, the dominant morphological features were neutrophil infiltration, intercellular edema and severe swelling of mithocondria. All these alterations, notably neutrophil infiltration, were attenuated by aminosteroid treatment. As for the biochemical findings, the changes in adenine nucleotides and nucleosides levels, thus the reduction of energy charge, were reversed in the treated, but not in the untreated group. Myocardial concentration of malondialdehyde, which was undetectable in the control group, was raised in all the animals after reperfusion, but this effect was significantly less marked with aminosteroid treatment. In addition, the higher myocardial content of ascorbic acid and the reduced serum potential peroxidation exhibited by the treated animals compared with untreated group indicate an enhanced antioxidant protection induced by aminosteroid administration. On the other hand, the serum levels of myoglobin, cardiac troponin I and creatine kinase MB isoenzyme suggest the ability of the aminosteroid to attenuate the modifications of membrane permeability induced by ischemia-reperfusion injury. All these results lead to the conclusion that aminosteroid treatment, at least in the conditions of the present study, is effective in reducing the morphological and biochemical alterations occurring in ischemic-reperfused myocardium.

Key Words: Ischemia; Reperfusion; Free radicals; Myocardial ultrastructure; Serum myocardial injury markers; Malondialdehyde; Energy metabolism.

Introduction

Myocardial disfunction induced by reperfusion after ischemia is a well known phenomenon. Several experimental models (Tennat *et al.*,1936; Jolly *et al.*, 1984; Johnson *et al.*, 1987) have been used to study the mechanisms involved in this process, which is of great clinical interest. Ischemia-reperfusion (IR) conditions may be realized in heart muscle in a number of circumstances, among them coronary thrombolysis, angioplasty, cardiac surgery of heart transplantation.

There is abundant evidence (Ceconi *et al.*, 1988; Zweier, 1988; Chambers *et al.*, 1989) dem-

Please address all correspondence to: Prof. P. Nassi, Dipartimento di Scienze Biochimiche, Viale Morgagni, 50, 50134 Firenze, Italia.

0022–2828/97/102825+11 $25.00/0 mc970516

onstrating that in myocardium, as well in other tissues, IR is associated with an enhanced production of reactive oxygen intermediates (ROIs). Among the various toxic effects induced by these chemical species, membrane phospholipid peroxidation has been recognized as one of the major mechanisms involved in the loss of structural integrity and in function impairment that occur upon reperfusion of ischemic myocardium (Schaper *et al.*, 1979; Guarnieri *et al.*, 1980; Taylor *et al.*, 1984; Ceconi *et al.*, 1991; Freedman *et al.*, 1991).

The pathogenetic role ascribed to ROIs in the IR syndromes has suggested many therapeutic approaches aimed to protect human tissues from the injuries due to these reactive substances, notably from lipid peroxidation. Among the proposed treatments the administration of 21-aminosteroids (Lazaroids) seems to fulfil many of the requirements needed for an effective protection. These compounds, in fact, can exert a many-fold action because they have an antioxidant effect, owing to their ability to act as free radical scavengers, and also a stabilizing effect on cell membranes where they can inhibit the propagation of lipid peroxidation by restricting the movement of peroxyl and alchoxyl radicals which are formed during this process (Jacobsen *et al.*, 1990; Hall and McCall, 1994). A number of reports point out the benefits resulting from aminosteroid treatment in various pathological conditions associated with an increased formation of ROls and, generally, free radicals. Particularly 21-aminosteroids proved to be very useful in the protection of neuronal viability in ischemic and post-traumatic lesions of central nervous system (Tanno *et al.*, 1992). On the other hand, the effectiveness of aminosteroid treatment in reducing IR myocardial damage is rather controversial, since the beneficial effects described in several reports have not been confirmed by other authors (Holzgrefe *et al.*, 1990; Ovize *et al.*, 1991; Carrea *et al.*, 1992; Levitt *et al.*, 1994)

In this connection, we have previously demonstrated (Perna *et al.*, 1996) a cardioprotective effect of a 21-aminosteroid compound, U 74389 G, using a model of heart global ischemia followed by reperfusion. In the present study, we explored the ability of the same compound to protect pig myocardium against the IR damage induced by transient coronary occlusion.

Materials and methods

All the chemicals were of analytical grade. U-74389G was a generous gift from Upjohn Company, Kalamazoo, MI, USA.

Animal model and experimental protocol

Twenty farm pigs, of either sex, weighing 30–50 kg, fasted overnight, were used. All animals received human care in compliance with the guide for the care and use of Laboratory Animals as stated from European Law.

Animals were randomly assigned to three groups: control ($n=6$), untreated ($n=7$) and treated ($n=7$). The animals of the control group were operated as described below but they were not submitted to IR. The untreated group consisted of animals subjected to IR without treatment with aminosteroid. The animals of the treated group received the aminosteroid at a dose of 4 mg/kg both 10 min before coronary occlusion, and at the time of reperfusion.

The animals were pre-medicated with intramuscular ketamine (15 mg/kg) and diazepam (5 mg/kg) and anesthetized with sodium penthobarbital (20 mg/kg). Orotracheal intubation was performed and animals were ventilated using oxygen and fluothane such as to maintain an arterial pO_2 greater than 100 mmHg and normal pH. Peripheral electrocardiography (ECG) leads were tied, femoral vessels were exposed and cannulated for the measurement of hemodynamic parameters and fluid infusion. Left ventricular pressure was evaluated by means of Miller catheter introduced through the femoral artery. All data were recorded on a six-channel Esa-Ote Recorder. The chest was entered through a median sternotomy, the pericardium opened and the heart suspended in a pericardial craddle. A catheter was positioned, through a purse string on the right atrial appendage, into the coronary sinus for blood sample drawing and drug infusion. The left anterior descending (LAD) artery was dissected free from the vein and epicardial tissue and was encircled with an heavy silk distal to the first major diagonal branch. Heparin was then administered (2 mg/kg). Coronary occlusion was achieved by clamping with a small bulldog clamp the LAD artery where it was dissected free creating an ischemic area which corresponded to about one third of the left ventricle, without significant difference between the experimental groups. In fact, the extension of the ischemic areas was $31 \pm 5\%$ of the left ventricle in the untreated group and $33 \pm 7\%$ in the treated group, as assessed by the triphenyl tetrazolium chloride-Evan's blue technique (Klein *et al.*, 1981), which also indicated the absence of a infarcted area within the ischemic region. After 30 min of ischemia, small specimens (50–100 mg) of myocardial tissue were taken with an 11 surgical blade from the ischemic area of the

Table 1 Scoring method of myocardial and microvascolar injury

Endothelial injury
0—Normal endothelial cell
0.5—Mild to moderate endothelial swellings with decrease of pynocytotic vesicles
1—Severe endothelial swellings with loss of pynocytotic vesicles
2—Platelet adhesion to altered endothelial cell swellings
3—Leukocyte adhesion with and without endothelial cell swellings
4—Loss or disintegration of the endothelial lining
Myocyte injury
0—Normal myocyte
1—Mild to moderate mitochondrial swellings
1—Intercellular edema
1—Sarcomere contraction
2—Severe mitochondrial swellings with loss of cristae and matrix density

anterior wall of the left ventricle and used for biochemical and histological evaluation; the clamp was then released and the reperfusion continued for 30 min, afterwards a new set of samples was taken from the same area.

Histological methods

For the morphological analysis, a myocardial sample was taken in the above indicated area from each animal and immediately fixed by immersion in cold 4% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, at room temperature, and post-fixed in 1% OsO_4 in 0.1 M phosphate buffer, pH 7.4, at 4°C. The specimens were dehydrated in a graded acetone series, passed through propylene oxide and embedded in Epon 812. Semithin sections 1–2-μm thick were cut and stained with toluidine blue-Na tetraborate and observed under light microscope. Ultrathin sections were also cut, stained with uranyl acetate and alkaline bismuth subnitrate and examined under a transmission electron microscope. To quantify the ultrastructural damage, 20 consecutive capillaries from untreated and aminosteroid treated areas were photographed at 2000 × magnification and scored by the criteria of Kolodgie *et al.* (1991), with slight modifications (Table 1). The observer was blinded as to treatment.

Biochemical assays

Myocardial bioptic specimens of untreated, treated and control animals, taken from the same ischemic area of the left ventricle, were immediately frozen in liquid nitrogen and homogenized for 60 s in 10% trichloroacetic acid using an Ultraturrax homogenizer at half maximal speed. After 10 min of centrifugation at $8000 \times \mathbf{g}$ the resultant acid supernatant was assayed for malondialdehyde (MDA) by adding an equal volume of 0.67% (w/v) thiobarbituric acid (TBA). After 30 min of incubation at 100 °C the mixture was cooled, the absorbance was read at 532 nm and the concentration of MDA was calculated on an ε value of 153 000 (Esterbauer and Cheeseman, 1990).

Myocardial adenine nucleotides, nucleosides (adenosine plus inosine) and ascorbic acid were determined in the same neutralized supernatant by the high performance liquid chromatography procedure of Lazzarino *et al.* (1991).

MPO activity was evaluated as an index of neutrophil accumulation by a specific assay for this enzyme (Grisham *et al.*, 1990). Cardiac tissue samples were rapidly frozen in liquid nitrogen, pulverized and homogenized in 20 mM potassium phosphate buffer (pH 7.4) to 1:5 (w/v). After 30 min of centrifugation at $20000 \times \mathbf{g}$ the supernatants were discarded and the pellets were resuspended in 0.5% hexacyltrimethylammonium bromide dissolved in 50 mM potassium phosphate buffer (pH 6). Each sample was sonicated for 1 min in ice and then centrifugated for 30 min at $20000 \times \mathbf{g}$. An aliquot of the supernatants was assayed spectrophotometrically for MPO activity. Changes in absorbance at 655 nm were measured with 3,3′, 5, 5′-tetramethylbenzidine-hydrogen peroxide as the substrate. One unit of MPO activity was defined as the amount of enzyme that produces a change in absorbance per min of 1.0 at 37°C.

To determine the ratio of dry tissue to frozen tissue weight, a piece of frozen tissue was weighed and dried at 150°C for 24 h. The dry tissue weight was then estimated. The mean value for dry tissue weight was $20 \pm 0.4\%$ of the corresponding frozen tissue weight ($n = 6$) (Liu *et al.*, 1993).

Blood samples taken from the coronary sinus before the ischemia and at the end of reperfusion were used to measure levels of serum myoglobin, creatine kinase MB isoenzyme (CK-MB) and of cardiac troponin I (cTnl). These protein were assayed with fluorimetric enzyme immunoassay (Dade, Miami, FL, USA) using a Baxter Stratus II analyser. Serum susceptibility to peroxidative process was determined as "potential peroxidation" by measuring MDA concentration in 20% trichloroacetic acid deproteinized samples (serum: TCA; 1:7) with the above mentioned procedure, before and after incubation with 2 mM copper sulphate at 37°C for 24 h (Özdemirler *et al.*, 1995).

Statistical analysis

All values were expressed as mean ± S.E.M.. Statistical analysis of the data was performed with Student's *t*-test. Differences were considered statistically significant when P was <0.05.

Results

Hemodynamic measurements

In both untreated and treated animals mean artherial blood pressure (MABP), heart rate (HR) and left ventricular dP/d*t* (LV dP/d*t*) were measured before coronary occlusion at the end of ischemic period and after reperfusion.

In the untreated group MABP dropped with LAD coronary occlusion to about 70% of its initial value (from 105 ± 15 to 80 ± 12 mmHg) and a parallel reduction was observed for LVdP/d*t* (from 1670 ± 475 to 1485 ± 380 mmHg/s); HR decreased from 112 ± 17 to 100 ± 14 beats/min at the end of ischemic period. Similar changes were found in the treated animals: MABP decreased from 102 ± 13 to 76 ± 11 mmHg; LVdP/d*t* from 1725 ± 462 to 1430 ± 345 mmHg/s; HR from 106 ± 18 to 97 ± 11 beats/min. In any case, for all the considered hemodynamic parameters the differences between the treated and untreated group were not significant. ECG patterns suggestive for ischemia, that is a slight elevation in the ST segment, were evident in all the animals after coronary occlusion; in this period, two animals in the treated and one in the untreated group fibrillated, but they were successfully defibrillated at 150 W/s. All of the 20 randomized animals completed the protocol.

With reperfusion, ECG and all the hemodynamic parameters gradually returned to normal. Sporadic premature atrial and vetricular contractions were observed at the beginning of reperfusion in both treated and untreated animal groups. Besides these findings, it is noteworthy that the myocardial area, which during coronary occlusion appeared violet and hypokinetic, rapidly recovered normal color and contractility when the coronary clamp was released. This provides direct visual evidence for the effectiveness of our experimental model to realize hypoperfusion/reperfusion conditions.

Morphological results

Control myocardium

Myocardial tissue obtained from the left ventricle of sham operated pigs showed normal myocardial cells as well as a normal microvasculature. Within the myofiber, sarcomeres were in register and mitochondria contained tightly packed cristae.

Ischemic myocardium

During ischemia, the myocardial tissue showed essential normal morphology except for the presence of low amplitude, focal mitochondrial swellings (data not shown).

Ischemic reperfused myocardium

Reperfusion after coronary artery occlusion resulted in severe microvascular as well as parenchymal injuries. The dominant feature was represented by an increased neutrophil adhesion to the microvascular wall and emigration out of vasculature within the interstitial space [Fig. 1(A)]. The microvascular endothelium in the areas of intense neutrophil infiltration, as well as in those not directly involved in such process, showed signs of marked damage consisting in the appearance of localized areas of swelling, as testified by intraluminal blebs and focal loss of endothelial integrity [Fig. 1(B)]. As a result of the endothelial damage, extensive perivascular areas of intercellular edema were common findings. Within the myofiber, mitochondria demonstrated severe swellings with decrease in matrix density and loss of cristae. Glycogen granules appeared less abundant than in the control samples. In addition, sarcomeres were characteristically in the state of contraction, often showing disappearance of I-bands [Fig. 1(C)].

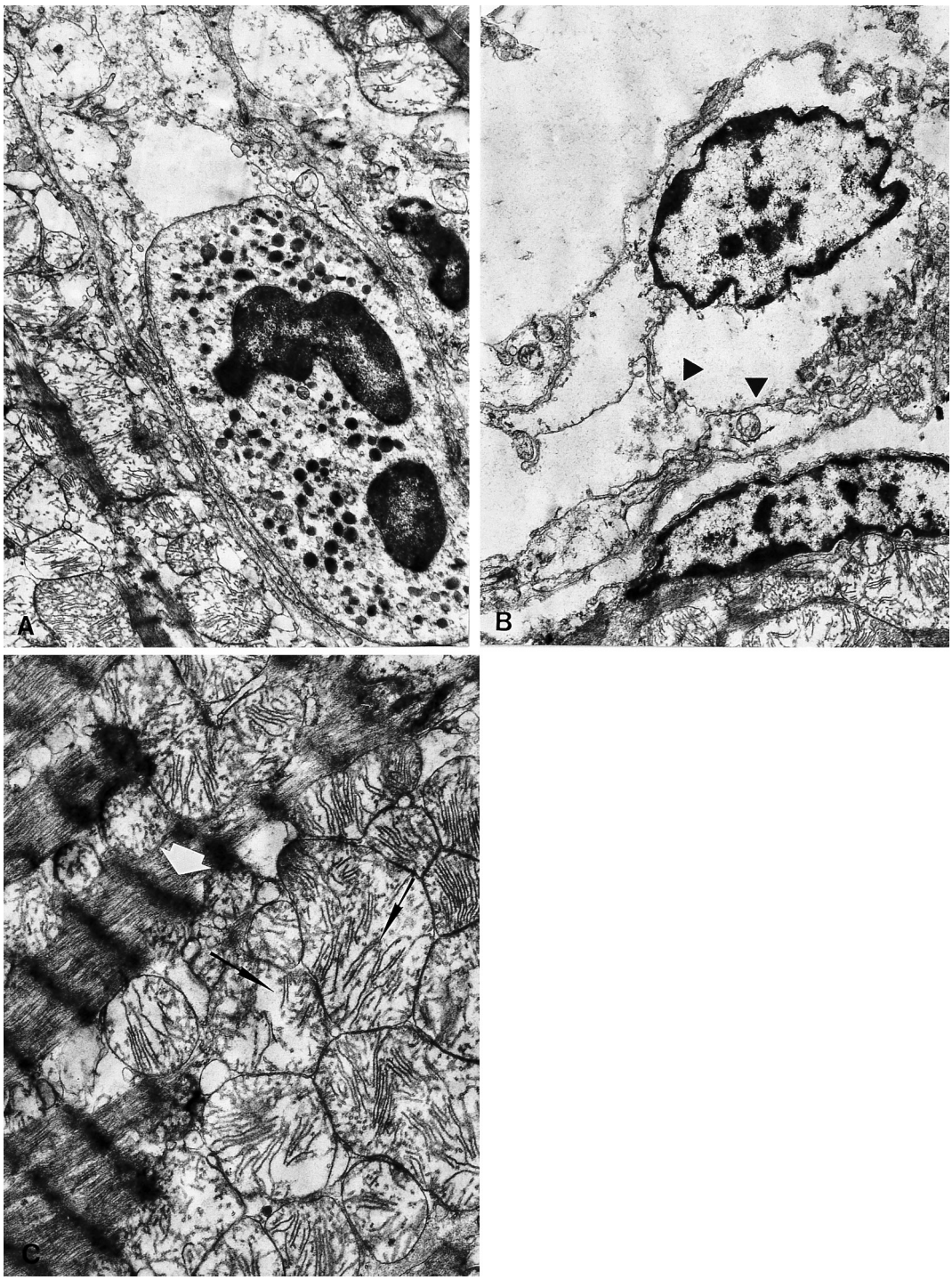

Figure 1 Ischemic and reperfused myocardium. (A) a neutrophil is seen to adhere to the luminal surface of small capillary. The myocardial fibers adjacent to the vessel show signs of severe damage. TEM × 99 000. (B) extensive areas of cell destruction are seen at the endothelial lining of a small capillary (arrowheads). TEM × 5940. (C) mitochondria show high amplitude swelling, separated cristae and clear matrix (arrows). Sarcomers are in a severe state of contraction (white arrow). TEM × 11 880.

Aminosteroid treated ischemic reperfused myocardium

After treatment with aminosteroid, the neutrophil accumulation within the myocardium was markedly attenuated, as was the myocardial reperfusion injury. In fact, the ultrastructural alterations at the endothelial level, scored as indicated in Materials and Methods, were significantly lower than those

observed in the untreated ischemic reperfused samples (0.22 ± 0.07 *v* 3.47 ± 0.22; $P<0.01$). Indeed, the endothelium maintained its integrity in most of the examined areas and no appreciable endothelial disruptions or swellings were found along the microvascular lining of the treated samples (Fig. 2). Upon aminosteroid treatment, the myofiber damage was also significantly lowered in comparision with that observed in the untreated ischemic reperfused samples (0.76 ± 0.19 *v* 2.38 ± 0.11; $P<0.01$) In fact, the intercellular edema was quite reduced in size and mitochondria often showed a quite normal ultrastructure with dense matrix and well arranged cristae. Only in focal areas of the myofibers mitochondria exhibited localized and low amplitude swellings [Figs 2(B) and 2(C)]. Finally, some myofibrils appeared still contracted, even after aminosteroid treatment [Fig. 2(C)].

Biochemical modifications

To study the effects of aminosteroid treatment on the energy metabolism, we measured adenine nucleotides and nucleosides in myocardial bioptic specimens taken at the end of ischemia and at the end of reperfusion in the untreated and treated animals. The energy charge of adenilic system was calculated on the basis of ATP, ADP and AMP concentrations. The results of these determinations are reported in Table 2, together with the corresponding data observed in the control animals. It is evident that the contents of ATP and ADP (the nucleotides containing high energy phosphate group) significantly decreased in the ischemic conditions both in the untreated and, albeit to a lesser extent, in the treated animals. Likewise, the increase in AMP and nucleoside levels observed in the ischemic myocardium was lower in the treated than in the untreated group. Such differences between ischemic treated and untreated animals were statistically significant, as it was the difference in the energy charge that we calculated for these groups.

Further to reperfusion, adenine nucleotide and nucleoside levels were partially restored, an effect which was more marked for the treated group, where AMP level and the value of energy charge were not even significantly different from the control. Table 3 shows the results of experiments aimed to examine the effects of aminosteroid treatment on the status of antioxidant defenses. MDA, a well known marker of lipid peroxidation, was undetectable in the control myocardium. In the ischemic condition we found detectable MDA levels which were not significantly different in the treated and in the untreated animals. After reperfusion, MDA myocardial concentration rose markedly in both groups, but this increase was significantly reduced owing to the aminosteroid treatment. Compared with the control value, the myocardial content of ascorbic acid was significantly reduced in the untreated group after ischemia and, at a greater extent, after reperfusion; on the contrary, the treated animals exhibited ascorbic acid levels which both in ischemic and after reperfusion were not significantly different from those of the controls.

After reperfusion, myocardial levels of MPO activity significantly ($P<0.05$) increased compared to the control value ($32.50 \pm 3.12\ \mathrm{U} \times 10^{-3}$ /g dry tissue) but, in agreement with histological findings, they were significantly lower in the treated than in the untreated group (66.30 ± 4.22 *v* $106.20 \pm 8.65\ \mathrm{U} \times 10^{-3}$ /g dry tissue; $P<0.05$).

Serum potential peroxidation, that is the susceptibility of serum to undergo peroxidation according to its antioxidant defenses, was determined in the treated and untreated animals after reperfusion. In the treated animals serum potential peroxidation was found to be lower not only with respect to that of untreated group, but also compared to that of the control group (Fig. 3)

As shown in Figure 4, serum levels of myoglobin, CK-MB and cTn I, were considerably higher compared to the control values in all the animals subjected to ischemia-reperfusion. However, the levels of all these proteins were significantly lower in the treated than in the untreated group: an effect which was expecially marked for myoglobin, whose concentration in the treated animals was 40% compared with that observed in the absence of treatment.

Discussion

Early reperfusion is the most effective way to avoid or to reduce myocardial necrosis and to improve ventricular function in ischemic myocardium. However, the introduction of cellular elements, particularly neutrophils, calcium and oxygen into the previously ischemic bed may initiate cellular changes that worsen a potentially reversible myocardial damage (Forman *et al.*, 1989, 1990). The hypothesis that neutrophils mediate IR injury has been reported by studies performed in a variety of models (Hernandez *et al.*, 1987; Otamiri, 1989; Mileski *et al.*, 1990; Formigli *et al.*, 1992). The morphological results here reported show that upon reperfusion a large number of tissue infiltrating

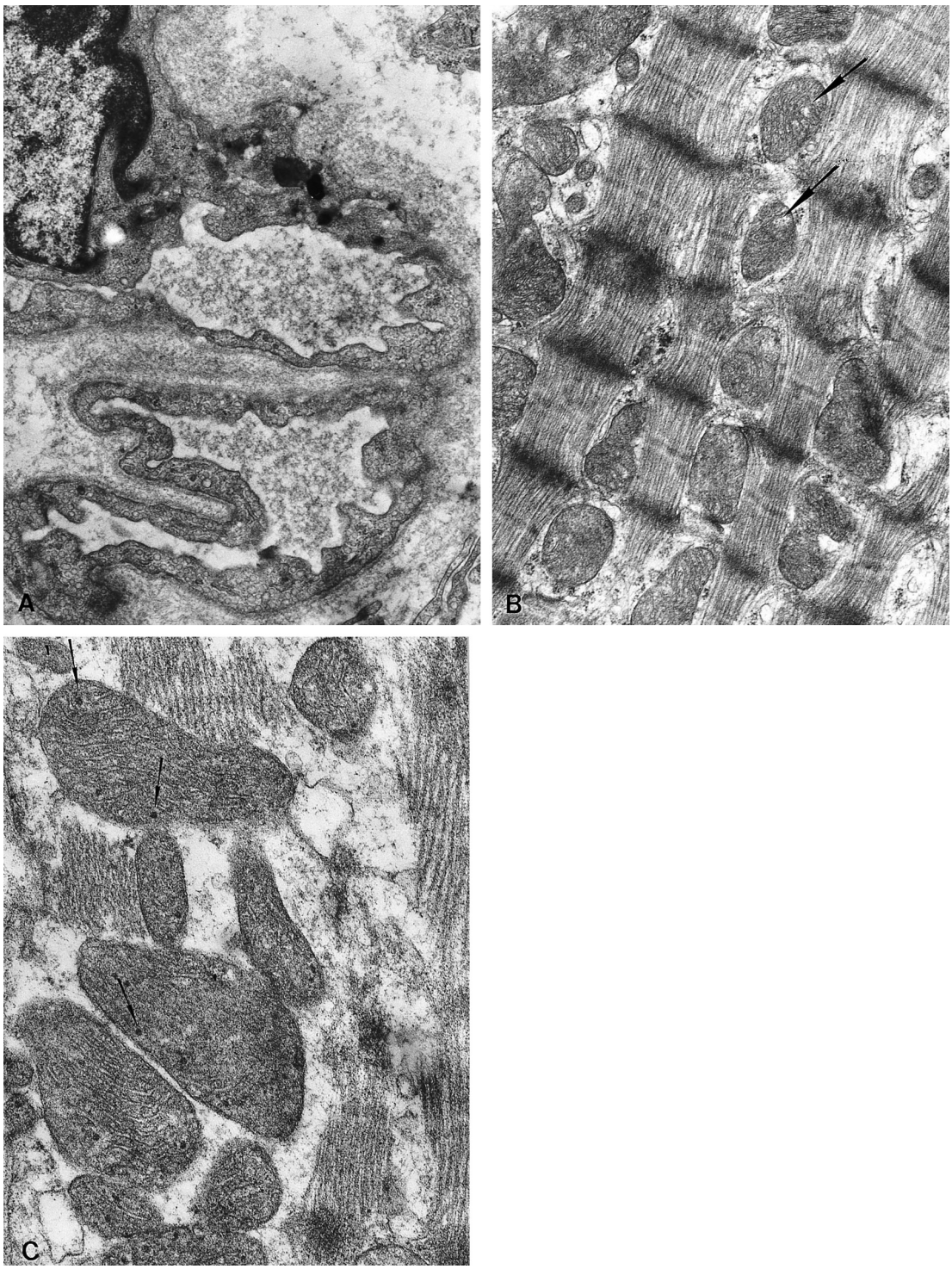

Figure 2 Aminosteroid-treated ischemic and reperfused myocardium. (A) a capillary shows a well preserved endothelial lining. TEM × 11 880. (B) a myocardial fiber shows mitochondria with focal areas of swelling (arrows) and normal cristae. Myofibrils exhibit areas of contraction. TEM × 15 840. (C) at higher magnification, mitochondria contain small-sized electron dense bodies within their matrix (arrows). TEM × 15 840.

neutrophils was associated with severe ultrastructural alterations of the myocardial tissue. These findings confirm and extend previous reports showing that neutrophils accumulation within IR tissue represent a major contribution to the reperfusion injuries, by actively producing ROIs via

Table 2 Myocardial adenine nucleotides, nucleosides and energy charge in ischemic and reperfused pig hearts with and without aminosteroid treatment

	ATP	ADP	AMP	Ino + ado	EC
Control	11.84 ± 0.61	6.03 ± 0.69	1.90 ± 0.15	1.10 ± 0.15	0.75 ± 0.01
Ischemic:					
Untreated	2.99 ± 0.36*	2.55 ± 0.22*	3.86 ± 0.28*	5.45 ± 0.42*	0.45 ± 0.02*
Treated	4.56 ± 0.50*‡	3.75 ± 0.20*‡	2.75 ± 0.24*‡	3.49 ± 0.36*‡	0.58 ± 0.02*‡
Reperfused:					
Untreated	3.92 ± 0.30*	2.97 ± 0.18*	2.64 ± 0.16*	2.96 ± 0.19*	0.57 ± 0.01*
Treated	7.20 ± 0.51*‡	4.14 ± 0.30*‡	1.93 ± 0.17†‡	1.84 ± 0.25*‡	0.70 ± 0.02†‡

Values indicate mean ± S.E.M. and expressed as μmol/g dry tissue except energy charge which is obtained from the following calculation: (ATP + 1/2 ADP)/ (ATP + ADP + AMP).
ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; Ino + ado, inosine + adenosine; EC, energy charge.
* $P<0.05$ *v* control; †N.S. *v* control; ‡$P<0.05$ *v* untreated. $n=6$ for control; $n=7$ for, untreated and treated animals.

Table 3 Oxidative processes and antioxidant defences in ischemic and reperfused pig hearts with and without aminosteroid treatment

	MDA (nmol/g dry tissue)	Ascorbic acid (μmol/g dry tissue)
Control	N.D.	1.05 ± 0.10
Ischemic:		
Untreated	15.30 ± 2.73	0.74 ± 0.08*
Treated	8.89 ± 3.44§	1.19 ± 0.14†‡
Reperfused:		
Untreated	128.49 ± 11.74	0.54 ± 0.06*
Treated	86.70 ± 7.43‡	0.83 ± 0.05†‡

Values are mean ± S.E.M. MDA, malondialdehyde; N.D., not detectable
* $P<0.05$ *v* control; †N.S. *v* control; ‡$P<0.05$ *v* untreated; §N.S. *v* untreated. $n=6$ for control; $n=7$ for untreated and treated animals.

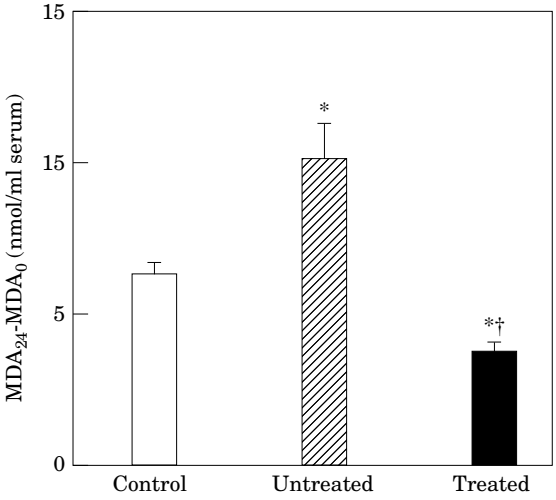

Figure 3 Serum peroxidation potential. Data are expressed as mean ± S.E.M. of six determinations for each group and were estimated by taking the difference between MDA values of 24 h and 0 h incubated with 2 mM copper sulphate. MDA content was assayed as described under Materials and Methods and are expressed as nmol/ml serum. *$P<0.05$ *v* control: †$P<0.05$ *v* untreated.

the membrane associated NADPH oxidase (Engler *et al.*, 1983; Horgan *et al.*, 1990; Walden *et al.*, 1990; Formigli *et al.*, 1992; Siminiak and Ozawa, 1993). It is possible that neutrophil tissue accumulation is the consequence of an increased expression of specific endothelial adhesion proteins for circulating granulocytes by the endothelium of small blood vessels. Accordingly, recent studies have shown that hypoxia-reoxygenation stimulates the endothelial cells of capillaries and post-capillary venules to express E-selectin and ICAM-1; such molecules are known to be responsible for the initial adhesion of neutrophils to the endothelial lining and for the subsequent diapedesis of these cells across the vascular wall (Palluy *et al.*, 1992; Youker *et al.*, 1992; Formigli *et al.*, 1995). Besides neutrophil infiltration, microvascular endothelium damage and ultrastructural mitochondrial alterations were the most prominent morphological features observed in IR myocardium. The endothelial damage may be the result of peroxidative processes which enhance capillary permeability, eventually leading to intercellular edema which was clearly evident in examined samples.

The mitochondrial alterations that we found in IR myocardium were probably not compatible with a maintained functionality of the organelles. These lesions may be the consequence of a peroxidative damage, even if mitochondrial disfunction could represent by itself an additional source of ROIs through an enhancement of the "univalent leakage" in the respiratory chain.

All of the above morphological changes were

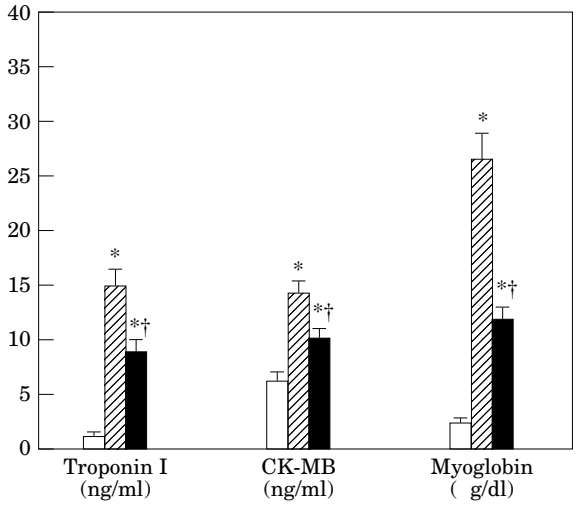

Figure 4 Myocardial markers of injury. Data are expressed as mean ± S.E.M. of six determinations for each group. Assays of the marker proteins are described in Materials and Methods. *P<0.05 *v* control; †P<0.05 *v* untreated. Control (□), untreated (▨); treated (■).

attenuated by aminosteroid treatment: only mild alteration occurred at the endothelial level, edema among the myocardial fibers was reduced, mitochondrial injuries were less evident and neutrophil tissue accumulation was markedly attenuated as it was also indicated by MPO levels. With regards to this latter finding, it is conceivable that the aminosteroid, in addition to protect endothelium, acting as an antioxidant and membrane stabilizer, may also inhibit—with an effect similar to that described for α-tocopherol—the expression and or the upregulation of adhesion molecules for circulating neutrophils (Campo *et al.*, 1994; Formigli *et al.*, 1997; Novelli *et al.*, 1997).

As for the results of biochemical determinations, the effectiveness of the aminosteroid in preserving the cellular antioxidant defences and in reducing the peroxidative processes induced by IR is indicated by the ascorbic acid and MDA levels found in cardiac tissue. In the present study, MDA was measured by means of a classical thiobarbituric acid (TBA) assay. Although the specificity of this test is questionable (Ceconi *et al.*, 1991), we thought that, in our experimental conditions, an overall determination of MDA and other TBA reactive substances may represent a more suitable marker of lipid peroxidation. A slight increase in MDA concentration was also shown at the end of ischemia, indicating that free radical production began during this phase. This is not surprising, since it has been reported that the production of oxygen free radicals and lipid peroxides may take place even at the very low O_2 tensions attained in the ischemic tissues (Rao *et al.*, 1983). In any case, the low level of lipid peroxidation during this period correlated well with the lack of substantial ultrastructural alterations of the ischemic myocardium. As expected, MDA concentration markedly increased after reperfusion, but this effect was significantly lower in the animals submitted to aminosteroid treatment. Also, in serum, where MDA was measured to determine the peroxidation potential, the increase in MDA levels induced by copper was reduced in the treated animals, indicating an enhanced antioxidant protection and a lower susceptibility of serum to lipid peroxidation.

The protective effect of aminosteroid against the IR induced ultrastructural alterations of mitochondria was associated with a functional improvement of these organelles, as judged by the concentration of high energy phosphate and the value of energy charge we found in the untreated and treated animals. In fact, the oxygen replenishment was able to normalize myocardial energy store only with the pharmacological treatment. Without aminosteroid treatment this did not happen, probably because ATP metabolites (adenosine and inosine) that are the substrates for salvage synthesis of ATP were depleted or degradated (Liu *et al.*, 1993). The membrane stabilizing effect of aminosteroids is a property that, on one hand, contributes to the protection against lipid peroxidation, thus to a reduction of cell damage, by restricting the propagation of this process within the membranes where it may rise up; on the other hand, this property could be associated with a reduction of the leakage of intracellular components, such as enzymes and other proteins, which is a frequent event in IR syndromes. To verify this effect in our experimental system, we determined the serum concentrations of three commonly measured markers of myocardial cell damage: myoglobin, CK-MB and cTn I. CK-MB and cTn I were measured to overcome the low specificity of myoglobin which, owing to the precocity of its increase in serum, appeared as a very suitable marker of myocardial injury in our experimental conditions (Keffer, 1996). The levels reached by these proteins after IR in the treated and untreated animals suggest that aminosteroid administration attenuated membrane permeability alteration and the release of myocardial proteins.

In conclusion, the results reported here in provide evidence that, at least in the condition used in the present study, aminosteroid treatment is remarkably effective in reducing the morphological and biochemical alterations occurring in ischemic-

reperfused myocardium. Further studies will be of interest to confirm such a beneficial effect in other experimental models and to ascertain the clinical relevance of these findings.

Acknowledgements

We thank Dr Agostino Ognibene for help with protein fluorimetric immunoassays. This work was supported by grants from the Ministero dell' Università e della Ricerca Scientifica e Tecnologica, Progetto Nazionale Insufficienza Cardiaca.

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