Free Rad. Res., Vol. 27, pp. 577–590
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Antioxidant Activity of U-83836E, A Second Generation Lazaroid, During Myocardial Ischemia/Reperfusion Injury

GIUSEPPE M. CAMPO[a,*], FRANCESCO SQUADRITO[a], SALVATORE CAMPO[b], DOMENICA ALTAVILLA[a], ANGELA AVENOSO[a], MARCELLA FERLITO[a], GIOVANNI SQUADRITO[b] and ACHILLE P. CAPUTI[a]

[a]*Institute of Pharmacology, School of Medicine, University of Messina, Piazza XX Settembre n°4, 98122, Messina, Italy and* [b]*Department of Internal Medicine, School of Medicine, University of Messina, Viale Gazzi, 98100, Messina, Italy*

Accepted by Prof. B. Halliwell

(Received 14 April 1997; In revised form 1 July 1997)

The 21-aminosteroid compounds are potent lipid peroxidation inhibitors belonging to a new class of antioxidants given the collective name of "lazaroids". They protect cells from oxidative damage induced by oxygen-based free radicals in a variety of in vitro and in vivo test systems. U-83836E is one of the second-generation lazaroids that are based on a non steroidal structure characterized by a ring portion of α-tocopherol bonded with various amine groups. We investigated the ability of U-83836E to reduce myocardial damage in rats undergoing left coronary artery occlusion for 60 min followed by 6 hours of reperfusion.

This ischemia/reperfusion model produced wide heart necrosis, membrane lipid peroxidation, ventricular arrhythmias, tissue neutrophil infiltration and a marked decrease in endogenous antioxidants.

Intravenous administration of U-83836E, (7.5, 15 and 30 mg/kg) at onset of reperfusion, reduced myocardial necrosis, expressed as a percentage of either the area at risk or the total left ventricle ($p < 0.001$), improved haemodynamic conditions by decreasing ventricular arrhythmias ($p < 0.005$), limited membrane lipid peroxidation (evaluated by assessing conjugated dienes, $p < 0.001$; and 4-hydroxynonenal, $p < 0.001$) restored the endogenous antioxidants vitamin E ($p < 0.001$), and superoxide dismutase ($pt < 0.001$). Furthermore, the lazaroid inhibited the derimental hydroxyl radical formation ($p < 0.001$), evaluated indirectly by a trapping agent and reduced heart neutrophil infiltration, measured by testing cardiac tissue elastase ($p < 0.001$) that is released from the stimulated granulocytes at the site of injury. These data suggest that this compound could be a new useful tool to study the mechanisms of oxidative damage during myocardial infarction.

Keywords: Antioxidants, lazaroids, free radicals, lipid peroxidation, vitamin E, myocardial injury

INTRODUCTION

In recent years, although several mechanisms have been proposed to explain the pathogenesis of ischemia/reperfusion injury, most attention has focused on a role for reactive oxygen metabolites and inflammatory leukocytes.[1,2]

Early restitution of blood flow to the ischemic myocardium is essential to halt the progression of

* Corresponding author. Tel.: 39 90 712533. Fax: 39 90 661029.

cellular injury associated with decreased oxygen and nutrient delivery. However, reperfusion in itself may have a detrimental effect on reversibly injured cells.[3,4] Several data indicate that free radical-induced lipid peroxidation of cell membranes is thought to be a major mechanism in the evolution of myocardial reperfusion damage.[5,6] Associated with reperfusion of the ischemic myocardium is a cellular reaction characterized by infiltration of leukocytes and the production of inflammatory mediators in the cardiac tissue with subsequent increase of fibro-cellular injury.[7] Endogenous defence mechanisms have been identified which use antioxidants or free radical scavengers to neutralize reactive species-generated lipid peroxidation; however, the extensive generation of free radicals appears to overwhelm the natural defence mechanisms, dramatically reducing the levels of endogenous antioxidants.[8] This results in the uncontrolled progression of peroxidative damage to cellular membranes. The endogenous chain-breaking antioxidant, vitamin E (α-tocopherol) is of great importance in the antioxidant defence system. Vitamin E deficiency exacerbates myocardial injury due to oxidative stress.[9] Previous studies indicate that myocardial ischemia/reperfusion is associated with a decrease in the tissue α-tocopherol levels.[10] Acute administration of vitamin E resulted in a high plasma concentration, but did not protect the heart after coronary artery occlusion/reperfusion in the pig.[11] In fact, the marked lipophilicity of the vitamin limits its incorporation into tissues.

U-83836E (–)-2-((4-(2,6-di-1-pyrrolidinyl-4-pyrimidinyl)-1-piperazinyl) methyl)-3-,4-dihydro-2,5,7,8-tetramethyl-2h-1-benzopyran-6-dihydrochloride, is one of the second-generation compounds belonging to a family of antioxidants, given the collective name of "lazaroids", which are potent inhibitors of iron-mediated lipid peroxidation, cell membrane stabilizers and scavengers of hydroxyl radicals in a variety of in vitro and in vivo models.[12–14] These new lazaroids are based on a non steroidal structure characterized by a ring portion of α-tocopherol bonded with various active groups. This compound would have a better pharmacokinetic profile due to its low hydrophobicity. The aim of the present study was to assess the possible ability of this drug in reducing the damage of rat heart after myocardial ischemia and reperfusion.

MATERIALS AND METHODS

Surgical Procedures

Male Sprague-Dawley rats (230–250 g body weight) underwent myocardial ischemia which

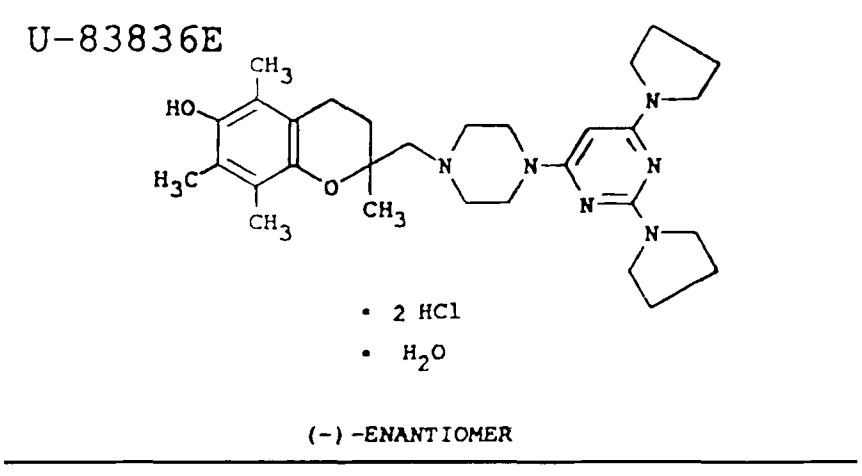

FIGURE 1 Chemical structure of U-83836E.

was induced by a temporary occlusion of the left main coronary artery.[15] Rats were anaesthetized with sodium pentobarbital (50 mg/kg, i.p.) and placed on a heated operating table. Polyethylene catheters (PE 50) were inserted into the common carotid artery for the measurement of blood pressure and heart rate, as previously reported.[16] After tracheotomy, the animals were ventilated with room air by a respirator for small rodents (model 7025, Ugo Basile, Varese, Italy) with a stoke volume of 15 ml/kg and a rate of 54 stroke/min to maintain normal PO_2, PCO_2 and pH parameters. An incision was made on the left side of the chest and the fourth intercostal space was exposed. Sutures were placed through the overlapping skin and muscles to permit rapid closure of the chest wall following the surgical procedure. The chest was then opened and the ribs were gently spread. The heart was quickly expressed out of the thoracic cavity, inverted and a 4.0 silk ligature was placed under the visualized left main coronary artery. The ligature was then tied. The heart was returned quickly to the thoracic cavity and the tips of the suture, used to produce the coronary ligation, were exteriorized through the chest wall. After the removal of air in the chest by syringe, the incision was closed by tying the previously placed sutures. The occlusion period lasted 1 hour, then the tips of the sutures were removed and the heart was reperfused for 6 hours (MI/R groups). Sham animals underwent all the previously described surgical procedures apart from the fact that the sutures, passing around the left coronary artery, were not tied (Sham MI/R groups).

Animals were treated with U-83836E (7.5, 15, and 30 mg/kg) or vehicle (NaCl 0.9%) acutely intravenously at onset of the reperfusion.

Myocardial Damage Quantification

Infarcted and perfused areas were determined by the triphenyl tetrazolium chloride Evan's blue technique.[17] At the end of the reperfusion period, the ligature around the left main coronary artery was retightened; 2 ml of Evan's blue dye (2 mg/ml solution) was injected into the jugular vein to stain the area of the myocardium perfused by the patent coronary arteries. The area at risk was determined by negative staining. The atria, right ventricle, and major blood vessels were subsequently removed from the heart. The left ventricle was then sliced into section 3-mm-thick parallel to the atrioventricular groove. The unstained portion of the myocardium (*i.e.*, the area at risk) was separated from the stained portion (*i.e.*, the area not at risk). The unstained portion was again sliced into 1-mm-thick sections and incubated in a 1% solution of triphenyl-tetrazolium chloride (TTC) stain in phosphate buffer 20 mM, pH 7.4 and 37°C for 20 min. The area at risk of infarction was colored brick red due to the formation of a precipitate that results from the reaction of TTC with dehydrogenase enzymes. The loss of these enzymes from the infarcted myocardium prevents formation of the precipitate; thus, the infarcted area within the risk region remains pale yellow (*i.e.*, necrotic area). Samples from all three portions of left ventricular cardiac tissue (*i.e.*, nonischemic, ischemic non necrotic, and ischemic necrotic) were weighed and stored at –70°C for subsequent analysis.

Haemodynamic Measurements

In order to monitor blood pressure, a cannula (PE 50) was inserted into the left common carotid artery. The arterial catheter was connected to a pressure transducer. The pressure pulse triggered a cardiotachometer, and arterial blood pressure was displayed on a polygraph. Arterial blood pressure is reported as mean arterial blood pressure (MABP) in mmHg. The Changes in the electrical activity of the myocardium were detected by the electrocardiogram (ECG) in lead II (Biocard, model BC-1, Florence, Italy).

Lipid Peroxidation Determination

Estimation of the plasma conjugated dienes (CD) and the plasma 4-hydroxynonenal (HNE) was carried out to evaluate lipid peroxidation in cardiac membranes. Samples (0.5 ml) of arterial blood were drawn from the carotid catheter before occlusion and at the end of reperfusion. The blood removed was replaced at a ratio of 1:2 with saline solution (NaCl 0.9%). The blood was collected in polyethylene tubes with previous addition of 20 µl of heparin solution (16,000 IU) and 10 µl of butylated hydroxytoluene (BHT) (1 mg/ml in phosphate buffer).

The plasma samples obtained after centrifugation at 3,000 × g for 10 min at 4°C were divided in two different tubes and were frozen at –70°C until the analysis. For assay of CD lipid were extracted from plasma samples by chloroform/methanol (2:1) and dried under nitrogen atmosphere and then dissolved in cyclohexane. Plasma contents of CD was performed at 232 nm (Uvikon mod.860, Kontron instruments, Everett, USA) by using a spectrophotometric technique.[18] The amount of plasma CD was expressed as ΔABS/ml. HNE plasma levels were assessed by using a commercial colorimetric kit (Lipid peroxidation assay kit, Cat. n°437634, Calbiochem-Novabiochem corporation, San Diego CA, USA). The concentration of plasma was expressed in mM.

Vitamin E Evaluation

Cardiac and plasma vitamin E (VE) levels were measured in order to determine the oxidative state of the heart and the blood following the ischemia/reperfusion injury. Samples of blood (0.3 ml) were drawn from the carotid catheter before occlusion and at the end of reperfusion. The blood removed was replaced at a ratio of 1:2 with saline solution (NaCl, 0.9%), then collected in dark polyethylene tubes, to avoid the direct action of the light, with previous additions of 10 µl of heparin solution (8,000 IU) and 5 µl of BHT (1 mg/ml in phosphate buffer). The blood samples were centrifugated at 3,000 × g for 10 min at 4°C. Myocardial VE levels were determined in the area not at risk and in the area at risk samples obtained after excision of the heart. Plasma and myocardial specimens were stored at –70°C until the assay. The assay was performed using a high performance liquid chromatography (HPLC) method with some modifications.[19] Briefy, 0.15 ml of plasma or tissue homogenate, contained in dark polyethylene tubes, was treated with 150 µl of tocopherol acetate (25 µg/ml in ethanol) (Sigma Chemical Co., St Louis, MO, USA), which has been used as an internal standard, and with 300 µl of butanol/ethyl acetate (1:1, V:V) (Acros Chemical, Geel, Belgium). After vortexing for 20 s, 15 mg of sodium sulfate were added (Acros Chemical, Geel, Belgium) and shaken on a vortex mixer for additional 60 s. After centrifugation at 15,000 × g for 5 min at 4°C the organic layer was recovered and 50 µl was injected into the HPLC apparatus. The HPLC equipment consisted of a solvent delivery module (Mod. 422 Master, Kontron Instruments, Everett, USA), a programmable variable wavelength detector (Spectromonitor 4100, Thermo Separation Products, Florida, USA), connected to an automatic integrator (Mod. CR-3A, Shimadzu, Kyoto, Japan). The column used was a ultratechsphere C_{18}, 250 × 4.6 mm, 5µ (HPLC Technology LTD, Macclesfield, Cheshire, UK), attached to a precolumn (Guard column, Water-Millipore, Milford, USA). The mobile phase was methanol/water (97:5, V:V) at a flow rate of 1 ml/min at room temperature. The detector was set at wavelength of 280 nm. The concentration of plasma vitamin E was expressed in µM.

Cardiac Superoxide Dismutase Assessment

Superoxide dismutase (SOD) activity was evaluated to estimate endogenous defences against superoxide anions. The analysis was carried out in the area not at risk, the area at risk and necrotic area of the left ventricle. SOD activity was determined spectrophotometrically at 505 nm by

using a commercial kit (Ransod assay kit, cat. n°Sd 125, Randox Laboratories, Crumlin, U.K.). SOD activity was expressed as Units/mg protein.

Measurement of Hydroxyl Radical ($OH^{\cdot}$) Formation

In order to quantify $OH^{\cdot}$ production during occlusion and reperfusion in the heart, we used the aromatic trap technique.[20] Sodium salicylate serves as a specific trap for hydroxyl radicals because it can react chemically with $OH^{\cdot}$ radicals produced, yielding 2,5-dihydroxybenzoic acid (2,5-DHBA), 2,3-dihydroxybenzoic acid (2,3-DHBA) and catechol as its hydroxylation derivatives in an approximate proportion of 40%, 49% and 11%, respectively.[21] In the present study, we measured both 2,5-DHBA and 2,3-DHBA.[22,23] To allow the chemical reaction, each group of animals received sodium salicylate (100 mg/kg i.p.)[24] (Janssen Chemical, Beerse, Belgium), one hour before surgical procedures. Another experimental group, which underwent myocardial ischemia plus reperfusion, was not administered with sodium salicylate with the aim to study any possible direct action of the acid on the considered parameters. We did not observe any interaction between salicylic acid and the compound U-83836. Samples of blood (0.5 ml) were drawn from the carotid catheter before occlusion, 15 min after the reperfusion, 180 min after the reperfusion and at the end of reperfusion. The blood removed was replaced at a ratio of 1:2 with saline solution (NaCl 0.9%). The blood was collected in polyethylene tubes with previous addition of 20 µl of heparin solution (16,000 IU). The plasma samples obtained after centrifugation at 3,000 × g for 10 min at 4°C were frozen at –70°C until the assay. To measure 2,5-DHBA and 2,3-DHBA formation, an HPLC method was used.[21,25] Briefly, 250 µl of plasma was treated with 10 µl of 100 µM 2,4-dihydroxybenzoic acid (2,4-DHBA) (Janssen Chemical, Beerse, Belgium), which has been used as an internal standard and 10 µl of 40% $HClO_4$ than the plasma was extracted with 2.5 ml HPLC grade diethylether (Janssen Chemical, Beerse, Belgium) and mixed on a vortex for 2 minutes. After centrifugation for 15 min at 15,000 × g at 4°C, the diethylether layer was separated and was then evaporated in a vacuum concentrator system (Heto Lab Equipment, Denmark). The residue obtained was dissolved in 30 µl of 0.1 N HCl and 32.5 µl of mobile phase, and 50 µl of the solution was injected into the HPLC apparatus. The HPLC equipment consisted of a solvent delivery module (Mod. 422 Master, Kontron Instruments, Everett, USA), a programmable wavelength detector (Mod. 165, Beckman Instruments, San Ramon, USA), connected to an automatic integrator (Mod. CR-3A, Shimadzu, Kyoto, Japan). The column used was a Lichrosorb-10-RP18, 10µ 250 × 4.6 mm (Labservice Analytica, Milano, Italy), attached to a precolumn (Guard Column Water-Millipore, Milford, USA). The mobile phase was 80% 0.03 M citric acid, 0.03 M acetic acid buffer (pH 3.6) and 25% methanol (Janssen Chemical, Beerse, Belgium) at a flow rate of 1.3 ml/min. The detector was set at a wavelength of 315 nm. The concentrations of 2,3-DHBA and 2,5-DHBA were expressed in µM.

Cardiac Elastase Content

Elastase (ELA) levels were evaluated as an index of PMNs accumulation and activation in the jeopardized tissue because this enzyme is released from the stimulated granulocytes at the site of injury.[26] The analysis was carried out in the area not at risk, the area at risk and necrotic area of the left ventricle by using a specific immunoassay kit (PMN elastase, IMAC, cat. n° 11332, Merk, Darmstadt, Germany). ELA activity was expressed in Units/g tissue.

Drug

U-83836E was supplied by Upjohn SpA, Caponago (MI), Italy. The compound was administered intravenously in saline solution (NaCl, 0.9%). All

substances were prepared fresh daily and administered in a volume of 1 ml/kg.

Statistical Analysis

Data are expressed as means ± S.D. and were analyzed by analysis of variance for multiple comparison of results; Duncan's multiple range test was used to compare group means. In all cases, a probability of error of less than 0.05 was selected as criterion for statistical significance.

Statement of Humane Care

The studies reported in this manuscript have been carried out in accordance with the declaration of Helsinki and with the guide for the Care and Use of Laboratory Animals.

RESULTS

Myocardial Infarct Size

The area at risk showed no significant differences between each of the experimental groups (Table I), indicating that a similar amount of tissue (about 70%) was jeopardized by the occlusion of the left coronary artery in each group. In the MI/R vehicle group, the necrotic area, expressed either as a percentage of the area at risk or as a percentage of the left ventricle was significantly increased indicating that a large amount of cardiac tissue at risk became necrotic. Administration of U-83836E reduced significantly myocardial necrosis extension. This reduction was observed either in the necrotic area/area at risk or in the necrotic area/total left ventricle.

Cardiovascular Measurements

At the end of the experiment mean arterial blood pressure (MABP) averaged 58.3 ± 8.6 mmHg (n = 13) in MI/R rats treated with vehicle. Administration of U-83836E, with all doses, improved MABP (63.1 ± 6.5 mmHg, n = 9; 64.4 ± 7.5 mmHg, n = 9; 66.2 ± 8.5 mmHg, n = 9; with the doses of 7.5, 15 and 30 mg, respectively) ($p < 0.05$ vs. MI/R + vehicle group) in rats subjected to myocardial ischemia/reperfusion injury. No significant differences were found among Sham MI/R vehicle group and Sham MI/R + U-83836E (114.6 ± 5.8 mmHg, n = 7 and 116.2 ± 6.1 mmHg, n = 7, respectively).

MI/R rats showed a significant presence of ventricular arrhythmias (Fig. 2). U-83836E reduced the occurrence of ventricular arrhythmias.

Conjugated Dienes

Table II shows the changes in plasma CD concentrations before occlusion, 15 min after reperfusion and at the end of reperfusion in the considered experimental groups. Low CD levels were assayed in each group before occlusion. (<2 ΔABS/ml) and there were no significant differences among any of them. Instead, a large increase of this marker was found in the plasma of MI/R rats given vehicle at the end of reperfu-

TABLE I Obtained percentage (%) of infarct size after ischemia/reperfusion injury

Experimental group	Area at risk / Total	Necrotic / Area at risk	Necrotic / Total
MI/R + vehicle (n = 13)	68.7 ± 3.4	64.6 ± 5.2	39.7 ± 4.5
MI/R + U-83836E (7.5 mg/kg) (n = 9)	70.4 ± 2.6	40.8 ± 5.8*	27.1 ± 7.4*
MI/R + U-83836E (15 mg/kg) (n = 9)	69.6 ± 3.6	31.7 ± 4.7*	19.1 ± 5.8*
MI/R + U-83836E (30 mg/kg) (n = 9)	70.1 ± 3.7	20.2 ± 5.4*	13.2 ± 6.3*

The total number of animals in each group is indicated in parentheses. Values are mean ± S.D.; *$p < 0.001$ vs MI/R + vehicle group.

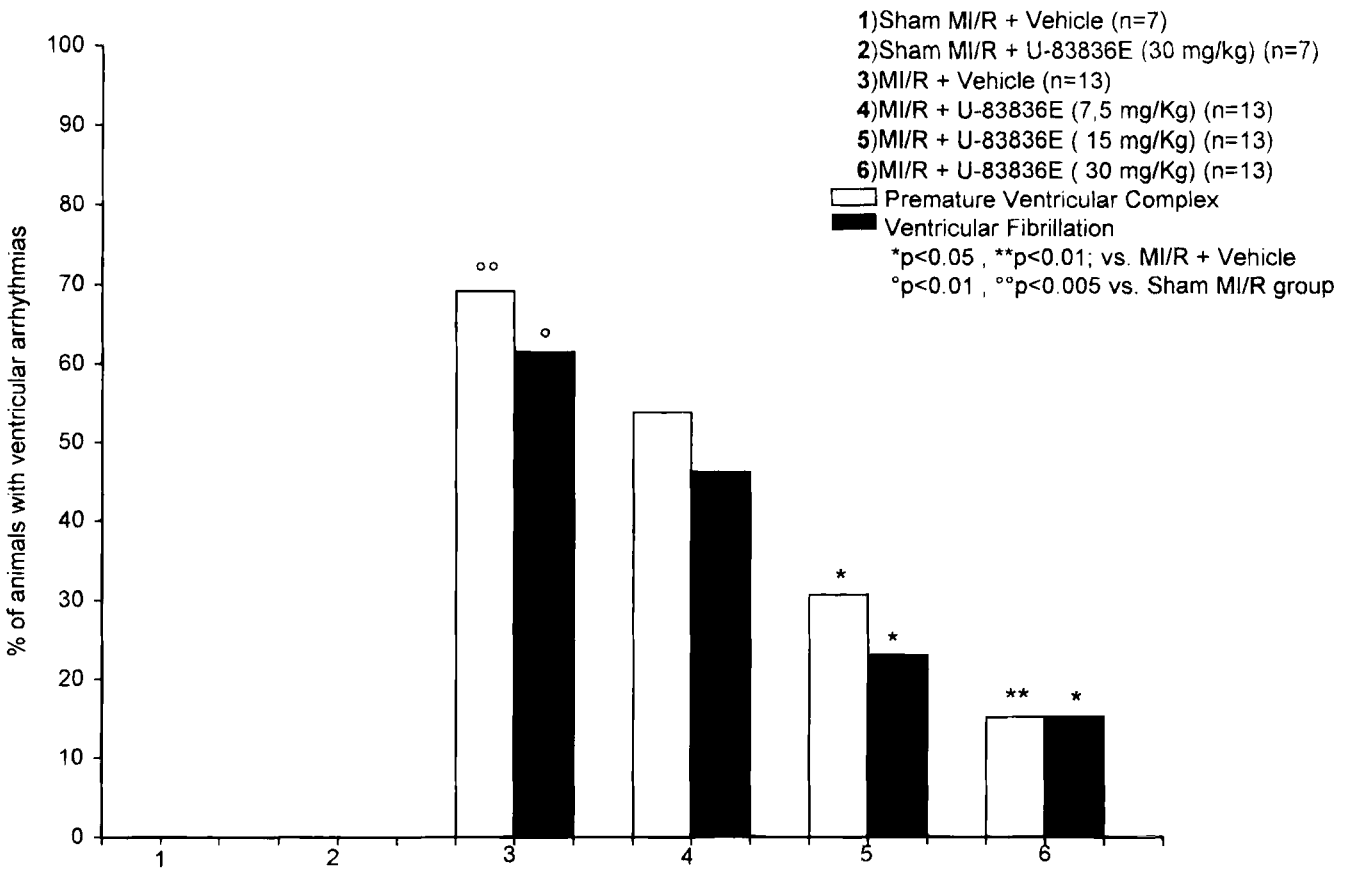

FIGURE 2 % of animals with ventricular arrhythmias. Statistical analysis was performed by using Fisher's exact probability test. Statistical significance is reported at the top of the bars, the total number of animals in each group is indicated in parentheses.

TABLE II Plasma conjugated dienes (ΔABS/ml) assessed in basal conditions and at the end of the reperfusion

Experimental group	Basal	End of reperfusion
Sham MI/R + vehicle (n = 7)	1.71 ± 0.48	3.02 ± 0.42
Sham MI/R + U-83836E (30 mg/kg) (n = 7)	1.81 ± 0.62	3.13 ± 0.39
MI/R + vehicle (n = 13)	1.73 ± 0.68	9.82 ± 1.61
MI/R + U-83836E (7.5 mg/kg) (n = 9)	1.96 ± 0.61	8.11 ± 1.43*
MI/R + U-83836E (15 mg/kg) (n = 9)	1.83 ± 0.62	6.92 ± 1.1**
MI/R + U-83836E (30 mg/kg) (n = 9)	1.79 ± 0.84	5.34 ± 1.2**

The total number of animals in each group is indicated in parentheses. Values are mean ± S.D.; *$p < 0.05$ and **$p < 0.001$ vs MI/R + vehicle group.

sion. The administration of U-83836E resulted in a blunting of the CD formation. All the doses were effective.

4-Hydroxynonenal

Before occlusion, very low HNE levels were measured in all the groups (<3 μM). In contrast, a marked increase in HNE was observed in plasma withdrawn at the end of reperfusion in the MI/R vehicle group. In Table III it is clearly represented how the compound U-83836E significantly reduced this increase. Only a small increase in HNE concentration was found in Sham MI/R groups at the end of reperfusion (<6.5 nmol/ml) likely due to the surgical procedures. However, the administration of U-83836E in Sham rats did not modify plasma HNE.

Vitamin E Analysis

Figure 3 summarizes graphically the plasma VE values measured in the basal conditions, and at the end of reperfusion. Before occlusion, plasma VE levels ranged from 0.150 to 0.175 μmol/mg of cholesterol, and there were no significant differences among the groups. In contrast, at the end of reperfusion, a marked decrease was noted in the plasma of MI/R rats given vehicle. The treatment with the lazaroid significantly restored VE. Myocardial VE contained in the areas not at risk ranged between 50.0 and 60.0 nmol/g tissue. Instead, decreased VE concentrations (19.0 ± 4.1 nmol/g tissue; n = 7, $p < 0.001$) were found in the areas at risk of MI/R vehicle rats. The administration of the drug partially restored VE levels (32.0 ± 8.0 nmol/g tissue, n = 9, $p < 0.005$; 35.4 ± 9.0 nmol/g tissue, n = 9, $p < 0.001$; 48.6 ± 13.0 nmol/g tissue, n = 9, $p < 0.001$, with the doses of 7.5, 15 and 30 mg/kg, respectively).

SOD Activity

Table IV shows the variations of SOD activity evaluated in the area not a risk, the area at risk

TABLE III Plasma 4-hydroxynonenal (μM) analysed in basal conditions and at the end of the reperfusion

Experimental group	Basal	End of reperfusion
Sham MI/R + vehicle (n = 7)	2.94 ± 0.42	6.21 ± 0.78
Sham MI/R + U-83836E (30 mg/kg) (n = 7)	2.84 ± 0.73	5.41 ± 0.88
MI/R + vehicle (n = 13)	2.78 ± 0.56	30.4 ± 4.94
MI/R + U-83836E (7.5 mg/kg) (n = 9)	2.48 ± 0.61	20.5 ± 3.21*
MI/R + U-83836E (15 mg/kg) (n = 9)	2.29 ± 0.63	16.9 ± 2.85*
MI/R + U-83836E (30 mg/kg) (n = 9)	2.26 ± 0.83	13.8 ± 3.22*

The total number of animals in each group is indicated in parentheses. Values are mean ± S.D.; *$p < 0.001$ vs MI/R + vehicle group.

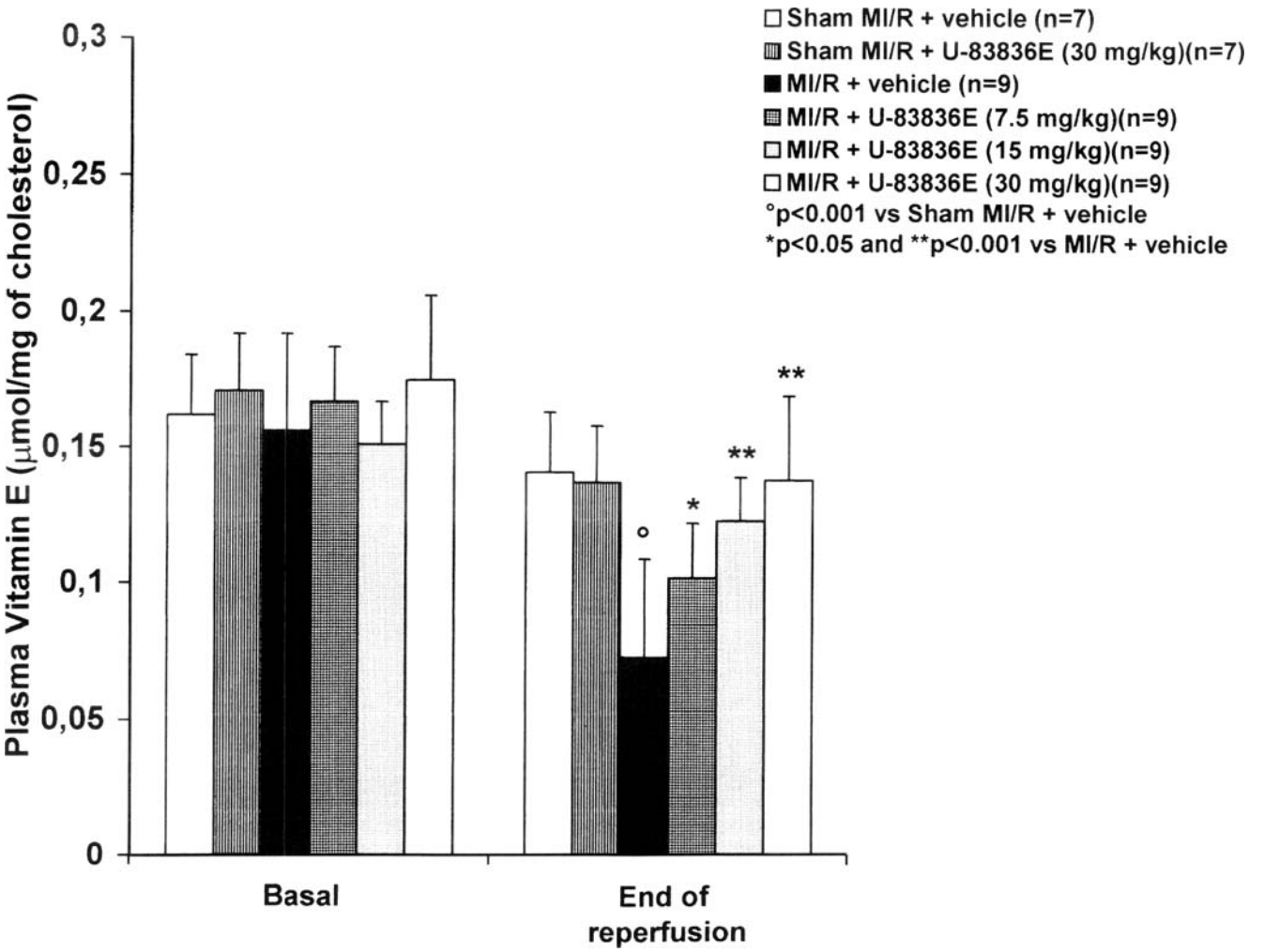

FIGURE 3 Plasma vitamin E assayed in basal conditions and at the end of reperfusion. Bar heights represent mean ± S.D. Statistical significance is reported at the top of the bars, the total number of animals in each group is indicated in parentheses.

TABLE IV Cardiac superoxide dismutase activity (U/mg protein) determined in the considered areas

Experimental group	Area not at risk	Area at risk	Necrotic area
Sham MI/R + vehicle (n = 7)	23.4 ± 3.8	—	—
Sham MI/R + U-83836E (30 mg/kg) (n = 7)	25.6 ± 4.1	—	—
MI/R + vehicle (n = 13)	24.7 ± 3.2	12.3 ± 4.4°	8.7 ± 3.5°
MI/R + U-83836E (7.5 mg/kg) (n = 9)	25.8 ± 4.5	17.2 ± 3.6*	12.6 ± 2.9*
MI/R + U-83836E (15 mg/kg) (n = 9)	23.5 ± 3.2	19.4 ± 3.8***	14.2 ± 3.1**
MI/R + U-83836E (30 mg/kg) (n = 9)	24.6 ± 4.3	20.8 ± 6.1***	15.7 ± 3.7***

The total number of animals in each group is indicated in parentheses. Values are mean ± S.D.; °$p < 0.001$ vs area not at risk; *$p < 0.01$; **$p < 0.005$ and ***$p < 0.001$ vs MI/R + vehicle group.

and in the necrotic area in each experimental group. In the area not at risk, SOD activities ranged between 23 and 26 U/mg protein in all groups and these values were considered normal. In contrast, a marked decrease in SOD activity was found in both the area at risk and in the necrotic area of the MI/R group treated with vehicle. The treatment with U-83836E significantly inhibited the reduction in SOD activity both in the area at risk and in the necrotic area.

$OH^{\cdot}$ Production

Figure 3 shows the time course of 2,3-DHBA and 2,5-DHBA produced in each group and measured under basal conditions, 15 min after reperfusion, 180 min after reperfusion and at the end of reperfusion. Very low amounts of the acids were detected before ischemia in all groups studied (<0.5 μM for 2,3-DHBA and <1.5 μM for 2,5-DHBA). A high amount of both acids was seen 15 min after the beginning of reperfusion in all infarcted groups, thus confirming the critical role of free radicals played during the early period of reoxygenation. After 180 min of reperfusion and at the end of reperfusion, both 2,3-DHBA and 2,5-DHBA levels were nearly similar to basal values. Treatment with U-83836E showed a reduction in $OH^{\cdot}$ production during reperfusion. No important differences were observed at 180 min and at the end of reperfusion.

Elastase Levels

Table V reports the time course of ELA amount evaluated in the area not a risk, the area at risk and in the necrotic area. High ELA activities were found in both the area at risk and in the necrotic area of the MI/R group treated with vehicle. However, U-83836E, at all the used doses, reduced tissue PMNs accumulation both in the area at risk and in the necrotic area. ELA values in the area not at risk ranged between 0.013 ± 0.01 and 0.025 ± 0.012 μg/g tissue in each group, and there were no significant differences in ELA content among groups.

TABLE V Myocardial elastase activity (μg/g tissue) assayed in the reported areas

Experimental group	Area not at risk	Area at risk	Necrotic area
Sham MI/R + vehicle (n = 7)	0.28 ± 0.012	—	—
Sham MI/R + U-83836E (60 mg/kg)(n = 7)	0.25 ± 0.017	—	—
MI/R + vehicle (n = 13)	0.21 ± 0.012	1.24 ± 0.14	1.72 ± 0.15
MI/R + U-83836E (7.5 mg/kg) (n = 9)	0.30 ± 0.014	1.15 ± 0.13*	1.41 ± 0.11**
MI/R + U-83836E (15 mg/kg) (n = 9)	0.26 ± 0.016	0.94 ± 0.12**	1.22 ± 0.10**
MI/R + U-83836E (30 mg/kg) (n = 9)	0.24 ± 0.010	0.67 ± 0.14**	0.93 ± 0.13**

The total number of animals in each group is indicated in parentheses. Values are mean ± S.D.; *$p < 0.01$ and **$p < 0.001$ vs MI/R + vehicle group.

DISCUSSION

Myocardial ischemia/reperfusion injury is a dynamic and complex pathophysiological mechanism that induces severe malfunction in the heart. Previous findings have shown a burst of oxygen free radicals during the first minutes of reperfusion.[14,27,28] The exact mechanisms of free radicals production during myocardial ischemia/reperfusion is not fully clear, but several sources have been identified,[29–32] including the xanthine oxidase pathway, activated leukocytes, arachidonic acid metabolism and electron leakage from the transport chain within mitochondria. Peroxidation of membrane phospholipid produces damage of membrane integrity and is considered a major mechanism of free radical-mediated reperfusion injury.[5,6] Endogenous heart defence mechanisms have been characterized, they use antioxidants or free radical scavengers to neutralize reactive species-generated lipid peroxidation. Among them vitamin E is the most important natural lipid soluble chain-breaking antioxidant. During reperfusion the extensive generation of free radicals appears to overwhelm the natural defence mechanisms, dramatically reducing the content of endogenous antioxidants. Thus, following free radical attack the levels of these substances and vitamin E particularly,[10] have been shown to be severely depleted.[14] Several reports have shown that the administration of vitamin E in the setting of myocardial ischemia and reperfusion is positive and endogenous α-tocopherol levels have been found to be diminished in experimental models characterized by oxidative injury.[9,33] However, α-tocopherol, to achieve sufficiently high tissue concentration for adequate protection, must be administered for several days, before the ischemic event.[33,34] In fact, acute administration of vitamin E has many limitations due to its high lipophilicity resulting in slow incorporation;[34] moreover, pharmacological doses of α-tocopherol may result in side effects such as hypotension and tachycardia.[34,35] U-83836E is a member of the second-generation lazaroids that are based on a nonsteroidal structure characterized by a ring portion of α-tocopherol. The lipophilicity of this new compound is much less than that of vitamin E.

The present study was designed to evaluate the effect of acute treatment with U-83836E in a rat model of ischemic and reperfused myocardium. In our model, U-83836E reduced infarct size. The data were positive whether the necrotic area was expressed as a percentage of the area at risk or as a percentage of the total left ventricle. This indicates that a large amount of myocardial tissue was spared by the drug intervention.

Elastase data demonstrated that a strong reduction of PMNs infiltration occurred in cardiac tissue. This reduction was observed both in the area at risk and in the necrotic area, confirming the protective effects of the compound. We think that the decrease in neutrophil accumulation induced by U-83836E may be due to inhibition of the lipid peroxidation and the consequent reduction in the chemotactic peroxide reduction.[32]

Since it has been shown that the main cause of death is due to ventricular fibrillation,[36] and in light of the fact that the drug inhibits the development of ventricular arrhythmias, it might be supposed that U-83836E, by improving energy state of myocardial cells, may limit intracellular calcium overload, in turn reducing the incidence of ventricular arrhythmias and improving survival rate.

Lipid peroxidation is considered a critical mechanism of the injury occurring during reperfusion of the myocardium following a period of ischemia. The evidence supporting these biochemical changes is based on analysis of a wide number of intermediate products.[37] Two indicative methods extensively used for evaluating lipid peroxidation are the plasma assay of HNE and CD. We carried out these analysis before coronary artery ligation, 15 min after reperfusion and at the end of reperfusion. The large amount of CD and HNE, found at the end of reperfusion, strongly emphasizes the damage due to reperfusion. Administration of U-83836E caused a sig-

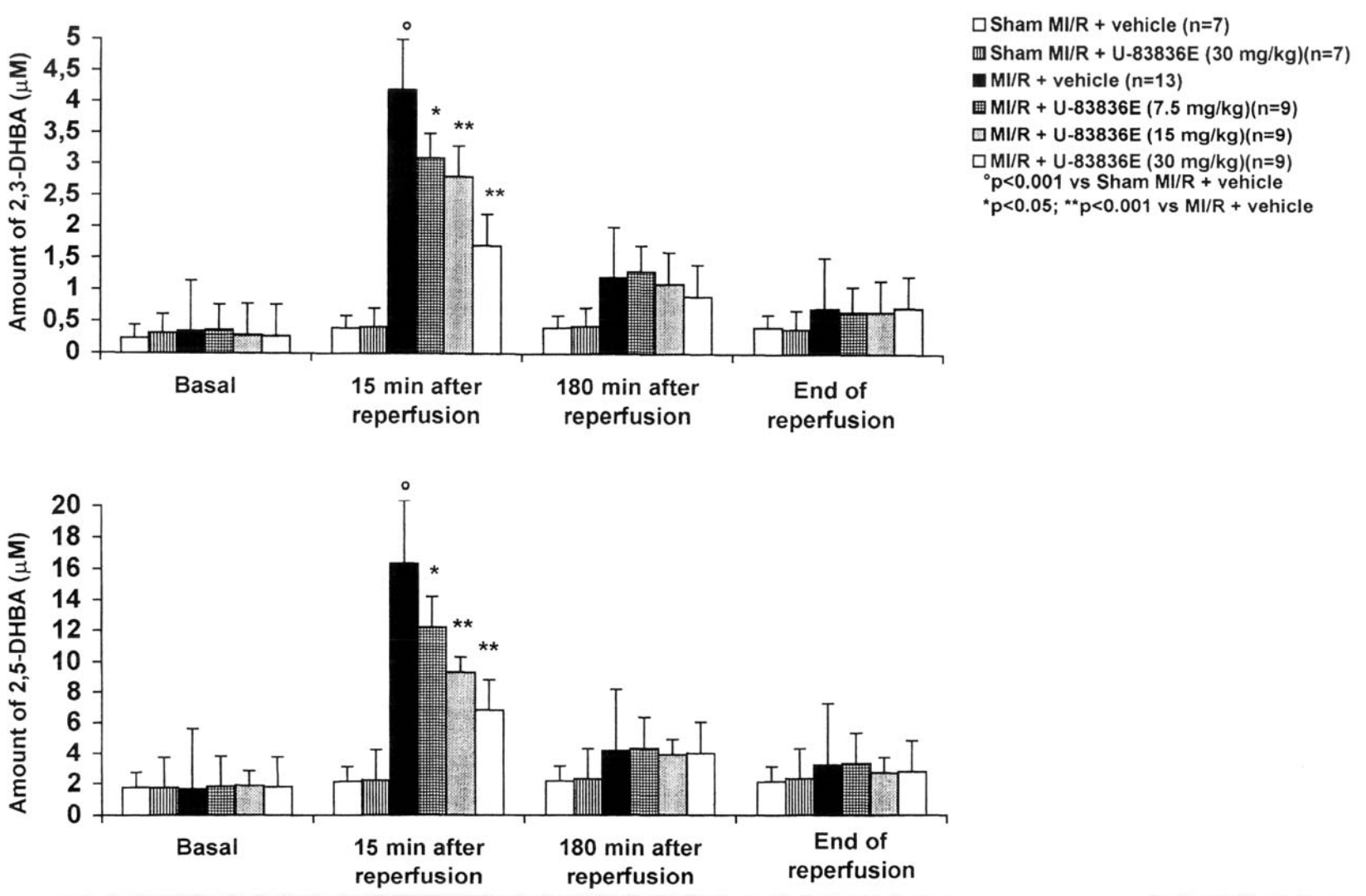

FIGURE 4 Time course of the plasma amount of 2,3-DHBA and 2,5-DHBA during the experiment. Bar heights represent mean ± S.D. Statistical significance is reported at the top of the bars, the total number of animals in each group is indicated in parentheses.

nificant attenuation of membrane injury, as demonstrated by CD and HNE levels.

The burst of oxygen free radicals production, that occurs upon the reperfusion in the heart, leads to a decrease in VE and SOD data concentrations as consequence of consumption during oxidative stress.[38,39] This reduction contributes to increase cellular damage by favouring free radicals attack. U-83836E blunted the depletion of these endogenous antioxidants by competing in free radicals neutralization.

The detection of 2,3-DHBA and 2,5-DHBA is usually considered a bona fide reporter for the flux of hydroxyl radicals which reacted with salicylic acid during oxidative stress.[22,38] The data obtained show that a large amount of $OH^{\cdot}$ radical was produced after 15 min of reperfusion. U-83836E reduced both the acids 2,3-DHBA and 2,5-DHBA formation by directly trapping the $OH^{\cdot}$ radical and by inhibiting their production. Because salicylic acid makes the stable derivatives by trapping $OH^{\cdot}$, it may theoretically be considered as a scavenger of $OH^{\cdot}$. Therefore, we compared the effect of salicylic acid on the considered parameter against control and treated ischemic/reperfused groups (data not shown). Salicylic acid failed to exert any beneficial effect on the postischemic injury. This suggests that salicylic acid does not have a significant scavenging effect because it can trap only a small portion (<11%) of produced $OH^{\cdot}$.[21]

Finally, a significant reduction in neutrophil recruitment in the damaged cardiac tissue was demonstrated by the ELA data. This further confirmed the beneficial effect of the drug. We suggest that the U-83836E induced reduction in neutrophil infiltration, may be due to inhibition of the lipid peroxidation and the consequent reduction in the chemotactic intermediates formation.

In conclusion our results obtained by using the antioxidant drug U-83836E suggest that it may have therapeutic potential for cardioprotective use in acute myocardial infarct.

Acknowledgements

This work was partially supported by CNR (National Research Council), Italy (grant n°9502181CT04) and MURST 40%. We gratefully acknowledge the Upjohn SpA, Caponago (MI), Italy for the generous supply of U-83836E.

References

[1] Zimmerman, B. J., Grisham, M. B. and Granger, D. N. (1990). Role of oxidants in ischemia/reperfusion induced granulocyte infiltration. *American Journal Physiology*, **258**, 185–190.
[2] Granger, D. N. and Korthuis, R. J. (1995). Physiologic mechanisms of postischemic tissue injury. *Annual Review Physiology*, **57**, 311–332.
[3] Hearse, D. J. and Bolli, R. (1992). Reperfusion induced injury: manifestations, mechanisms and clinical relevance. *Cardiovascular Research*, **26**, 101–108.
[4] Becker, L. C. and Ambrosio, G. (1987). Myocardial consequences of reperfusion. *Progress Cardiovascular Disease*, **30**, 23–44.
[5] Ferrari, R., Ceconi, C., Curello, S., Cargnoni, A., De Giuli, F. and Visioli, O. (1992). Occurrence of oxidative stress during myocardial reperfusion. *Moecular Cellular Biochemistry*, **111**, 61–69.
[6] Nohl, H., Stolze, K., Napetschnig, S. and Ishikawa, T. (1991). Is oxidative stress primarily involved in reperfusion injury of the ischemic heart? *Free Radical Biology & Medicine*, **11**, 581–588.
[7] Seekamp, A. and Ward, P. A. (1993). Ischemia-reperfusion injury [review]. *Agents Actions*, **41**, 137–152.
[8] Roth, E., Torok, B., Bar, W. and Pollak, S. (1987). Antioxidant protection against free radical-mediated myocardial injury. *Progress Clinical Biology Research*, **236**, 633–640.
[9] Klein, H. H., Pich, S., Lindert, S., Nebendahl, K., Niedmann, P. and Krevzer, H. (1989). Combined treatment with vitamins E and C in experimental myocardial infarction in pigs. *American Heart Journal*, **118**, 667–674.
[10] Weisel, R. D., Mickle, D. A. G., Finkle, C. D., Tumiati, L. C., Madonik, M. M., Ivanov, J., Burton, G. W. and Ingold, K. V. (1989). Myocardial free-radical injury after cardioplegia. *Circulation*, **80(suppl.II)**, 14–23.
[11] Klein, H. H., Pich, S., Lindert-Heimberg, W., Nebendahl, K. and Niedmann, P. (1993). Failure of chronic, high dose, oral vitamin E treatment to protect the ischemic reperfused porcine heart. *Journal Molecular Cellular Cardiology*, **26**, 103–112.
[12] Monyer, H., Hartley, D. and Choi, D. W. (1990). 21-aminosteroids attenuate excitotoxic neuronal injury in cortical cell cultures. *Neuron*, **5**, 121–126.
[13] Krantis, A., Harding, R. K., McKay, A. E. and Morris, G. P. (1993). Effects of compound U-74500A in animal models of gastric and duodenal ulceration. *Digestive Disease Science*, **38**, 722–729.
[14] Campo, G. M., Squadrito, F., Altavilla, D., Squadrito, Giov., Avenoso, A., Canale P., Ioculano, M.,

Sperandeo, A. and Caputi, A. P. (1996). Protection of ischemic and reperfused rat myocardium by the nonglucocorticoid 21-aminosteroid U-74389G a new inhibitor of lipid peroxidation. *Journal Pharmacology Experimental Thererapeutic*, **277**, 333–340.

[15] Griswold, D. E., Hillegass, L. M., Hill, D. E., Egan, J. W. and Smith, E. F. III (1988). Method for quantification of myocardial infarction and inflammatory cell infiltration in rat cardiac tissue. *Journal Pharmacological Methods*, **20**, 225–235.

[16] Squadrito, F., Altavilla, D., Zingarelli, B., Ioculano, M., Calapai, G., Campo, G. M. and Caputi, A. P. (1993). Tumor Necrosis Factor involvement in myocardial reperfusion injury. *European Journal Pharmacology*, **237**, 223–230.

[17] Klein, H. H., Puschmann, S. and Schaper, Schaper, W. (1981). The mechanism of the tetrazolium reaction in identifying experimental myocardial infarction. *Archives Analytical Pathology*, **393**, 287–297.

[18] Corongiu, F., Lai, M. and Milai, A. (1983). Carbon tetrachloride, bromotrichlormethane and ethane acute intoxication. *Biochemical Journal*, **212**, 625–631.

[19] Nieremberg, D. W. and Lester, D. C. (1985). Determination of vitamins E and A in serum and plasma using a simplified clarification method and high-performance liquid chromatography. *Journal Chromatography*, **345**, 275–284.

[20] Floyd, R. A., Watson, J. J. and Wong, P. K. (1984). Sensitive assay of hydroxyl free radical formation utilizing high pressure liquid chromatography with electrochemical detection of phenol and salicylate products. *Journal Biochemistry Biophysical Methods*, **10**, 221–235.

[21] Takemura, G., Onodera, T. and Ashraf, M. (1992). Quantification of hydroxyl radical and its lack of relevance to myocardial injury during early reperfusion after graded ischemia in rat heart. *Circulation Research*, **71**, 96–105.

[22] Powell, S. R. (1994). Salicylate trapping of $OH^{\cdot}$ as a tool for studying post-ischemic oxidative injury. *Free Radical Research*, **21**, 355–370.

[23] Wolff, S. P., Jiang, Z. Y. and Hunt, J. U. (1991). Protein glycation and oxidative stress in diabetes mellitus and ageing. *Free Radical Biology & Medicine*, **10**, 339–352.

[24] Ohkuwa, T., Sato, Y. and Naoi, M. (1995). Hydroxyl radical formation in diabetic rats induced by streptozotocin. *Life Science*, **57**, 1789–1798.

[25] Onodera, T. and Ashraf, M. (1991). Detection of hydroxyl radicals in the postischemic reperfused heart using salicylate as a trapping agent. *Journal Molecular & Cellular Cardiology*, **23**, 365–370.

[26] Lang, H., Jochum, M., Fritz, H. and Redl, H. (1989). Validity of the elastase assay in intensive care medicine. In: G. Shlag and H. Redl; (eds.), *Second Vienna shock forum*, (Alan R. Liss, New York, Inc.), pp, 701–706.

[27] Thompson, J. and Hess, M. (1986). The oxygen radical system: a fundamental mechanism in the production of myocardial necrosis. *Progress Cardiovascular Disease*, **28**, 449–462.

[28] Kloner, R., Przyklenk, K. and Whittaken, P. (1989). Deleterious effects of oxygen radicals in ischemia/reperfusion: resolved and unresolved issues. *Circulation*, **80**, 1115–1127.

[29] Kukrejia, P., Kontos, H., Hess, M. and Ellis, E. (1986). PGH synthase and lipoxygenase generate superoxide in the presence of NADH. *Circulation Research*, **59**, 612–629.

[30] Muxfeld, M. and Shaper, W. (1987). The activity of xanthine oxidase in heart of pigs, guinea pigs, rabbits, rats and humans. *Basic Research Cardiology*, **82**, 486–492.

[31] Grisham, M. B. and Granger, D. N. (1989). Metabolic sources of reactive oxygen metabolites during oxidant stress and ischemia and reperfusion. *Clinical Chest Medicine*, **10**, 71–81.

[32] Lucchesi, B. M. (1990). Modulation of leukocyte-mediated myocardial reperfusion injury. *Annual Review Physiology*, **52**, 561–583.

[33] Shum-tim, D., Tchervenkov, C. I. and Chiu, R. C. (1992). Oral vitamin E prophylaxis in the protection of newborn myocardium from global ischemia. *Surgery*, **112**, 441–450.

[34] Massey, K. D. and Burton, K. (1989). α-tocopherol attenuate myocardial membrane-related alterations resulting from ischemia and reperfusion. *American Journal Physiology*, **256**, H1192–H1199.

[35] Buchwald, A., Klein, H. H., Lindert, S., Pich, S., Oberschmidt, R., Nebendahl, K. and Kreuzer, H. (1989). Effect of α-tocopherol (vitamin E) in a porcine model of stunned myocardium. *Journal Cardiovascular Pharmacology*, **14**, 46–52.

[36] Hearse, D. J. and Tosaki, A. (1988). Free radicals and calcium: simultaneous interacting triggers as determinants of vulnerability to reperfusion-iduced arrhythmias in the rat heart. *Journal Molecular & Cellular Cardiology*, **20**, 213–223.

[37] Wills, E. D. (1987). Evaluation of lipid peroxidation in lipid and biological membranes. In: K. Snell, and B. Mullock, (eds.) *Biochemical Toxicology; a practical approach*, (IRL press, Oxford), pp. 127–152.

[38] Grootveld, M. and Halliwell, B. (1986). Aromatic hydroxylation as a potential measure of hydroxyl-radical formation in vivo: identification of hydroxylated derivatives of salicylate in human body fluid. *Biochemical Journal*, **237**, 499–504.