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Original Contribution

Acute administration of vitamin E triggers preconditioning via K_{ATP} channels and cyclic-GMP without inhibiting lipid peroxidation

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Abstract

Vitamin E (VitE) is considered an antioxidant agent. One or more brief periods of ischemia (isc), followed by short reperfusion (rep), increase the tolerance of the heart to a subsequent prolonged ischemia, a phenomenon known as ischemic preconditioning (PC). Mitochondrial K_{ATP} channels (mitoK_{ATP}), cyclic-GMP (cGMP), and free radicals are involved in the mechanism of PC, whereas some antioxidants abolish this benefit. The purpose of this study was to evaluate the effect of VitE on infarct size, PC, and the oxidative status in vivo. Male rabbits were divided into seven groups and were subjected to myocardial ischemia (isc) and reperfusion (rep) with the following interventions: (1) control (no intervention); (2) E_{150} (iv VitE at a dose of 150 mg/kg for 75 min, starting 40 min before index isc and lasting through 5 min of rep); (3) E_{300} (iv VitE 300 mg/kg as previously described); (4) PC (two cycles of 5 min isc and 10 min rep), (5) combined E_{150}–PC; and (6) combined E_{300}–PC. In the last two groups VitE was given 40 min before index ischemia. Blood samples were taken for malondialdehyde (MDA) and conjugated dienes (CDs) measurement. In a second series of experiments heart tissue samples were taken at the time of long ischemia for MDA and CD determination and for cGMP assay. In order to test whether combined treatment with VitE (as the E_{150} group) and the mitoK_{ATP} blocker 5-hydroxydecanoic acid (5-HD) changes the infarct size, an additional group was assessed in the first series of experiments. Tissue VitE concentration was evaluated in myocardium. VitE at both doses reduced the infarct size (19.7±2.8% for E_{150} and 18.8±4.9% for E_{300} vs 47.4±2.6% in control, $P<0.05$) without attenuating the effect of PC (10.2±3.1% for E_{150}–PC, 12.4±2.2% for E_{300}–PC, vs 13.5±3.3% for PC). Combined VitE and 5-HD treatment abrogates this benefit (37.4±6.5%, $P<0.05$ vs E_{150} and NS vs control). VitE increases intracellular cGMP and CDs levels ($P<0.05$ vs control) to the same extent as PC ($P<0.05$ vs control), with no effect on MDA (P=NS between all the groups). Peripheral markers of oxidative stress are increased during reperfusion in all groups ($P<0.05$ vs baseline). Overall, VitE limits infarct size via mitoK_{ATP} and cGMP, while preserving the benefit of ischemic PC.

Keywords: Vitamin E; Ischemic preconditioning; Infarct size; Ischemia/reperfusion; Lipid peroxidation

Introduction

Ischemic preconditioning is an endogenous protective mechanism in which one or more brief periods of myocardial ischemia and reperfusion render the heart resistant to a subsequent potentially lethal ischemic insult [1]. Although the hard end point of myocardial protection is the limitation of infarct size [2], the mechanism of preconditioning still remains

Abbreviations: mitoK_{ATP}, mitochondrial K_{ATP} channels; cGMP, cyclic-GMP; PC, preconditioning; ROS, reactive oxygen species; NO, nitric oxide; I/R, infarct to risk area; CDs, conjugated dienes; MDA, malondialdehyde; 5-HD, 5-hydroxydecanoic acid.

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obscure with several unknown facts with respect to the intracellular signaling pathways triggered [3]. The opening of mitochondrial K_{ATP} channels, with subsequent generation of reactive oxygen species (ROS), is considered to be a pivotal step in the mechanism of preconditioning [4]. It is of interest that NO, guanylate cyclase, cGMP, and PKG are significant mediators, which result in the opening of mitoK_{ATP} channels [3]. Infusion of free radical scavengers, superoxide dismutase and *N*-2-mercaptopropionyl glycine, prevents the protection from preconditioning in rabbits [5] and rats [6]. Ascorbic acid which is known to scavenge oxygen-derived free radicals has also been shown to abolish the benefit of ischemic preconditioning in pigs [7]. However, we have previously shown that the administration of melatonin and *N*-acetylcysteine to rabbits does not prevent the protection from ischemic preconditioning via limiting the infarct size but it does protect from oxidative damage during ischemia and reperfusion [8].

Vitamin E belongs to the most powerful group of lipid-soluble chain-breaking antioxidants that prevent lipid peroxidation and disruption of membrane integrity [9]. Epidemiologic evidence supports an inverse relation between vitamin E intake and risk of coronary artery disease [10]. However, this general statement is much debated today, since previous epidemiologic findings were not supported in a recent large, multicenter trial of patients with increased risk of coronary artery disease [11]. Furthermore, the findings of a recent large trial do not support vitamin E supplementation for cardiovascular disease or cancer prevention among healthy woman [12].

In the present study we sought to determine the effect of vitamin E as an antioxidant agent on (a) the beneficial effect of ischemic preconditioning by reducing myocardial infarct size, (b) lipid peroxidation and oxidative stress during ischemia reperfusion, (c) the effect of vitamin E on tissue cGMP levels as well as the effect of combined vitamin E and PC on cGMP levels.

Materials and methods

Surgical preparation

New Zealand white male rabbits weighing between. 2.3 and 3.1 kg were used. All animals received proper care in compliance with the *Principles of Laboratory Animal Care*, published by the National Society for Medical Research, and the *Guide for the Care and Use of Laboratory Animals*, prepared by the Academy of Sciences and published by the National Institutes of Health (Institute of Laboratory Animal Resources Commission on Life Sciences, 1996).

All animals were anesthetized by slowly injecting pentobarbital (30 mg/kg) into an ear vein. They were then subjected to tracheal incision and intubation for mechanical ventilation with a respirator for small animals (MD Industries, Mobile, AL). The ventilator was properly adjusted at a rate of approximately 35 respirations/min, in order to maintain blood gases and pH within the normal range. Two polyethylene catheters were inserted; one was positioned in the carotid artery for continuous blood pressure monitoring, and the other in the jugular vein for fluid infusion (1 ml of normal saline containing 1000 IU heparin/100 ml was administered every 30 min), drug administration, and additional anesthesia when necessary. A bipolar chest lead was used for continuous electrocardiographic recording. Blood pressure and heart rate were continuously monitored. The chest was opened via a left thoracotomy in the fourth intercostal space and, after pericardiotomy, the beating heart was exposed. A 3-0 silk thread was passed through the myocardium around a prominent branch of the left coronary artery and by pulling the ends of the suture through a small segment of a soft tube induced regional ischemia; the tube was then firmly attached against the artery with a clamp. The successful induction of ischemia was verified by visual inspection (cyanosis) and by ST elevation on the electrocardiogram. Reperfusion was achieved by unclamping the tube [13].

Experimental protocol

Forty six rabbits were randomly divided into seven groups; all animals were subjected to the following interventions prior to 30 min regional ischemia of the heart and 3 h reperfusion.

Control group (CTL) ($n=10$); no intervention.

E_{150} group ($n=6$): Vitamin E (DL-all-*rac*-α-tocopherol, Sigma Aldrich Chemical Co., St. Louis, MO) was administered intravenously as 10 ml of an oil-in-water emulsion containing DL-all-*rac*-α-tocopherol with a few drops of Tween 80 in normal saline at a total dose of 150 mg kg^{-1} for 75 min, starting 40 min before index ischemia and lasting up to 5 min of reperfusion. Vitamin E has been previously administered intravenously as an oil-in-water emulsion to humans [14].

E_{300} group ($n=5$): Vitamin E was administered intravenously, as described before, at a total dose of 300 mg kg^{-1} for 75 min, starting 40 min before index ischemia and lasting up to 5 min of reperfusion. The 300 mg kg^{-1} dosage of vitamin E has been administered intravenously to pigs in an earlier protocol [15].

Preconditioning group (PC, $n=7$): Two cycles of preconditioning, each including 5 min of regional ischemia and 10 min of reperfusion.

Combined E_{150}–PC group ($n=6$): Vitamin E at a total dose of 150 mg kg^{-1} was administered as in group E_{150}, and all animals were subjected to two cycles of preconditioning.

Combined E_{300}–PC group ($n=6$): Vitamin E at a total dose of 300 mg kg^{-1} was administered as in group E_{300}, and all animals were subjected to two cycles of preconditioning.

In a second series of experiments 24 additional rabbits (four in each group) were subjected to the above interventions up to 20 min of sustained ischemia and tissue samples were taken from the ischemic and nonischemic regions for analysis of tissue lipid peroxidation products (malondialdehyde (MDA) and conjugated dienes (CDs)) and determination of cGMP levels.

In order to investigate the role of mitoK_{ATP} channels on infarct size, a final group ($n=6$) was treated as the E_{150} group but with the addition of the mitoK_{ATP} blocker 5-hydroxydecanoic acid (5-HD). 5-HD (Sigma Aldrich Chemical Co.) was dissolved in normal saline (10 mg kg^{-1}) and was given as a

bolus dose of 5 mg kg^{-1} into the jugular vein 40 min before sustained ischemia as previously described [16].

Blood samples were taken at different time points (baseline, before sustained ischemia, and at 20 min of reperfusion), and plasma was obtained for MDA and CD measurement as lipid peroxidation markers.

Also, in order to determine the tissue concentrations of vitamin E, 10 additional rabbits were treated with vitamin E 150 or 300 mg kg^{-1} ($n=5$ in each group) as previously described until 20 min of sustained ischemia while 5 rabbits were used as controls. In total 90 rabbits were used to complete this study. The experimental protocol is shown in Fig. 1.

Risk area and infarct size

At the end of the experiment, hearts were removed, mounted on a perfusion apparatus, and perfused for 2 min retrogradely via the aorta with normal saline (20 ml/min, 50 mm Hg, room temperature). When all residual blood had been removed from the arteries, the coronary ligature was retightened to the same extent as before and 5 ml of green fluorescent microspheres (2–9 μm diameter; Duke Scientific Corp., Palo Alto, CA, suspended in saline) were slowly infused over 5 min for the delineation of the normally perfused tissue from the risk zone. Hearts were then frozen for 24 h at –20°C and sliced into 3-mm-thick sections from the apex to the base. The slices were then incubated in 1% triphenyl tetrazolium chloride solution (TTC) for 20 min at 37°C and the infarcted area was defined as the negatively stained region. The heart slices were then immersed in 10% formaldehyde solution for 24 h to delineate the infarcted (tetrazolium chloride negative) areas more clearly. To clarify the borders between the risk zone and the normal area, slices were examined under ultraviolet light (wavelength 366 nm). Infarcted, risk, and normal areas were traced onto an acetate sheet, photographically enlarged, and quantified by planimetry (Scion Image Program, Epson Perfection 1200S scanner, Adobe Photoshop 6.0). The areas of infarction and myocardial tissue at risk for infarction were automatically transformed to volumes by multiplying by the slice thickness (3 mm). Infarct and risk area volumes were expressed in cubic centimeters and the percentage of infarct to risk area (I/R) was calculated as previously described [17].

Measurement of malondialdehyde

MDA plasma concentrations at different time points (under basal conditions, before sustained ischemia, and at 20 min of reperfusion) were determined spectrophotometrically and expressed as micromolar using a commercial kit (Oxford Biomedical Research Colorimetric Assay for lipid peroxidation) with some modifications [8,18]. The method we used is based on the reaction of the chromogenic reagent *N*-methyl-2-phenylindole with MDA. One molecule of free MDA combines with two molecules of the chromogenic agent to produce a stable chromophore with a maximal absorbance at 586 nm.

Briefly, 0.65 ml of 10.3 mM *N*-methyl-2-phenylindole in acetonitrile was added to 0.2 ml of plasma. After vortexing for 3–4 s and adding 0.15 ml of HCl 37%, samples were mixed well and incubated at 45°C for 60 min. The samples were then cooled on ice and centrifuged at 3500*g* for 20 min and the absorbance at 586 nm was measured. A standard curve of an accurately prepared standard MDA solution (from 2 to 20 μmol/ml) was also run for quantitation. Measurements for each group were performed in triplicate.

Conjugated dienes evaluation

Peroxidation of polyunsaturated fatty acids leads to the formation of a conjugated diene system, with a characteristic UV absorption maximum of 234 nm. Briefly, plasma (100 μl) was mixed with 1 ml water, and 3 ml chloroform:methanol (2:1 v/v), and was vortexed vigorously for 2 min. Samples were centrifuged

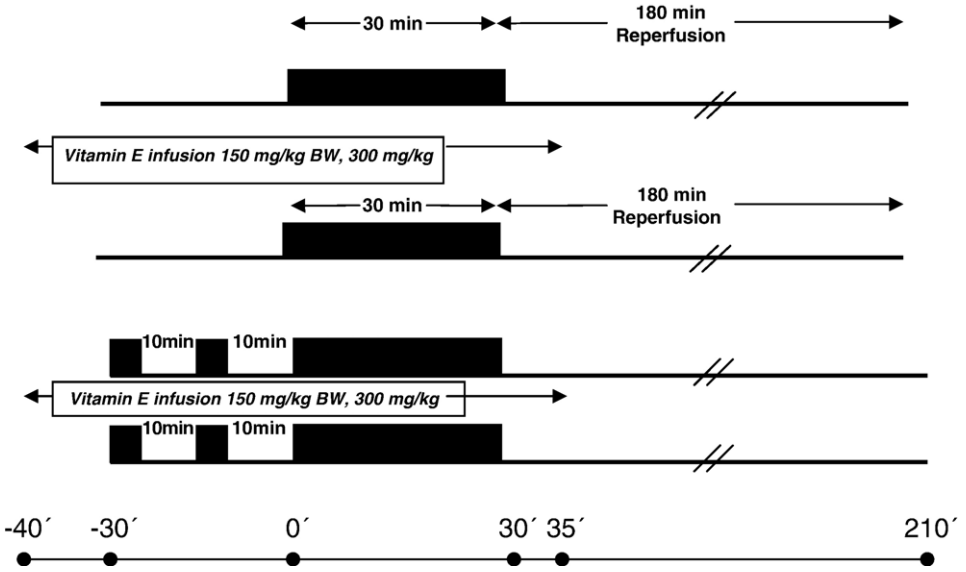

Fig. 1. Diagrammatic presentation of the experimental protocol used to evaluate the effect of acute administration of vitamin E and preconditioning on infarct size.

for 5 min at 2000*g*; the lower organic layer was removed and dried under nitrogen. The dry residue was solubilized in 1 ml hexane and the absorbance was measured at 234 nm, with hexane used as a blank. All measurements were performed in duplicate [19,20]. The amount of plasma CDs was expressed as ΔABS/ml.

Determination of MDA and CDs in tissue preparation

Samples of the ischemic and nonischemic areas were obtained at 20 min of sustained ischemia for each group, frozen, and kept at −70°C until assay. On the day of analysis, tissue samples were washed in ice-cold NaCl 0.9% (w/v), blotted on absorbent paper, and weighed. Each sample was then minced in a small volume of ice-cold 20 mM Tris-HCl buffer, pH 7.4, and homogenized, in a ratio 1:10 (w/v), using a Teflon pestle. After centrifugation at 3000*g* for 10 min at 4°C [20,21], the clear homogenate supernatant was used for the biochemical assays as described above. The results for MDA were expressed as micromolar per milligram protein. For the determination of CD the results were expressed as nanomole hydroperoxide per milligram protein using ε_{max}=25,200 M^{-1} cm^{-1} [22]. Protein concentrations were determined using the BioRad Bradford assay.

cGMP measurement

Heart powders were homogenized with 5 ml/g absolute ethanol and extracted on ice for 10 min. The samples were centrifuged (10,000 *g*, 5 min, 4°C) and supernatants were used for cGMP determination by radioimmunoassay using the cGMP [^{3}H] assay Biotrak system (TRK 500) according to the manufacturer's protocol (Amersham Biosciences, Buckinghamshire, HP7 9NA, England). cGMP concentration was normalized to tissue weight.

Vitamin E determination

Myocardial levels of vitamin E were determined with high performance liquid chromatography (HPLC) using the method of Good et al. [23] for tissue sample preparation and the method of Talwar et al. [24] for HPLC analysis at a wavelength of 295 nm for concentrations between 1 and 90 μg/ml. Within- and between-run precision was calculated to be <10%, while the limit of detection and limit of quantification were found to be 0.553 and 1.675 μg/ml, respectively.

Data analysis and statistics

All results are presented as mean±standard error (SE). Comparisons of absolute values of variables from the groups were analyzed using one-way analysis of variance model (ANOVA) with Bonferroni correction and with Duncan post hoc analysis. A calculated *P* value of less than 0.05 was considered to be statistically significant.

A one-factor repeated measures ANOVA model was used to compare each variable separately during the treatment period (baseline until 20 min of reperfusion). All tests were two-sided with a 95% significance level. Statistical analysis was carried out using the statistical package SPSS ver 10.00 (Statistical Package for the Social Sciences).

Results

Infarct size

The infarct-to-risk zone ratio, which is the most reliable index of protection [4,7,8], was 47.4±2.6% in the control group as shown in Fig. 2. Administration of vitamin E at a dosage of either 150 mg kg^{-1} or 300 mg/kg/BW reduced the infarct size, to 19.7±2.8 and 18.8±4.9%, respectively ($P<0.01$ vs control). The PC group had a significantly smaller infarct size 13.5±3.3% ($P<0.01$ vs control). Administration of vitamin E did not affect the reduction in myocardial infarct size obtained in the PC group: the mean infarct sizes in the E_{150}–PC group and the E_{300}–PC group were 10.2±3.1, and 12.4±2.2%, respectively ($P<0.01$ vs control), (Fig. 2). Combined treatment with vitamin E and 5-HD abrogated the benefit obtained by VitE treatment (37.4±6.5%, $P<0.05$ vs E_{150} and NS vs control values).

Mean blood pressure and heart rate were not affected by administration of 150 mg/kg BW or 300 mg/kg/BW vitamin E and /or PC.

Measurement of circulating MDA

MDA production at baseline, before sustained ischemia, and at 20 min of reperfusion as an index of lipid peroxidation is shown in Table 1. There were no significant differences between the different study groups compared at each time point. However, there were significant increases in MDA concentration at 20 min of reperfusion when compared to

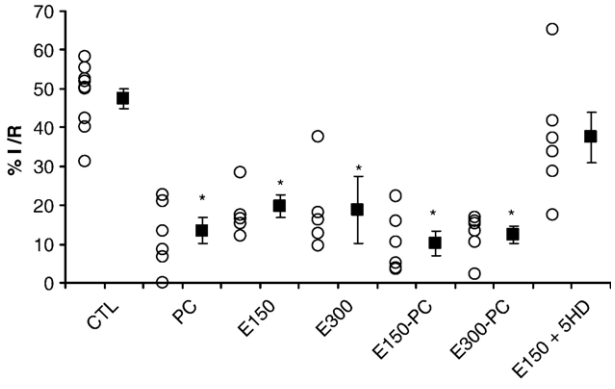

Fig. 2. The effect of various interventions on infarct size (expressed as a percentage of risk zone) in rabbit hearts following 30 min of ischemia and 180 min of reperfusion. CTL, control group; E_{150}, group treated with vitamin E 150 mg/kg/BW; E_{300}, group treated with vitamin E 300 mg/kg/BW; PC, preconditioning with 2 cycles of 5 min ischemia–10 min reperfusion; E_{150}-PC, group treated with vitamin E 150 mg/kg/BW and subjected to preconditioning; E_{300}-PC, group treated with vitamin E 300 mg/kg/BW and subjected to preconditioning; E_{150}+5-HD, group treated with vitamin E150 mg/kg/BW and 5-HD 10 mg/kg/BW *$P<0.01$ versus control group and E_{150}+5-HD group.

Table 1
Plasma malondialdehyde (MDA) assessed under basal conditions, at the time before sustained ischemia, and at 20 min of reperfusion

Group	Baseline	Before ischemia	20 min rep
Control	1.23±0.11	1.49±0.22	3.66±0.28 *
E_{150}	1.28±0.17	1.70±0.40	3.06±0.33 *
E_{300}	1.20±0.44	1.75±0.36	2.94±0.61 *
PC	1.30±0.08	1.80±0.47	3.15±0.89 *
E_{150}–PC	1.15±0.37	1.69±0.40	2.60±0.45 *
E_{300}–PC	1.02±0.28	1.72±0.07	2.59±0.34 *
E_{150}+5–HD	1.25±0.29	1.93±0.31	3.37±0.57 *

Data are means±SE.
* $P<0.05$ compared to baseline values.

baseline values in all study groups (*$P<0.05$ vs baseline values) including those treated with vitamin E.

Plasma-conjugated dienes

Table 2 shows plasma CD concentrations at baseline, at the time before sustained ischemia, and at 20 min of reperfusion. There were significant increases in CD concentrations at 20 min of reperfusion when compared to baseline values in the CTL and PC groups. Both doses of vitamin E resulted in an increase in CDs at 20 min of reperfusion ($P<0.05$ vs baseline values).

Cardiac MDA

The ratio of cardiac MDA levels (μM/mg protein) in ischemic/nonischemic areas is shown in Fig. 3. Increased MDA levels were noted in all areas at risk in all study groups. Vitamin E and PC exhibited a tendency to increase MDA concentrations during ischemia but not to a statistically significant extent.

Cardiac-conjugated dienes

Fig. 4 shows CD concentrations expressed as nanomole hydroperoxide per milligram protein obtained in the different study groups at 20 min of sustained ischemia. In the CTL group a significant decrease in the ratio of CD concentrations in ischemic and nonischemic areas was obtained when compared

Table 2
Plasma-conjugated dienes (ΔABS/ml) assessed under basal conditions, at the time before sustained ischemia, and at 20 min of reperfusion

Group	Baseline	Before ischemia	20 min rep
Control	0.117±0.02	0.12±0.03	0.43±0.05 *
E_{150}	0.129±0.03	0.159±0.02	0.286±0.08 *
E_{300}	0.140±0.02	0.144±0.01	0.313±0.02 *
PC	0.120±0.008	0.149±0.04	0.349±0.08 *
E_{150}–PC	0.117±0.01	0.122±0.02	0.197±0.09 *
E_{300}–PC	0.145±0.03	0.268±0.04	0.725±0.09 *,[a]
E_{150}+5-HD	0.132±0.02	0.188±0.035	0.392±0.05 *

Data are means±SE.
[a] $P<0.05$ compared to E_{150}, E_{300}, PC, and E_{150}–PC groups at 20 min of reperfusion.
* $P<0.05$ compared to baseline values.

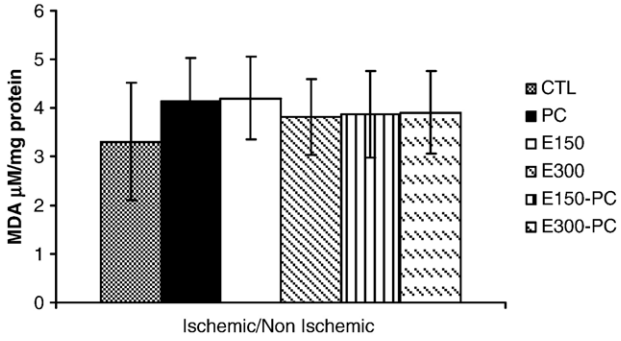

Fig. 3. The ratio of cardiac malondialdehyde (MDA) levels (μM/mg protein) determined in the ischemic and nonischemic areas.

to the PC group and to the groups treated with different doses of vitamin E ($P<0.05$ vs CTL group).

cGMP levels

Fig. 5 shows the ratio of cGMP levels in ischemic/nonischemic areas. PC and vitamin E-treated groups or combined PC with vitamin E was found to enhance the cGMP levels in the ischemic myocardium after 20 min of sustained ischemia (*$P<0.05$ vs CTL group).

Concentration of vitamin E in myocardium

The myocardial concentration of vitamin E in the control group was 0.150±0.06 nmol/mg tissue. Acute intravenous treatment with 150 mg kg^{-1} vitamin E increased the myocardial vitamin E concentration to 11.46±1.7 nmol/mg tissue, whereas treatment with 300 mg kg^{-1} increased the vitamin E concentration to 21.6±5.8 nmol/mg tissue at 20 min of sustained ischemia.

Discussion

The present study demonstrates that the acute administration of vitamin E reduces infarct size and maintains the beneficial effect of ischemic preconditioning without altering plasma MDA or cardiac CDs during ischemia and reperfusion. Vitamin E increases the levels of tissue cGMP in the ischemic

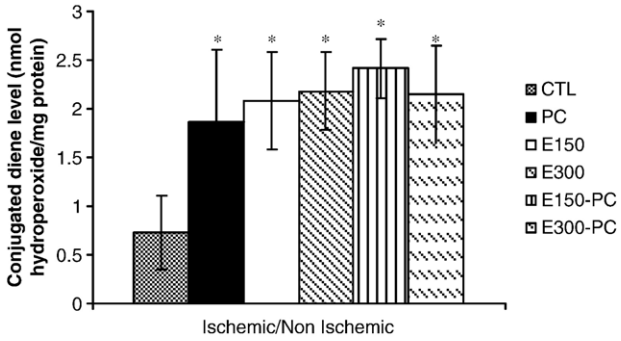

Fig. 4. The ratio of cardiac-conjugated dienes (nmol hydroperoxide/mg protein) determined in the ischemic and nonischemic areas. * $P<0.05$ versus CTL group.

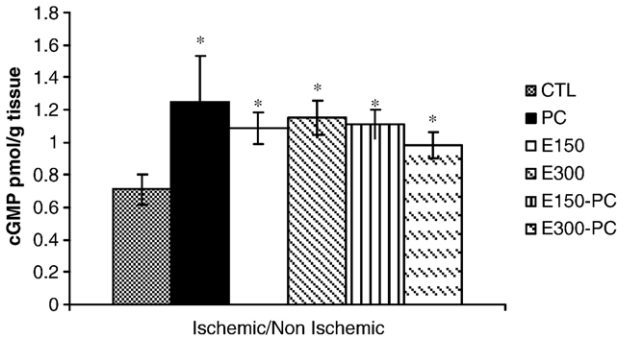

Fig. 5. The ratio of cGMP levels (pmol/g tissue) in the ischemic and nonischemic areas. * $P<0.05$ versus CTL group.

myocardium to the same extent that preconditioning does. Finally, the coadministration of vitamin E with the mitoK_{ATP} blocker 5-HD abrogates the reduction of infarct size.

The mediators involved in the mechanism of preconditioning have been extensively investigated in many studies with the use of different models [5,25,26]. However, regardless of the experimental model, there is a great deal of evidence that intracellular free radicals play a pivotal role, as triggers or mediators, in the observed protection [27–29]. Vitamin E is thought to exert its therapeutic properties against cardiovascular diseases by means of its antioxidative properties, as it is considered to be one of the most effective natural hydrophobic scavengers of peroxyl radicals. Although lipid peroxidation plays a central role in the development of atherosclerosis, the ability of vitamin E to prevent this process and the progression of in vivo lesions remains uncertain [30]. Apart from its antioxidative properties, vitamin E alters several intracellular agents, interferes in enzymatic activities, promotes apoptosis, and contributes to novel gene expression as has been reported recently [31–33]. Due to the above conflicting evidence and considering the effect of vitamin E on free radicals, we tested its effect on infarct size, on preconditioning, on several intracellular mediators, and on the oxidative status in anesthetized rabbits.

Acute intravenous infusion of vitamin E at two different doses was found to reduce the infarct size, in agreement with previous studies [15,34,35]. Importantly, we noted for the first time in vivo that vitamin E does not abolish the beneficial effect of PC in limiting the infarct size. Vitamin E has no effect on the antioxidant response element (ARE)—so signaling may be different from thiols, etc. However, conflicting findings regarding the antioxidant effects of vitamin E in animals and humans have been reported in previous studies [36–40].

MDA [8,15,20] and CDs [20,41] are some of the most widely used indexes for the evaluation of lipid peroxidation. The measurement of MDA and CDs in plasma using a photometric method has been criticized as notoriously unreliable [42,43]. However, it should be emphasized that the method we used for MDA determination showed similar results when it was compared with GC-MS measurements in previous studies [43].

Based on the above one of the main purposes of the present study was to evaluate the effect of vitamin E on intracellular lipid peroxidation. The large amount of MDA, found in the ischemic area at the time of sustained ischemia, indicates the development of damage mediated by free radicals. It is of interest that pretreatment with vitamin E does not prevent lipid peroxidation within the cardiac tissue under these conditions. In particular, vitamin E and PC exhibited a tendency to increase MDA concentrations during ischemia. The importance of prooxidation reactions of α-tocopherol has been established in previous studies [31]. Lipid peroxidation of LDL is faster in the presence of α-tocopherol and is substantially accelerated by enrichment of vitamin E in LDL in vitro [44,45]. It is assumed that peroxidation is propagated by the vitamin E radical, α-tocopheroxyl, if the latter is not reduced by vitamin C or ubiquinol-10 [46]. However, there are no data to support the role of vitamin E as a prooxidant in vivo.

Furthermore, there is a significant increase in the circulating levels of the lipid peroxidation products MDA and CDs at 20 min of reperfusion in all study groups compared to baseline values.

Tissue CDs may degrade and therefore decreased in the myocardium of the control group during sustained ischemia where a more extensive myocardial infarction was observed compared to the PC and vitamin E-treated groups. Free radicals and lipid peroxidation products, which are produced after short-lasting ischemia, generate reversible changes in cellular unsaturated fatty acids responsible for activation of phospholipases and subsequent release of substrates for the lipoxygenase pathway [47]. Starkopf et al. [47] found that preconditioned hearts had a significant content of free fatty acids at the end of a sustained ischemic period compared to controls before ischemia. Moreover, they also noted that with respect to phosholipids, ischemic preconditioning with 5 min ischemia and 5 min reperfusion increases hydroxyl-conjugated dienes during ischemia. This is in agreement with our finding, since we observed at the same time point a significant increase of CDs in the myocardium of the PC group and furthermore, in the vitamin E-treated groups.

Thus, with respect to free radical generation during PC, it appears that vitamin E does not interfere with lipid peroxidation products, which are elevated by the opening of mitochondrial K_{ATP} channels, and therefore does not block the protective effect of PC. Based on this finding and in order to further investigate the hypothesis that vitamin E may possibly act through mitochondrial K_{ATP} channels opening, we included in our study a group in which vitamin E was coadministered with the K_{ATP} channel blocker 5-HD. The addition of 5-HD abrogated the beneficial effect of vitamin E, indicating that the cardioprotective effect of vitamin E is probably related to the opening of the mitoK_{ATP} channels.

Another source of free radical generation during PC springs from the Akt-NO-PKG pathway. We have previously shown that circulating cyclic-GMP levels increase in preconditioned rabbit hearts in vivo [48]. Qin et al. [49] have shown that NO triggers preconditioning through the guanylyl cyclase-GMP cascade and these events reside upstream of the ROS-producing event. Other studies have shown that sildenafil, which elevates cGMP levels in cells, reduces infarct size in rabbit hearts [50]

and α-tocopherol increases NO-dependent relaxation [51]. Moreover, the formation of cGMP increases when human umbilical vein endothelial cells are pretreated with 10–200 μM vitamin E for 24 h [52]. In addition, Watanabe et al. [53] have demonstrated in a double-randomized clinical trial that the response in forearm blood flow and production of platelet cGMP after sublingual administration of nitroglycerin was decreased in the placebo group but not in the vitamin E group. Vitamin E was found to prevent the attenuation of the response in vasodilation and the intracellular production of cGMP. Our results support and extend these prior observations as we demonstrated that acute administration of vitamin E preserves NO bioavailability by enhancing cGMP levels in the ischemic myocardium.

Furthermore, in order to determine whether the above results are intra- or extracellular events due to vitamin E we evaluated the myocardial concentration of vitamin E. Acute intravenous treatment resulted in large increases in myocardial vitamin E levels during sustained ischemia. This finding is in agreement with previous studies that found high myocardial levels after acute or pretreatment with vitamin E [15,54].

Vitamin E administration up to 20 min of ischemia does not appear to interfere with lipid peroxidation products and increase cGMP levels. These properties of vitamin E and its ability to increase cGMP levels may induce and activate intracellular signaling pathways, which subsequently mediate the protective mechanism of ischemic preconditioning. Interestingly, the coadministration with the mitoK$_{ATP}$ channels blocker 5-HD was observed to abrogate the reduction of infarct size, indicating that vitamin E may exert its cardioprotective ability by favoring the opening of mitoK$_{ATP}$ channels.

Overall, acute intravenous administration of vitamin E was found to maintain the effect of ischemic preconditioning in vivo and reduce the infarct size but did not interfere with lipid peroxidation marker generation during ischemia and reperfusion. The preservation of lipid peroxidation and the intracellular increase in cGMP levels as well as the opening of mitoK$_{ATP}$ channels may probably contribute to the maintenance of the protective mechanism of preconditioning.

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