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Antioxidant properties of alpha-lipoic acid; effects on red blood membrane permeability and adaptation of isolated rat heart to reversible ischemia.

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Alpha lipoic acid (LA) is a natural dithiol compound which is known for its complex antioxidant properties. In the intracellular compartment, LA is reduced to dihydrolipoic acid (DHLA) under the action of certain enzymes. The aim of our work was to study 1) the antioxidant properties of LA and DHLA 2) the effects of treatment with lipoic acid on a) K^+efflux from human red blood cells challenged with a free radical generating system, b) post-ischemic recovery and oxidative stress in isolated perfused rat hearts challenged with an ischemia reperfusion (IR) sequence.

The antioxidant capacity of LA and DHLA was tested against an enzymatic superoxide-generating system and evaluated by electron paramagnetic resonance, following the kinetics of CPH spin probe oxidation into $CP^{\bullet}$ nitroxide. Erythrocytes were incubated at 37˚C with 20 mM 2, 2'- azobis (2-amidinopropane) hydrochloride (AAPH) in the presence of increasing concentrations of LA, DHLA or Trolox (10^{-4} M - 10^{-6} M). Male Wistar rats were treated with LA (50mg/kg/d, i.p) for 7 days. Isolated perfused hearts underwent 30 min of global total ischemia followed by 30 min of reperfusion during which functional parameters (heart rate, intra-ventricular pressures), were recorded.

While 5 mM of LA was ineffective in reducing the kinetics of $CP^{\bullet}$ nitroxide formation, DHLA was shown to lessen this rate in a dose-dependent manner, and, at 30 mM was even more efficient than 300 UI/ml SOD. DHLA provided more protection of red cells membranes at concentrations much lower than these of LA necessary to obtain the same effect; DHLA was comparable to Trolox for its antioxidant potency. Seven days of rats' treatment with 50 mg/kg/day of LA induced a slight increase in isolated perfused hearts coronary flow during reperfusion but this effect was not associated with an improvement in contractile function.

This finding suggests that LA might be more useful in the treatment of diseases associated with modifications in red cell behavior, than in these affecting directly the cardiovascular system.

1. Introduction

Alpha lipoic acid (LA) or thioctic acid (chemical name: 1,2 dithiolane-3-valeric acid or 6,8-dithio-octanoic acid) is a natural dithiol compound which is known as a co-factor in the α-ketoacid dehydrogenase mitochondrial complex and for its complex antioxidant properties [1-3]. Humans obtain LA from their diet and via *de novo* mitochondrial synthesis [2, 3]. Exogenous LA is quickly absorbed, transported to the intracellular compartment and reduced to dihydrolipoic acid (DHLA) under the action of certain enzymes: mitochondrial dihydrolipoyl dehydrogenase, cytosolic glutathione reductase and thioredoxin reductase [1, 4]. The reduction process results in two free thiol groups, which are responsible for the superior antioxidant effect of the reduced form (DHLA) as compared to the oxidized form (LA) [4]. Currently, only the oxidized form is used, especially as complement therapy in the treatment of diseases such as diabetic neuropathy [3, 5, 6] and ischemic stroke [7]. Recently, LA has been studied in a variety of experimental models because of its biological action and its amphiphilic character [8].

It is widely known that cardiac ischemia-reperfusion sequence might be associated with functional anomalies: reperfusion arrhythmia, myocardial stunning and degenerative processes affecting both vascular cells and cardiomyocytes [9, 10]. There are several mechanisms involved in the initiation and amplification of degenerative processes associated with the reperfusion phase, two of them appear to play an extremely important part: 1) changes in Ca^{2+} fluxes, inducing a cytosolic calcium overload and 2) free radical generation in a context of low antioxidant levels leading to the occurrence of local oxidative stress [9-12]. Oxygen free radicals (OFR) are usually formed during restoration of blood flow [10], and because of their high reactivity, these compounds will oxidize lipids and proteins in the membrane of cardiomyocytes and vascular cells [13, 14]. For these reasons, treatment with a combination of antioxidant

substances during a cardiac ischemia-reperfusion sequence either *in vitro* within an experimental environment or *in vivo* in patients, may limit myocardial injury [15-18]. Exploring membrane oxidative mechanisms in a cardiomyocyte model is complex and, for better understanding, much simpler experimental models are helpful. Erythrocytes are a very good model to investigate oxidative damage in biological membranes and to explore antioxidant protection. Indeed; erythrocytes are easy to collect, they are stable for several hours in phosphate buffers (pH=7.4) and because their membranes are rich in poly-unsatured fatty acids, one of the first targets of ROS, they are highly vulnerable to peroxidation [19-22]. The intracellular content of erythrocytes is rich in potassium and extra cellular K^+ efflux has been reported to be a good indicator of early erythrocytes membrane damage [23].

In this context, the aim of our work was to study 1) the antioxidant properties of LA and DHLA 2) the effects of treatment with lipoic acid on a) K^+efflux from human red blood cells challenged with a free radical generating system, b) post-ischemic recovery and oxidative stress in isolated perfused rat hearts challenged with an ischemia reperfusion (IR) sequence.

2. Materials and methods

2.1. Chemicals

All chemical were purchased from Sigma (Saint Quentin Fallavier, France) except for polyethylene glycol 300 and N-methyl-D-glucamine (Meglumine) from Fluka (Saint Quentin Fallavier, France); 2, 2'- azobis (2-amidinopropane) hydrochloride (AAPH) from Interchim (Montluçon, France) and alpha-lipoic acid (Thiogama 600) from Wörwag Pharma (Romania).

2.2 Antioxidant capacity of alpha-lipoic acid (LA) and dihydro-lipoic acid (DHLA)

2.2.1 Reduction of superoxide- induced oxidation of CPH spin probe

The antioxidant capacity of LA and DHLA was tested against an enzymatic superoxide-generating system. Briefly, the following reagents were added to 50 mM of pH 7.4 phosphate buffer containing 10^{-4} M of deferoxamine,: xanthine oxidase (0.05 IU/ml), catalase (500 IU/ml), the hydroxylamine spin probe CPH (10^{-4} M) and xanthine (5.10^{-4} M). This reaction generates superoxide anion which in turn oxidizes CPH into a very stable $CP^{\bullet}$ nitroxide free radical. Thirty microliters of the reacting solution were then immediately transferred into a quartz capillary tube which was placed into the resonator of an EMX-100 (Bruker, Wissembourg, France) Electron Paramagnetic Resonance (EPR) X-band spectrometer. The following EPR parameters were selected for optimal detection of the $CP^{\bullet}$ nitroxide at 310°K: field width = 80 G, microwave frequency = 9.44 GHz, microwave power = 20 mW, receiver gain = 10^{+4}, modulation frequency = 100 kHz, modulation amplitude = 2 G, conversion time = 41 ms, time constant = 82 ms. Signal acquisition was started exactly 2 minutes after the introduction of the enzyme substrate into the reacting solution, and followed sequentially for 12 minutes. The height of the characteristic triplet signal of the $CP^{\bullet}$ was measured and expressed in arbitrary units (AU) and a linear regression

curve was calculated from the height of the signal over time. The kinetics of superoxide-dependent oxidation of CPH were measured in the presence of superoxide dismutase (SOD, 300 IU/ml) and LA: 5 mM or DHLA: 5m M or 30 mM.

2.2.2 Potassium efflux from erythrocytes during oxidative challenge

Twenty ml of fresh blood taken by venous puncture from healthy volunteers was collected in dry tubes. Erythrocytes were separated from blood plasma by centrifugation (5 min at 1600 rpm at 4˚C) and washed five times with an isotonic saline solution (NaCl 0.9%). The supernatant and the buffy coat were carefully removed after each wash. After separation, 4 ml of packed erythrocytes were suspended in 16 ml of phosphate buffer (1.41 g l^{-1} Na_2PO_4H, 1.19 g l^{-1} $NaPO_4H_2$ and 8.8 g l^{-1} NaCl), at pH 7.4 in order to obtain a 20% suspension of erythrocytes.

The oxidative stress was obtained by the incubation of erythrocytes at 37˚C with 2, 2'- azobis (2-amidinopropane) hydrochloride 20 mM (AAPH). Three series, each series composed from 7 groups (n=12) were studied: 1) erythrocytes in phosphate buffer alone (Control, n=12) 2) erythrocytes in the presence of AAPH (AAPH, n=12), 3) erythrocytes submitted to an oxidative stress (AAPH 20 mM) in the presence of LA , DHLA or Trolox at different concentrations (10^{-4} M, 5×10^{-5} M, 2.5×10^{-5} M, 10^{-5} M, 10^{-6} M, n=12). Each sample was adjusted to a final volume of 5 ml by adding phosphate buffer as needed and then incubated and agitated at 37˚C. At T0 and every 30 min for 2 h, 0.4 ml of the suspension was drawn, added to 0.75 ml of saline solution and centrifuged at 1.600 rpm at 4˚C for 5 min. Then 0.25 ml of the supernatant was added to 4.75 ml of phosphate buffer and extra cellular potassium was measured by flame photometry (Flame Photometer 410, CIBA Corning, France). For each measurement, we used standard K^+ concentration curve with K^+ concentrations between 0-100 μM. By determining the K^+ emission

corresponding to each sample as well as the emission of the standard K^+ concentration curve, we were able to calculate the K+ values expressed in mM.

2.3. Effect of alpha-lipoic acid (LA) and dihydro-lipoic acid (DHLA) against ischemia-reperfusion induced myocardial injury

2.3.1. Perfusion Technique and Perfusion Medium

The local Ethic Committee approved the experimental protocol and the investigations in accordance with authorization 5406 from the French government, which agrees with the Guide for the Care and use of Laboratory Animals published by US National Institutes for Health. Male Wistar rats (280 g) were purchased from IFFA Credo Iffacredo (France). Two groups of rats were studied 1) (n=12). This solvent was an aqueous solution containing polyethylene glycol 300, N-methyl-D-glucamine or meglumine and distilled water. The rats were anaesthetized with sodium thiopental (60 mg/kg, i.p.) and heparin was intravenously injected (500 IU/kg). After 1 min the hearts were excised and placed in a cold (4˚C) perfusion buffer bath until contractions ceased. Each heart was then immediately cannulated through the aorta and perfused at 37˚C by the Langendorff method, at a constant perfusion pressure equivalent to 80 cm of water (8 kPa). The perfusion buffer consisted of a modified Krebs-Henseleit bicarbonate buffer (KH) (118 mM NaCl, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 4.5 mM KCl, 11 mM glucose and 1.5 mM $CaCl_2$). Before use, all solutions were filtered through a 0.8 μm Millipore filter to remove any particulate contaminants. The perfusion fluid was gassed with 95% oxygen and 5% carbon dioxide (pH 7.3-7.5 at 37˚C). An elastic water-filled latex balloon (n° 4 Hugo Sachs, Germany) was inserted into the left ventricle through the mitral valve and connected to a pressure transducer, the output of which was connected to a physiograph. The filling pressure was

individually adjusted to 4 to 10 mmHg. A TA 240 recover (Gould, Cleveland, OH) was used to measure heart rate (HR) and intraventricular pressures: left ventricular end diastolic pressure (LVEDP) and left ventricular systolic pressure (LVSP). The left ventricular developed pressure (LVDP) was calculated as the difference between LVSP and LVEDP (LVSP = LVSP- LVEDP). The rate-pressure product (RPP) was calculated from the product of LVDP (mm Hg) and HR (beats min^{-1}) and expressed in mmHg×beats×min^{-1}. Coronary flow was measured by the timed-collection of the effluent. After a stabilization phase of 15 min, the isolated hearts were perfused aerobically for 10 min (preischemic control period). Global normothermic ischemia was then induced by clamping aortic inflow for 30 min, during which a thermoregulated chamber maintained the heart temperature at 37˚C. After ischemia, aortic inflow was resumed for 30 min (reperfusion period).

2.3.2. Oxidative fluorescence histology

Dihydroethidium (DHE), an oxidative fluorescent probe, was used to localize superoxide anion ($O_2^{\bullet -}$**)**. Frozen heart tissues were fixed for 10 min in acetone and mounted on slides. The slides were incubated in a light-protected humidified chamber at room temperature with DHE (5 µmol/l) for 5 min and then immediately analyzed with a computer-based digitizing image system (Microvision, Evry, France) using a fluorescent microscope (Eclipse 600, Nikon, Champigny-Sur-Marne, France) connected to a video camera (Tri CCD, Sony, Paris). Fluorescence was detected with 510-560 nm excitation and 590 nm emission filters. Nuclei were counted using 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) which selectively binds to nuclei. Automatic computer-based analysis was performed with the same threshold for all sections (×500 magnification). Results are expressed as fluorescence intensity/number of nuclei.

2.4. Statistical analysis

All data are expressed as means ± S.E.M. The heart parameters for the control and LA group were compared using a Student test. The evolution of the extra cellular K^+ concentration was analyzed using the two-factor repeated measures analysis of variance (ANOVA) test (SigmaStat). To compare the protection of erythrocytes membranes after 30 and 120 minutes of incubation with AAPH in the presence of antioxidants, statistical analyses were performed with the one-factor analysis of variance (ANOVA) test (SigmaStat). ANOVA was followed, if necessary, by a Newman Keuls test. Significance was established at a value of $p < 0.05$.

3. Results

3.1 Antioxidant capacity of LA and DHLA

The kinetics of superoxide-dependent oxidation of CPH into $CP^{\bullet}$ in the presence of superoxide dismutase (SOD, 300 IU/ml), LA, 5 mM or DHLA, 5 mM and 30 mM are presented in Figure 1. The calculated regression curves were $y = 0.61x + 1.72$, $R^2 = 0.984$ for the superoxide-generating system alone, $y = 0.23x + 0.24$, $R^2 = 0.997$ in the presence of 300 IU/ml of SOD, $y = 0.59x + 0.94$, $R^2 = 0.976$ in the presence of 5 mM LA, $y = 0.38x + 0.45$, $R^2 = 0.977$ in the presence of 5 mM DHLA and $y = 0.11x + 0.23$, $R^2 = 0.975$ in the presence of 30 mM DHLA.

3.2. Effects of LA and DHLA on the membrane of red blood cells

The results showed that the K^+ erythrocytes efflux was weak when the erythrocytes were not subjected to oxidative stress with AAPH (Control group) as shown in Figure 2. LA did not modify this K^+ efflux (data not shown). When erythrocytes were incubated with 20 mM AAPH, the extra-cellular concentration of K^+ increased significantly during the 2 hours of the experiment (120min: AAPH: 2.55 ± 0.10 mM vs. Control: 0.28 ± 0.05 mM, $p<0.001$). In the presence of LA, the K^+ efflux decreased significantly, in a dose-dependent manner, beginning with a concentration of 10^{-5} M (120 min: LA 10^{-5} M: 2.31 ± 0.08 mM vs. AAPH: 2.55 ± 0.10 mM, $p<0.01$) (Figure 2).

Our experiments showed that LA, DHLA and Trolox all provided different degrees of protection. At the end of the 120 minutes of incubation (Figure 3), the efficacy of Trolox was superior only for the maximum tested concentration of 10^{-4}M (82.23 ± 23.61 Trolox vs. 63.33 ± 1.81 LA % Protection, $p<0.05$); *$p<0.05$ Trolox vs. DHLA: 82.23±23.61 vs. 72.56±1.15 % Protection). After this time, DHLA 5×10^{-5}M and 2.5×10^{-5}M afforded the best protection to erythrocytes membranes

(5×10^{-5} M: $ p<0.001 DHLA vs. LA (61.62 ± 2.02 vs. 39.87 ± 2.45 % Protection); # p<0.005 DHLA vs. Trolox (61.62 ± 2.02 vs. 51.60 ± 2.33 % Protection); 2.5×10^{-5} M: $ p<0.05 DHLA vs. LA (44.39 ± 2.99 vs. 20.24 ± 1.39 %Protection); # p<0.05 DHLA vs. Trolox :44.39 ± 2.99 vs. 21.51 ± 2.52 % Protection).

3.3. Effects of LA on myocardial post-ischemic recovery

3.3.1. Functional parameters

Myocardial recovery of functional parameters was evaluated during the 30 min of reperfusion, following 30 min of total global ischemia. The evolution of the pre-ischemic functional parameters was similar in the two groups.

Throughout the pre-ischemic period, the coronary flow (CF) of the isolated and perfused hearts was around 10.5 ml/min in the two groups. After the ischemic episode, CF had only partially recovered at the end of the reperfusion period. Chronic in vivo treatment with LA led to a significant increase in CF within the first 12 minutes (6.56 ± 0.42 vs. 5.54 ± 0.39 ml/min, p<0.05) and at the 22^{nd} minute (6.82±0.34 vs. 5.83±0.34, p<0.05) of the reperfusion as compared to control group (Figure 4).

The rate pressure product (RPP), representing the product of LVDP and HR is generally considered an index of the contractile activity of the myocardium. After reperfusion, the evolution in RPP was approximately the same for the two groups of hearts, RPP was not higher in the hearts of rats pretreated with LA (Figure 5).

Rhythm disturbances represented tachycardia and fibrillations were frequently noted after 30 minutes of total ischemia. LA pretreatment did not modify the occurrence and the duration of rhythm abnormalities.

3.1.2. In situ measurement of superoxide anion

Dihydroethidium (DHE) reacts with the superoxide radical to form ethidium bromide which induces intra-cellular nuclear' fluorescence in contact with the nuclear DNA. After a cardiac ischemia reperfusion sequence, oxidative stress assessed with DHE showed no differences on hearts samples from the LA group as compared to the control group ($0.757\pm0.053x10^{-3}$ vs. $0.983\pm0.168x10^{-3}$ % fluorescence/nuclei) (Figure 6).

4. Discussion

In the first part of our study, we set out to evaluate the antioxidant properties of LA and DHLA by analyzing their capacity to 1) scavenge superoxide radical 2) modify the permeability of red cell membranes caused by oxidative stress. EPR spectroscopy is one of the most specific techniques to identify and quantify radical species. We used a superoxide-generating system since this OFR is at the origin of secondary OFR-derived radicals produced in cells during situations such as ischemia-reperfusion sequences or diabetes. In our experimental conditions, we used xanthine and xanthine oxidase to generate superoxide anion, which is not directly measurable by EPR, but specifically oxidizes the spin probe CPH into an EPR-detectable long lasting $CP^{\bullet}$ nitroxide radical. The kinetics of $CP^{\bullet}$ formation can be followed for several minutes and, by adding sufficient amounts of the classic superoxide anion scavenging enzyme: superoxide dismutase (SOD), a significant reduction in $CP^{\bullet}$ radical formation was obtained, thus confirming the production of this OFR. While 5 mM of LA was ineffective in reducing the kinetics of $CP^{\bullet}$ nitroxide formation, DHLA was shown to lessen this rate in a dose-dependent manner, and, at 30 mM was even more efficient than 300 UI/ml SOD. These results are in agreement with the fact that DHLA is able to directly scavenge superoxide anion. LA is a complex antioxidant substance that can react at many levels: 1) it neutralizes free radicals formed by direct radical scavenging (hydroxyl radical: $HO^{\bullet}\cdot$), hypochlorous acid and singlet oxygen) 2) it regenerates endogenous antioxidants (GSH, vitamin C and vitamin E) from their oxidized forms and 3) it complexes transitional metals (especially iron and copper which are involved in $HO^{\bullet}\cdot$synthesis) [8, 21]. Concerning the clinical ways, dietary antioxidants have attracted attention as preventive and therapeutic agents [9,24,25].

Red cells are a good model to investigate oxidative damage in biological membranes [22], we used a suspension of erythrocytes which was incubated with 2,2'-azobis(2-amidinopropane) hydrochloride (AAPH) for 2 hours. AAPH is a water soluble azo-compound which can generate peroxyl, alkoxyl, phenoxyl radicals after thermal degradation at 37˚C [20, 34, 35]. The production of free radicals induces peroxidation of lipids and proteins on the erythrocyte membrane, which allows intracellular contents to leak out [22]. Potassium efflux is the earliest leakage process followed by the release of hemoglobin as a later phenomenon. The K^+ efflux may be influenced by the nature of the radical species formed but also by the presence of an antioxidant substance in a sufficient concentration [36]. This experimental model is widely used to evaluate the efficacy of several antioxidant substances in the prevention of membrane permeability [36, 37].

In the absence of AAPH, no K^+ efflux could be noticed, reflecting the stability of red cells' membranes in the buffer solution for 2 hours. While in the presence of AAPH alone, considerable potassium efflux was noticed. The addition of LA, DHLA or Trolox to the erythrocytes suspension induced a decrease in the concentration of extracellular K^+, reflecting an increase in membrane resistance to the oxidative aggression induced by AAPH. In our study, Trolox, a water-soluble vitamin E analogue, was introduced as an antioxidant standard [38, 39]. In identical experimental conditions, both LA and DHLA were active, starting with a concentration of 10^{-5} M, but Trolox offered earlier protection during the first 30 minutes (data not show). Moreover, DHLA provided more effective protection of red cells membranes at concentrations that were much lower than the concentrations of LA necessary to obtain the same effect; DHLA was comparable to Trolox for its antioxidant potency. Furthermore, after 2 hours of incubations, at concentrations lower than 10^{-4} M, the protection offered by DHLA was significantly higher than

that provided by Trolox. The protection of erythrocytes membrane induced by LA or DHLA could be explained by two mechanisms of action: direct neutralization of ROS and strengthening of the endogenous antioxidant status. The superior antioxidant effect of the reduced form of LA that we observed with both the EPR experiments and erythrocytes membrane protection could be explained by the presence of two free thiol groups within the DHLA structure, that are available for reducing reactions [2, 6, 21].

The second aim of our study was to describe the effects of LA on functional parameters of isolated perfused rat hearts during an ischemia-reperfusion sequence. Chronic treatment of rats with 50 mg/kg/day LA induced a slight increase in coronary flow in isolated perfused hearts, after 30 minutes a global total ischemia. This increase may allow better perfusion of the myocardium. In this context, oxygen and nutritive substances intake would be significantly improved. This effect was not associated with an improvement in contractile function. Our results agree with those of Ko *et al.,* who found that oral administration of LA reduced the release of lactate dehydrogenase, but with no improvement in cardiac contractile function, after 40 minutes of global total ischemia [26]. In another study, Coombes *et al*., showed that a combination therapy of vitamin E and lipoic acid improved contractile heart function in old rats, after an *in vivo* local ischemia reperfusion sequence [27]. However, in the same study, this combination of treatment was ineffective in adult female rats, a result that was attributed to differences in the basal antioxidant needs of the two sexes[28].

In this context, the apparently negative results of our study should not be interpreted as an absolute absence of LA action on myocardial and vascular cells during ischemia and reperfusion periods. In our experimental conditions, the isolated hearts were not perfused in the presence of

LA. The effects of LA could be more obvious in situations when the functions of membranes or cellular organelles are exposed to chronic stress. There is also the possibility that the chronic (seven days) treatment with a single antioxidant substance was not sufficient to protect the myocardium during an ischemia-reperfusion sequence. Moreover, ischemia-reperfusion injury is a complex situation in which the oxidative aggression is complicated by processes such as local inflammation [29, 30], calcium overload and disturbances in mitochondrial membrane permeability [31-33].

In conclusion, due to their ability to scavenge free radicals, LA and to an even greater degree DHLA, the reduced metabolite of AL, were able to protect the membranes of red blood cells. This finding suggests that LA might be useful in the treatment of metabolic syndrome and diabetes, two diseases associated with modifications in red cell behavior. The myocardium, however, was not sensitive to the antioxidant properties of LA and DHLA.

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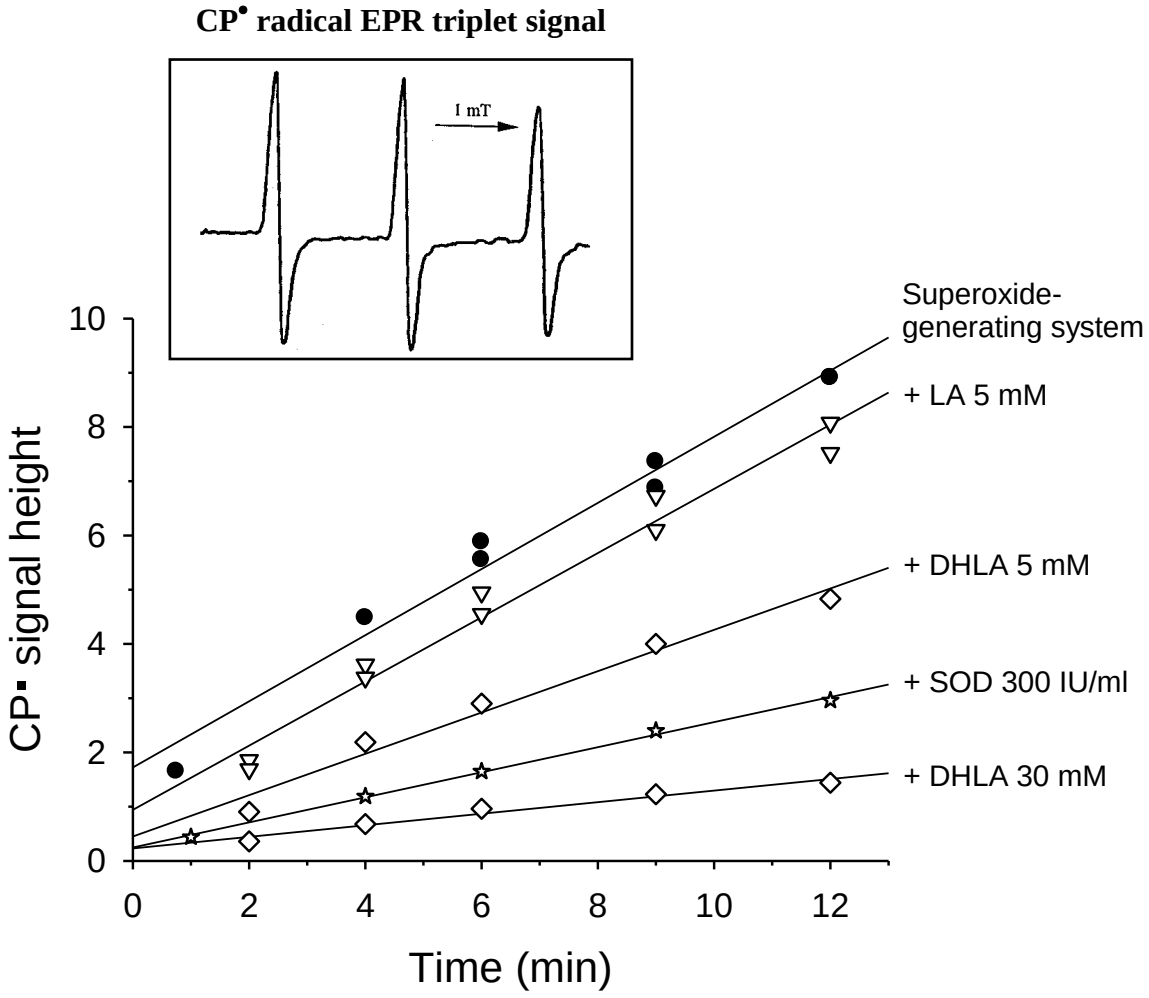


Figure 1: Kinetics of superoxide-dependent oxidation of CPH into CP$^{\bullet}$ in the presence of superoxide dismutase (SOD, 300 IU/ml), lipoic acid (LA, 5 mM) or dihydro-lipoic acid (DHLA, 5mM, and 30 mM). The superoxide-generating system, in a pH 7.4 50 mM phosphate buffer containing 10^{-4} M of deferoxamine, was composed of: xanthine oxidase (0.05 IU/ml), catalase (500 IU/ml), CPH (10^{-4} M) and xanthine (5.10^{-4} M).

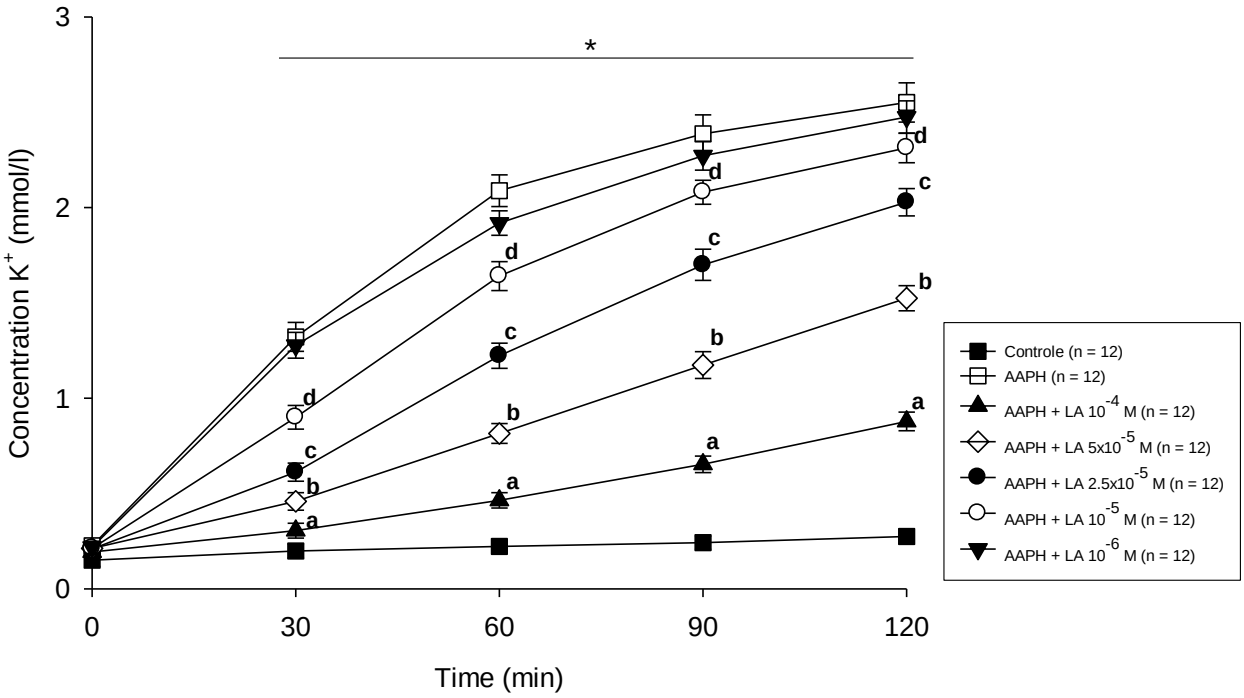


Figure 2: Evolution of extra-cellular K^+ concentration during 2 h of incubation of erythrocytes in the presence of AAPH: □ (n=12); ▲, AAPH + 10^{-4} M alpha-lipoic acid; ◊, AAPH + 5×10^{-5} M alpha-lipoic acid; ●, AAPH + 2.5×10^{-5} M alpha-lipoic acid; ○, AAPH + 10^{-5} M alpha-lipoic acid; ▼, AAPH + 10^{-6} M alpha-lipoic acid. ■: Control group (erythrocytes in buffer)
Significantly different from AAPH group, a,b,c,d, $p < 0.05$;
* Significantly different between control and AAPH group, $p < 0.001$.

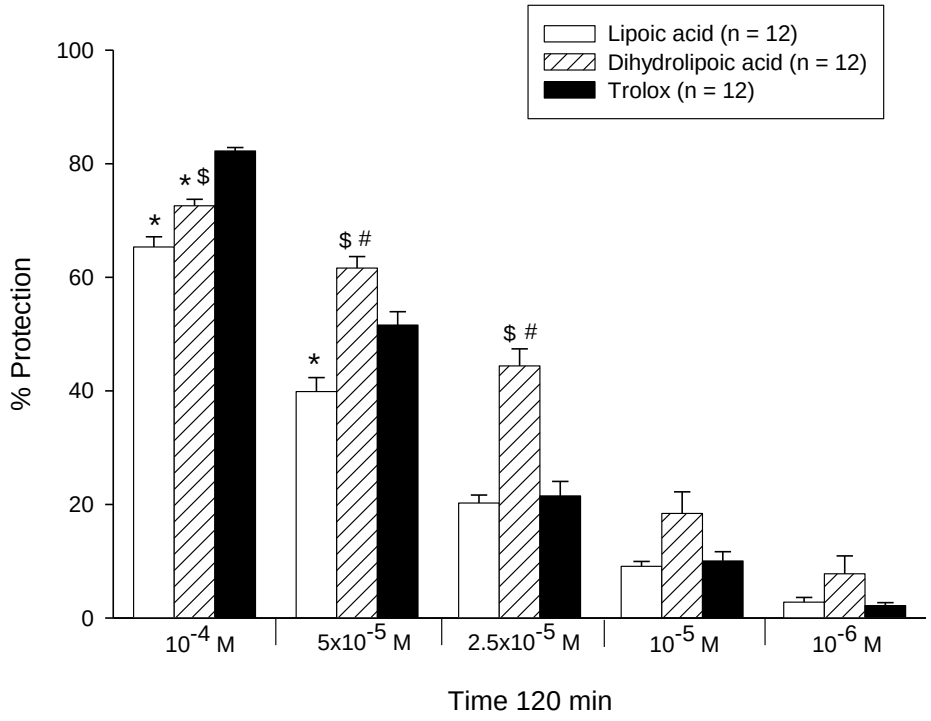


Figure 3: Protection of erythrocytes membranes afforded by lipoic acid ▭, dihydro-lipoic acid ▨ and Trolox ■ after 120 minutes of incubation with AAPH.
* $p<0.05$: LA, DHLA vs. Trolox; \$ $p<0.05$: LA vs. DHLA; # $p<0.05$: Trolox vs. DHLA.

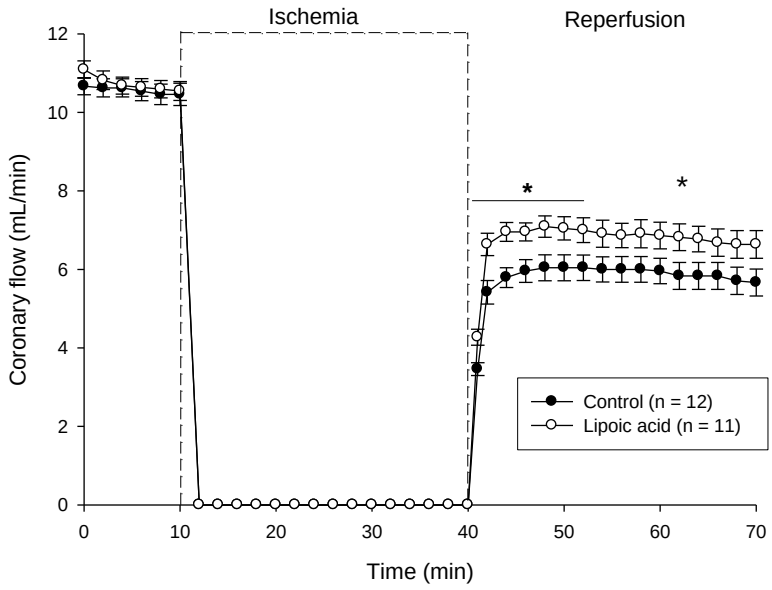


Figure 4: Evolution of coronary flow in isolated rat hearts before, during and after 30 min of global total ischemia. Results are presented as means ± S.E.M. ●, Control group (n=12); ○, hearts of rats treated with alpha-lipoic acid (n=11).

Significantly different from the control group, *, $p < 0.05$.

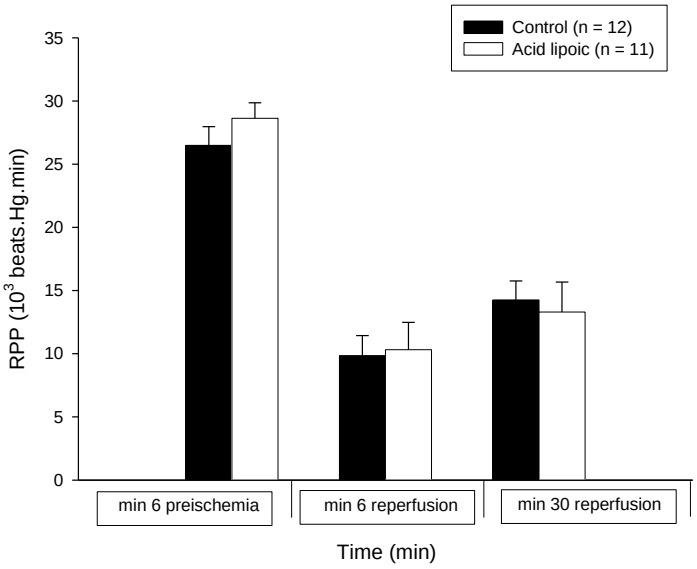
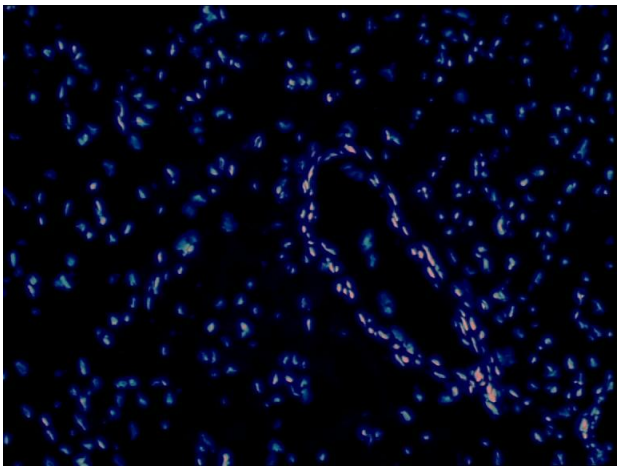


Figure 5: Level of Rate Pressure Product (RPP) 6 minutes before ischemia, and 6 and 30 minutes after global total ischemia. Results are presented as means ± S.E.M. ■, Control group - hearts of rats treated with the solvent of lipoic acid (n=12); □, hearts of rats treated with alpha-lipoic acid (n=11).

A :



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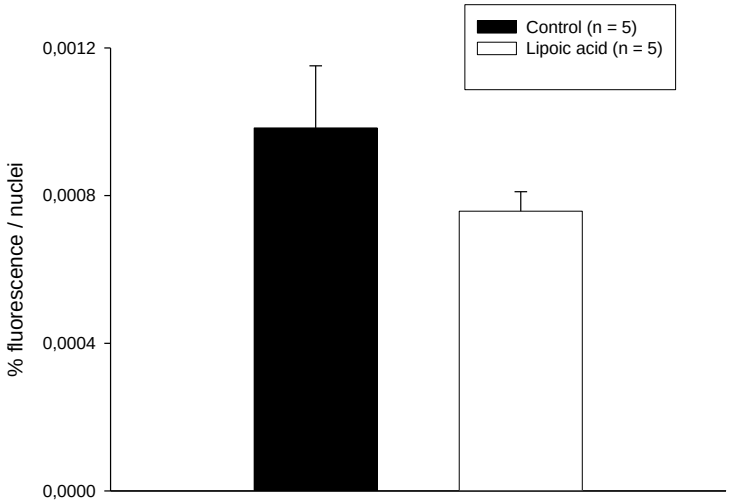

Figure 6: Measurement of superoxide anion emission in the cardiac tissue after a sequence of ischemia reperfusion. Results are presented as means ± S.E.M. ■, Control group - hearts of rats treated with the solvent of lipoic acid (n=12), □, hearts of rats treated with lipoic acid (n=12).