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# ALDA-1 protects myocardium from reperfusion damage due to its inhibitory action on mitochondrial permeability transition

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Reperfusion following an ischemic period represents an increased risk factor for cardiovascular morbidity and mortality. Heart reperfusion is characterized, mainly, by ventricular tachycardia, arrhythmias and a drop in blood pressure. The aim of this study was to explore the effect of the aldehyde dehydrogenase-2 agonist (ALDA-1), an inducer of the expression of the mitochondrial aldehyde dehydrogenase enzyme, on heart reperfusion damage; this is plausibly caused by the accumulation of aldehydes in the myocardium, as well as by mitochondrial permeability transition. Oxidative stress caused inhibition of *cis*-aconitase, increased generation of TBARs and disruption of mitochondrial DNA These adverse effects were avoided with ALDA-1 treatment.

**Keywords**: ALDA-1, Calcium, Heart ischemia; Heart reperfusion; Mitochondria, Permeability transition

Blood flow failure and subsequent oxygen deprivation in heart tissue by coronary occlusion resulted in a severe injury to the structure and function of myocardial cells[1,2]. Therapy hence aims at oxygen supply restoration through the use of proteolytic enzymes, implantation of stents or coronary artery bypass surgery[3,4]. Nevertheless, when a coronary artery occlusion is abruptly opened, heart damage occurs due to the increase of oxygen-derived free radicals[5,6] and to a cellular $Ca^{2+}$ overload[7-9].

It is well known that superoxide and hydroxyl anions induce membrane leakage through lipid or protein peroxidation[10]. Moreover, $Ca^{2+}$ accumulation is one of the causes of ventricular arrhythmias[11]. There are reports indicating a link between mitochondrial damage and cellular consequences in the pathophysiology of ischemia reperfusion[12,13]. Namely, this mechanism involves the opening of a non-specific transmembrane pore with a diameter of around 3-4 nm[14]. The opening of such pore allows for the release of ions and metabolites located in the mitochondrial matrix. This process depends on $Ca^{2+}$ overload[15] and is named permeability transition. Further, the loss of selective permeability causes uncoupling of oxidative phosphorylation and in consequence, the collapse of ATP synthesis. Studies from our laboratory and other groups have introduced a variety of compounds aimed at protecting myocardium from the damage induced by reperfusion; these compounds include cyclosporin A[16], octylguanidine[1,17], tamoxifen[18], and CDP choline[19,20]. This work addressed the possibility that the protection conferred by ALDA-1 would be due to the inhibition of mitochondrial permeability transition.

## Materials and Methods

ALDA-1, obtained from Sigma-Aldrich, was administered intravenously (IV) through a cannula located in the femoral vein at the dose of 20 mg/kg body weight, to 5 min before of the ischemia/reperfusion (I/R) period; this dose was chosen after performing a brief curve using 5, 10, 15, and 20 mg/kg body weight (not shown). To evaluate heart reperfusion damage/protection Wistar rats, 6 per group, *i.e.*, 6 controls without ischemia/reperfusion, and 6 treated with ischemia/reperfusion without ALDA-1 were anesthetized with sodium pentobarbital (55 mg/kg, *i.p.*) and maintained under assisted respiration through a tracheotomy. Meanwhile, heart rate was monitored with a left-II surface electrocardiograph, and blood pressure measured with a pressure transducer attached to a femoral cannula. The chest was opened by thoracotomy and the left coronary artery ligated near its origin with an intramural 6.0 silk loop. Occlusion of the artery was performed by passing a short tube over the vessel and clamping it firmly. The ischemic period lasted 5 min,

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in agreement with previous reports[16,18-20]. Reperfusion started by removing the clamp and also lasted 5 min[21]. Mitochondria were prepared by homogenizing tissues from the left ventricle in a 250 mM sucrose-1 mM EDTA solution adjusted to pH 7.3, and then following the standard centrifugation procedure. Protein levels were determined according to the Lowry Method[22]. Calcium uptake and release were tracked spectrophotometrically at 675-685 nm using the Arsenazo III indicator. The transmembrane electric gradient was assayed spectrophotometrically at 525-575 nm using Safranine dye. Mitochondrial swelling was analyzed at 540 nm. Oxygen consumption was assayed polarographically using a Clark type electrode. The incubation media are described in the respective figure legends. Mitochondrial DNA was isolated as described by García *et al.*[23]. The genetic material was analyzed in 0.8% agarose gel and visualized by adding ethidium bromide. Aconitase activity was analyzed according to the procedure reported by Hausladen and Fridovich[24]. Briefly, the mitochondrial protein was solubilized by adding 0.05% Triton X-100 containing 25 mM phosphate pH 7.2, followed by 0.6 mM manganese chloride, 1 mM citrate and 0.1 mM NADP. The *cis*-aconitate formed was measured spectrophotometrically at 240 nm. Mitochondrial membrane lipid peroxidation was determined spectrophotometrically as the concentration of thiobarbituric acid reactive substances (TBARS). A tetraethoxypropane curve was used as the standard. Infarct size was estimated after the reduction 2, 3, 5 triphenyltetyrazolium chloride. These reagents were obtained from Sigma-Aldrich.

## Results

Myocardial reperfusion is associated with a marked cytoplasmic $Ca^{2+}$ accumulation[25]. Actually, such a calcium overload induces the appearance of cardiac arrhythmias[11]. Fig. 1 shows the time course of cardiac frequency in control rats and those treated with ALDA-1. It is demonstrated that during the ischemic period, there was no difference in cardiac frequency between treated and untreated rats the values reached 380 ± 20 and 400 ± 20, respectively at 5 min. Alternatively, in the first minute of the reperfusion period, cardiac frequency in untreated rats increased considerably, while in ALDA 1-treated rats, a slight raise was observed. Thereafter, the cardiac frequency in untreated rats remained at high values until the end of the experiment. Remarkably, in Alda 1-treated rats ventricular tachycardia remained stable and at low levels throughout the rest of the experiment.

Figure 2 shows that the blood pressure of control rats diminished from 95 to 25 mm Hg during the ischemic reperfusion phase. In contrast, the magnitude of this variable in Alda 1-treated rats remained unchanged at 100 mm Hg, which may represent the efficiency of heart beats in these animals.

Permeability transition pore opening induced by calcium overload underlies reperfusion heart

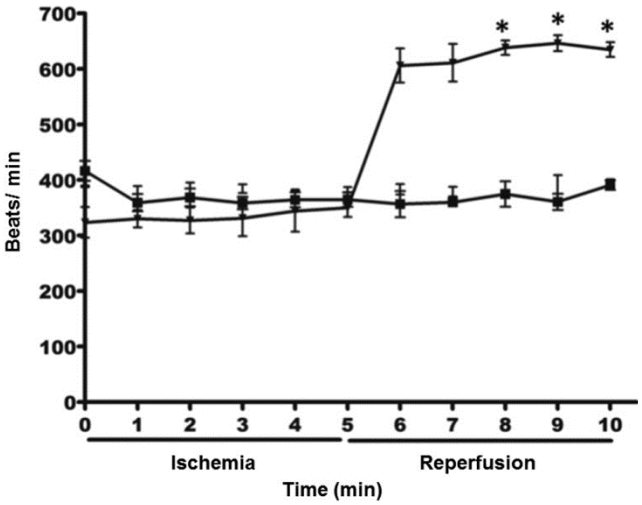


Fig. 1 — The time course of cardiac frequency in treated and untreated rats. When indicated, the left coronary artery was occluded and reperfusion was initiated. Heart frequency in treated rats is marked as ■. Heart frequency in untreated rats is indicated as ▼

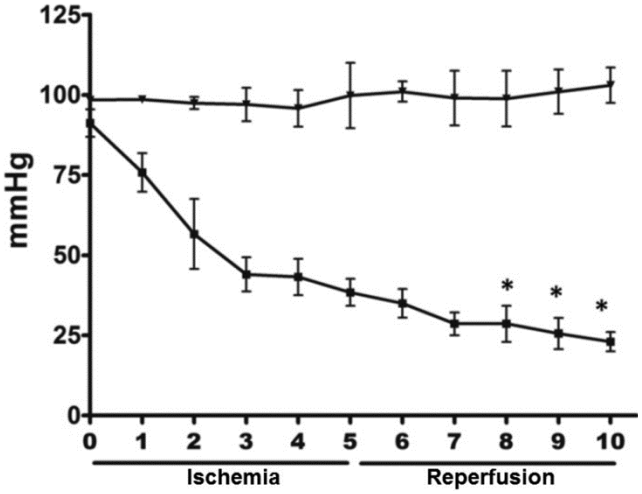


Fig. 2 — Analysis of blood pressure during ischemia and reperfusion periods. Blood pressure from untreated rats is indicated as ▼. Blood pressure from treated rats is indicated as ■

injury[15,26]. Therefore, the experiment in (Fig. 3) was performed with the purpose of assessing the protective effect of Alda-1 on $Ca^{2+}$-induced mitochondrial membrane leakage. As observed, mitochondria isolated from the reperfused left ventricle of untreated rats (I/R) were unable to retain $Ca^{2+}$ as a consequence of pore opening. The opposite occurred in mitochondria isolated from ALDA 1-treated rats (ALDA-1 + I/R); $Ca^{2+}$ remained in the mitochondrial matrix as demonstrated in (Fig. 3), indicating that the pore remained closed indeed. Interestingly, the behavior of these mitochondria resembles the control (CON).

Mitochondrial swelling is useful to analyze not only the ability of mitochondria to retain matrix $Ca^{2+}$ but also the mitochondrial membrane damage. Suppression of this process would indicate protection against oxidative stress membrane damage. Fig. 4 shows that heart mitochondria isolated from control rats (CON) underwent swelling neither before nor after the addition of $Ca^{2+}$. In contrast, heart mitochondria isolated from normal rats and subjected to ischemia/reperfusion did experience swelling after the addition of $Ca^{2+}$ (I/R). Lastly, heart mitochondria isolated from ALDA 1-treated rats did not undergo swelling or the opening of the non-specific pore (trace ALDA-1 + I/R) upon the addition of $Ca^{2+}$.

Analysis of membrane energization ($\Delta\Psi$) is a useful tool to evaluate the intactness of inner membrane after $Ca^{2+}$ accumulation. Fig. 5 shows that addition of the $Ca^{2+}$ to mitochondria isolated from heart subjected to ischemia/reperfusion (I/R) induced a fast collapse of the transmembrane electric gradient. The opposite was observed when $Ca^{2+}$ was added to heart mitochondria isolated from rats treated with ALDA-1, and subjected to ischemia reperfusion (ALDA-1 + I/R/). A similar result was obtained with mitochondria from control hearts (CON). Further examination of (Fig. 5) reveals that the addition of the uncoupler CCCP induced a complete fall of membrane potential.

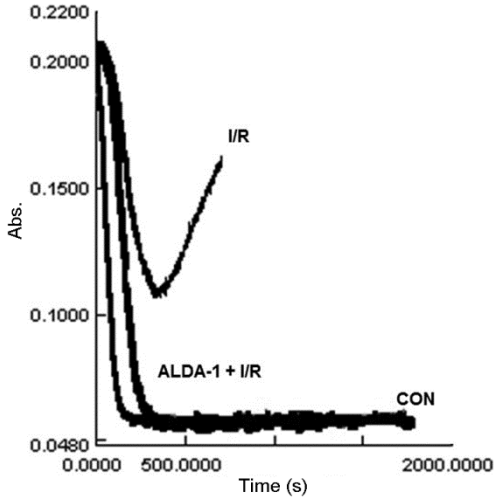


Fig. 3 — The protective effect of ALDA-1 on mitochondrial $Ca^{2+}$ retention. Where indicated, (I/R) mitochondria were isolated from untreated rat hearts. In the ALDA-1 + I/R regimen, mitochondria were isolated from hearts of ALDA-1-treated rats. Control mitochondria are indicated as CON

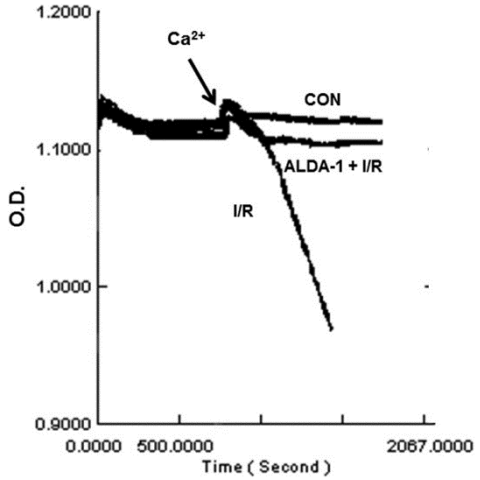


Fig. 4 — The protective effect of ALDA-1 on mitochondrial swelling induced after the addition of $Ca^{2+}$. Where indicated, 50 µM $Ca^{2+}$ was added. CON, heart mitochondria isolated from control rats. ALDA-1 + I/R, heart mitochondria isolated from hearts submitted to ischemia/reperfusion, and I/R, mitochondria isolated from hearts of untreated rats

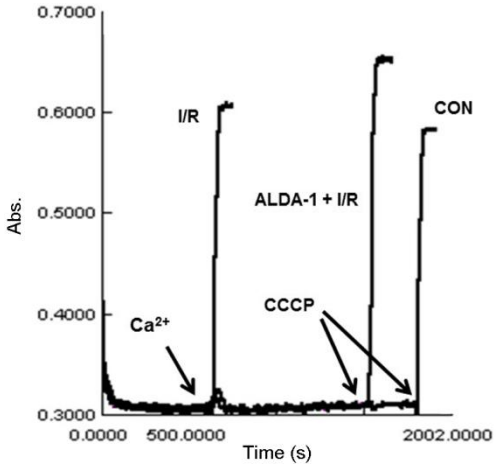


Fig. 5—The protective effect of ALDA-1 on the transmembrane electric gradient build-up by mitochondria submitted to ischemia reperfusion. Where indicated, either 50 µM $Ca^{2+}$ or 1 µM of the uncoupler CCCP was added. I/R trace illustrates the behavior of I/R heart mitochondria isolated from ALDA-1 untreated rats. CON trace demonstrates the behavior of mitochondria isolated from control rats. The trace ALDA-1 + I/R reveals the behavior of heart mitochondria isolated from ALDA-1 treated rats

Table 1—Respiratory control in mitochondria isolated from ischemia/reperfusion hearts of untreated and ALDA-1 treated rats. Mitochondria, 2 mg protein were incubated in 1.5 mL of a médium, Arsenazo III and Ca2+ were not added

| Mitochondrial oxygen consumption | | | | |
|---|---|---|---|---|
| | nAO/min/mg protein | | | |
| Condition | State 4 | State 3 | RC | ADP/O |
| Glutamate/Malate | 31±2 | 140±2.1 | 4.5±0.5 | 1.3±0.6 |
| I/R | 130±2.6 | 130±2.6 | 1 | - |
| ALDA-1 + I/R | 33±0.4* | 145±4.1* | 4.3±0.3* | 1.5±0.3 |
| Succinate | 100±3 | 435.2±60 | 4.3±0.5 | 1.2±0.5 |
| I/R | 80.14±1.6 | 80.14±1.6 | 1±0.2 | - |
| ALDA-1 + I/R | 100±3* | 411.6±20* | 4.1±0.3* | 1.3±0.4* |

The values are expressed ±S.D. of 7 experiments. *$P$ <0.01 *vs.* I/R

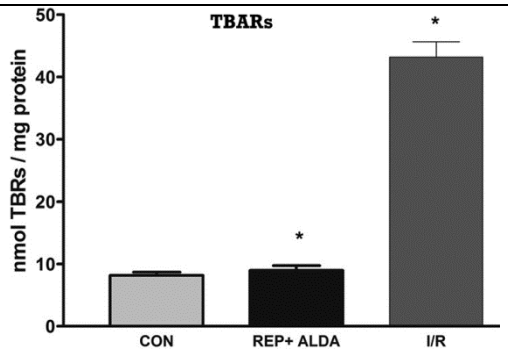


Fig. 6 — Protective effect of ALDA-1 on the generation of TBARs from heart mitochondria subjected to ischemia/reperfusion. Data are expressed as the average ± SD of 7 different preparations. *$P$ <0.05 *vs* I/R

Table 2—Protective effect of ALDA-1 on the activity of the enzyme aconitase from heart mitochondria submitted to sichemia/reperfusion isolated from control rats. Heart mitochondria isolated from hearts submitted to ischemia reperfusion of rats treated with ALDA-1 (I/R + ALDA-1). Heart mitochondria from untreated rats and submitted to ischemia reperfusion are indicated as I/R. The enzymatic activity was assayed in 150 µg protein

| Condition | nmoL *cis*-aconitate/min/mg |
|---|---|
| Control | 461±60 |
| I/R | 75±6* |
| I/R + ALDA-1 | 435±12* |

Values are expressed as the mean ± S.D. of 7 different experiments. *$P$ <0.001 *vs.* I/R

A dysfunction of oxidative phosphorylation may be provoked by an increased generation of reactive oxygen-derived species (ROS). Thus, we tested the possible protective effect of ALDA-1 against reperfusion induced damage. Respiratory control is defined as the ratio between the rate of oxygen consumption (nAtg $O_2$/min/mg protein) after the addition of ADP and the rate of oxygen consumption in the absence of ADP. As shown in (Table 1), heart mitochondria not subjected to I/R show a respiratory control (RC) value of 4.5 ± 0.5 with glutamate/malate as substrates, whereas RC from heart mitochondria subjected to I/R seemed to be almost lost. Alternatively, the RC value in heart mitochondria treated with ALDA-1 was 4.3 ± 0.3 after a 5 min ischemic treatment and a 5 min reperfusion. Importantly, RC values were very similar when succinate was used as the oxidized substrate.

The RC loss in mitochondria from an ischemic/reperfused heart is plausibly due to membrane leakiness, which would be induced by oxidation of membrane fatty acids. Then, the resulting increase in the concentration of malondialdehyde should be a useful marker to estimate oxidative stress[20]. Using thiobarbituric acid as a reagent, the highest amount of fatty acid derived species was generated in mitochondria from hearts under ischemic/reperfusion (Fig. 6). More importantly, the concentration of TBARS was about 80% less in mitochondria from rats treated with ALDA-1 than in those which did not receive this drug.

Aside from the analysis of TBARS, Aconitase activity is a reliable marker for assessing damage to mitochondria by oxidative stress[27]. As (Table 2) illustrates, this enzyme showed approximately 84% inhibition caused by reperfusion-induced oxidative stress. A statistically significant protection by ALDA-1 was observed since the activity was reduced only by about 6% (from 461 ± 60 nmoL/min/mg to 435 ± 12)

Mitochondrial DNA may also be susceptible to damage by oxidative stress[18,20]. Hence, we examined the ability of ALDA-1 to protect this polynucleotide in mitochondria isolated from hearts submitted to ischemia/reperfusion. Fig. 7 reveals that DNA isolated from mitochondria of untreated rats was considerably degraded compared to the genetic material extracted from heart mitochondria of ALDA 1-treated rats.

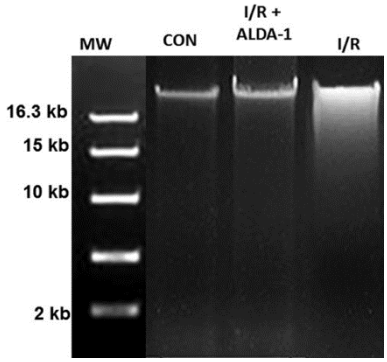


Fig. 7 — ALDA-1 protection on ischemia/reperfusion-induced DNA disruption. The lanes show molecular weight standards (MW). DNA from control of heart mitochondria (CON). DNA from heart mitochondria of rats treated with ALDA-1 and subjected to ischemia/reperfusion. Heart mitochondria of untreated rats subjected to ischemia-reperfusion (I/R)

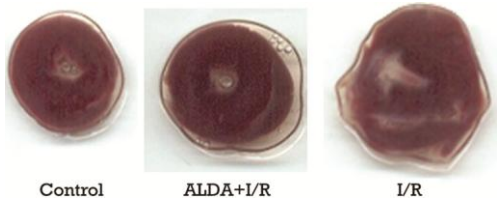


Fig. 8 — Infarct size determination. The hearts were excised and frozen at −20°C for 30 min, and then sliced into 2 mm transverse sections. The slices were incubated in 1% 2, 3, 5 triphenyltetrazolium chloride in sodium phosphate pH 7.4 at 37°C for 20 min with constant agitation. Then, the slices were immersed in a phosphate-buffered preservative solution plus 0.01% sodium azide to enhance the contrast between healthy tissue (black area) and infarcted area (white area)

The experiment shown in Fig. 8 was carried out to explore the extent of reperfusion-induced damage, and to further establish the protective effect of ALDA-1 on such damage. As expected, heart reperfusion resulted in a considerable absence of nitroblue tetrazolium labeling (I/R), while the dye was almost uniformly distributed in the ventricular tissue of ALDA 1-treated rats.

## Discussion

The biochemical characteristics of heart reperfusion damage have been extensively studied. Reperfusion following an ischemic period induces an increased rate of myocardial contractility, cardiac output, and predisposes to supraventricular and ventricular arrhythmias[20,21]. In other words, reperfusion is essentially characterized by the loss of sinusoidal rhythm. The deleterious effects of reperfusion on heart tissue frequently involve oxidative stress[28]. This process occurs when ROS generation overrides the ability of endogenous antioxidant enzymes. Indeed, there is substantial evidence indicating that oxygen-derived free radicals are an important factor in reperfusion heart injury[5,14,29]. There is also an increase in intracellular $Ca^{2+}$ for this reason. On this subject, the work of Bers[30] identified a close association between cellular $Ca^{2+}$ overload and alterations in heart rhythm.

In addition to $Ca^{2+}$ overload-induced arrhythmias, the harmful effects of oxidative stress cause the oxidation of membrane fatty acids. Membrane lipid peroxidation triggers the opening of the transmembrane pore; in turn, this prompts a transition in membrane permeability from a selective to a non-selective state. It is well established that permeability transition underlies myocardial reperfusion-induced injury[13,31,32]. Regarding this topic, dissimilar strategies have been implemented to prevent mitochondrial damage; specifically, we encounter the use of reagents, such as cyclosporin A[16], octylguanidine1, tamoxifen[18], and CDP-choline[19].

Further, membrane lipid peroxidation also evokes a high production of toxic aldehydes. In agreement with Mali *et al*.[31], toxic aldehydes are involved in ROS formation, anomalous $Ca^{2+}$ handling, and defective mitochondrial oxygen consumption of cardiomyocytes. As for the generation of toxic aldehydes, it should be mentioned that mitochondrial aldehyde dehydrogenase 2 has an important role in the clearance of such compounds. The beneficial role of ALDA-1 must thus be ascribed to the activation of this dehydrogenase. Previous reports have indicated that ALDA-1 is involved in the protection of heart and brain from ischemia/reperfusion injury[21], yet it is worthwhile to mention that the work of Gomes *et al*.[32] indicated that ALDA-1 protects from myocardial damage by avoiding mitochondrial dysfunction.

## Conclusion

The findings in the present work indicate that heart mitochondria isolated from ALDA-1-treated rats were able to sustain $Ca^{2+}$ accumulation and developed a high electric transmembrane gradient, unlike untreated mitochondria. Even more, ALDA-1 preserved the ability of mitochondria to synthesize ATP. This is inferred from the maintenance of respiratory control and the ADP/O ratio, whose values remained similar to those found in control mitochondria. Since ALDA-1 inhibits membrane leakage, it allows the inner membrane to maintain its

fully polarized state, which is in turn required for oxidative phosphorylation. In other words, ALDA-1 exerts its protection by inhibiting permeability transition.

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