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. Therapy hence aims at oxygen supply restoration through the use of proteolytic enzymes, implantation of stents or coronary artery bypass surgery. Nevertheless, when a coronary artery occlusion is abruptly opened, heart damage occurs due to the increase of oxygen-derived free radicals and to a cellular Ca$^{2+}$ overload.

It is well known that superoxide and hydroxyl anions induce membrane leakage through lipid or protein peroxidation. Moreover, Ca$^{2+}$ accumulation is one of the causes of ventricular arrhythmias. There are reports indicating a link between mitochondrial damage and cellular consequences in the pathophysiology of ischemia reperfusion. Namely, this mechanism involves the opening of a non-specific transmembrane pore with a diameter of around 3-4 nm. 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 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, octylguanidine, tamoxifen, and CDP choline. 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,
in agreement with previous reports\textsuperscript{16,18-20}. Reperfusion started by removing the clamp and also lasted 5 min\textsuperscript{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\textsuperscript{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 \textit{et al.}\textsuperscript{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\textsuperscript{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 \textit{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 triphenyltetryrazolium chloride. These reagents were obtained from Sigma-Aldrich.

Results

Myocardial reperfusion is associated with a marked cytoplasmic Ca\textsuperscript{2+} accumulation\textsuperscript{25}. Actually, such a calcium overload induces the appearance of cardiac arrhythmias\textsuperscript{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

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{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 ▼.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{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 ■.}
\end{figure}
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 (ΔΨ) 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.
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. 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.

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.

<table>
<thead>
<tr>
<th>Condition</th>
<th>State 4</th>
<th>State 3</th>
<th>RC</th>
<th>ADP/O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamate/Malate</td>
<td>31±2</td>
<td>140±2.1</td>
<td>4.5±0.5</td>
<td>1.3±0.6</td>
</tr>
<tr>
<td>I/R</td>
<td>130±2.6</td>
<td>130±2.6</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>ALDA-1 + I/R</td>
<td>33±0.4*</td>
<td>145±4.1*</td>
<td>4.3±0.3*</td>
<td>1.5±0.3</td>
</tr>
<tr>
<td>Succinate</td>
<td>100±3</td>
<td>435.2±60</td>
<td>4.3±0.5</td>
<td>1.2±0.5</td>
</tr>
<tr>
<td>I/R</td>
<td>80.14±1.6</td>
<td>80.14±1.6</td>
<td>1±0.2</td>
<td>-</td>
</tr>
<tr>
<td>ALDA-1 + I/R</td>
<td>100±3*</td>
<td>411.6±20*</td>
<td>4.1±0.3*</td>
<td>1.3±0.4*</td>
</tr>
</tbody>
</table>

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

Table 2 — Protective effect of ALDA-1 on the activity of the enzyme aconitase from heart mitochondria submitted to ischemia/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.

<table>
<thead>
<tr>
<th>Condition</th>
<th>nmoL cis-aconitate/min/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>461±60</td>
</tr>
<tr>
<td>I/R</td>
<td>75±6*</td>
</tr>
<tr>
<td>I/R + ALDA-1</td>
<td>435±12*</td>
</tr>
</tbody>
</table>

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

Mitochondrial DNA may also be susceptible to damage by oxidative stress. Hence, we examined the ability of ALDA-1 to protect this poly nucleotide 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.
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. ALDA-1 preserves the ability of mitochondria 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 function.

**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. 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. 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. There is also an increase in intracellular Ca^{2+} for this reason. On this subject, the work of Bers 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. Regarding this topic, dissimilar strategies have been implemented to prevent mitochondrial damage; specifically, we encounter the use of reagents, such as cyclosporin A, octylguanidine, tamoxifen, and CDP-choline.

Further, membrane lipid peroxidation also evokes a high production of toxic aldehydes. In agreement with Mali et al., 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, yet it is worthwhile to mention that the work of Gomes et al. 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 function.
fully polarized state, which is in turn required for oxidative phosphorylation. In other words, ALDA-1 exerts its protection by inhibiting permeability transition.

References

