Aldehyde dehydrogenase 2 activation in heart failure restores mitochondrial function and improves ventricular function and remodelling

Katia M.S. Gomes¹, Juliane C. Campos¹, Luiz R.G. Bechara¹, Bruno Queliconi², Vanessa M. Lima¹, Marie-Helene Disatnik³, Paulo Magno⁴, Che-Hong Chen³, Patricia C. Brum⁵, Alicia J. Kowaltowski², Daria Mochly-Rosen³, and Julio C.B. Ferreira¹,³*

¹Department of Anatomy, Institute of Biomedical Sciences, São Paulo, Brazil; ²Department of Biochemistry, Institute of Chemistry, São Paulo, Brazil; ³Department of Chemical and Systems Biology, Stanford University School of Medicine, Stanford, CA, USA; ⁴Heart Institute, São Paulo, Brazil; and ⁵School of Physical Education and Sports, University of São Paulo, São Paulo, Brazil

Received 9 September 2013; revised 14 March 2014; accepted 15 April 2014; online publish-ahead-of-print 9 May 2014

Time for primary review: 27 days

Aims
We previously demonstrated that pharmacological activation of mitochondrial aldehyde dehydrogenase 2 (ALDH2) protects the heart against acute ischaemia/reperfusion injury. Here, we determined the benefits of chronic activation of ALDH2 on the progression of heart failure (HF) using a post-myocardial infarction model.

Methods and results
We showed that a 6-week treatment of myocardial infarction-induced HF rats with a selective ALDH2 activator (Alda-1), starting 4 weeks after myocardial infarction at a time when ventricular remodelling and cardiac dysfunction were present, improved cardiomyocyte shortening, cardiac function, left ventricular compliance and diastolic function under basal conditions, and after isoproterenol stimulation. Importantly, sustained Alda-1 treatment showed no toxicity and promoted a cardiac anti-remodelling effect by suppressing myocardial hypertrophy and fibrosis. Moreover, accumulation of 4-hydroxynonenal (4-HNE)-protein adducts and protein carbonyls seen in HF was not observed in Alda-1-treated rats, suggesting that increasing the activity of ALDH2 contributes to the reduction of aldehydic load in failing hearts. ALDH2 activation was associated with improved mitochondrial function, including elevated mitochondrial respiratory control ratios and reduced H₂O₂ release. Importantly, selective ALDH2 activation decreased mitochondrial Ca²⁺-induced permeability transition and cytochrome c release in failing hearts. Further supporting a mitochondrial mechanism for ALDH2, Alda-1 treatment preserved mitochondrial function upon in vitro aldehydic load.

Conclusions
Selective activation of mitochondrial ALDH2 is sufficient to improve the HF outcome by reducing the toxic effects of aldehydic overload on mitochondrial bioenergetics and reactive oxygen species generation, suggesting that ALDH2 activators, such as Alda-1, have a potential therapeutic value for treating HF patients.

Keywords
Oxidant stress • Heart disease • Mitochondria • Pharmacological therapy • Bioenergetics

1. Introduction
Despite advances in clinical and pharmacological interventions, acute myocardial infarction with subsequent left ventricular dysfunction and heart failure (HF) continues to be a major cause of morbidity and mortality worldwide. Therefore, the identification of novel therapeutic targets that improve cardiac function in patients with myocardial infarction-induced HF remains a major priority. We recently found that mitochondrial aldehyde dehydrogenase 2 (ALDH2) plays a key role in mediating cardioprotection against acute ischaemic injury. Acute ALDH2 activation using Alda-1, a small molecule allosteric activator of this enzyme, is sufficient to protect heart against ischaemia/reperfusion injury.

Considering the pivotal role of ALDH2 in detoxifying mitochondrial reactive aldehydes that accumulate upon oxidative stress during chronic cardiac degenerative diseases, including the lipid peroxidation by-product (4-hydroxynonenal), we set out to determine the role of ALDH2 in HF. We tested here the possibility that selective...
pharmacological activation of mitochondrial ALDH2 in failed hearts counteracts the aldehydic load, preserves mitochondrial function, and inhibits the progression of cardiac dysfunction in myocardial infarction-induced HF in rats.

2. Methods

2.1 Animals and study design

This study was conducted in accordance with the ethical principles in animal research adopted by the Brazilian College of Animal Experimentation (www.cobea.org.br). The animal protocols were reviewed and approved by the Ethical Committee of Biomedical Sciences Institute of University of São Paulo (20012/36). A cohort of male Wistar rats (250–300 g) was selected for the study and maintained in a 12:12 h light–dark cycle and temperature-controlled environment (22 °C) with free access to standard laboratory chow (Nuvital Nutrients, Curitiba, PR, Brazil) and tap water.

2.2 Myocardial infarction-induced HF model

Myocardial infarction was induced by ligation of the left anterior descending coronary artery (LAD), as previously described.9 We have chosen this model since myocardial infarction is the underlying aetiology of HF in nearly 70% of patients.9 Male Wistar rats were anaesthetized with 3% isoflurane and intraperitoneally (ip) injected with sodium pentobarbital (100 mg/kg), and euthanized by decapitation. Forty-eight hours later, all rats were anaesthetized with sodium pentobarbital (100 mg/kg ip), and euthanized by decapitation. The pumps were inserted into the left ventricle without any clinical sign of pain, such as withdrawal reflex. The pumps were inserted in a mini-pump implantation model since myocardial infarction is the underlying aetiology of HF in nearly 70% of patients.9

Alda-1 in order to check drug toxicity. Subcutaneous pump implantation served as the control. Another group of healthy animals was treated with 0.9% saline (50% polyethylene glycol and 50% dimethyl sulfoxide by volume) alone (50% polyethylene glycol and 50% dimethyl sulfoxide by volume) and began 4 weeks after myocardial infarction and ended 10 weeks later.

2.3 In vivo treatment with Alda-1 (ALDH2 activator)

Four weeks after myocardial infarction surgery, physiological parameters were determined and animals were randomly assigned into three experimental groups: sham group (control, n = 22), placebo-treated myocardial infarction-induced HF group (n = 14), and Alda-1-treated HF group (HF + Alda-1, n = 16) (Figure 1A). Continuous infusion of Alda-1 (10 mg/kg per day) was achieved using Alzet osmotic pumps (2ML4 and 2ML2) and began 4 weeks after myocardial infarction and ended 10 weeks later (Figure 1A). A group of rats implanted with pumps containing the vehicle alone (50% polyethylene glycol and 50% dimethyl sulfoxide by volume) served as the control. Another group of healthy animals was treated with Alda-1 in order to check drug toxicity. Subcutaneous pump implantation was performed in 3% vaporized isoﬂurane-anaesthetized rats. This concentration provided deep anaesthesia, allowing mini-pump implantation without any clinical sign of pain, such as withdrawal reflex. The pumps were inserted in the back of the animals after making a sub-scapular incision.

At the end of the protocol, physiological parameters were re-analysed. Forty-eight hours later, all rats were anaesthetized with sodium pentobarbital (100 mg/kg ip) and euthanized by decapitation.

2.4 Cardiovascular measurements

Systolic blood pressure was determined non-invasively, using a computerized tail-cuff system (BP-2000, Visitech System, Apex, NC, USA). Evaluation of non-invasive cardiac function was performed by echocardiography using a Vevo 770 rodent ultrasound system (VisualSonics, Canada), equipped with a high-resolution mechanical transducer (17-MHz scanhead RMV716). Echocardiography measurements were performed in anaesthetized (isoﬂurane 3%) sham and HF rats, 4 and 10 weeks after surgery. Myocardial images were recorded using 2D-guided M-mode from the parasternal long axis. Mitral inflow pattern was recorded from the apical four-chamber view using pulsed-wave Doppler. Left ventricle systolic function was estimated by fractional shortening (FS) as follows: FS(%) = [(LVEDD – LVESD)/(LVEDD)] × 100, where LVEDD is the left ventricular end-diastolic diameter, and LVESD is the left ventricular end-systolic diameter. The observer was blinded to the treatment groups.

2.5 Isolated perfused rat heart model

Ten weeks after MI surgery, control, vehicle-treated HF, and Alda1-treated HF rats were injected with heparin (2000 U/kg ip), anaesthetized with sodium pentobarbital (100 mg/kg ip), and euthanized by decapitation. The hearts were rapidly excised and then perfused with an oxygenated Krebs–Henseleit solution containing (in mM) NaCl 120, KCl 5.8, NaHCO3 25, NaH2PO4 1.2, MgSO4 1.2, CaCl2 1.0, and dextrose 10, pH 7.4, at 37 °C in a Langendorff coronary perfusion system at a constant flow rate of 10 mL/min. The left atrium was then removed and a thin latex balloon was inserted into the left ventricle (LV) and connected to a pressure transducer (Utah Medical Deltran) for measurement of the isovolumic LV pressure. The balloon within the LV was initially inflated with water to a volume of 100 μL, and LV developed pressure (LVDP) was measured. The balloon was then deflated in 20 μL decrements to determine the relationship between LVDP and balloon volume. LVDP was determined under basal conditions and during the peak response of isoproterenol (10 μM).

2.6 Isolation of adult rat cardiomyocytes

Ten weeks after MI surgery, control, vehicle-treated HF, and Alda1-treated HF rats were injected with heparin (2000 U/kg ip), anaesthetized with sodium pentobarbital (100 mg/kg ip), and euthanized by decapitation. The hearts were rapidly excised and then perfused with low-Ca2+ solution 1 (100 mM NaCl, 10 mM KCl, 1.2 mM KH2PO4, 5 mM MgSO4, 20 mM glucose, 50 mM taurine, 10 mM HEPES, and 100 μM CaCl2), then with digestion solution containing low-Ca2+ solution 1, collagenase (0.5 mg mL−1, Worthington Type 1A), and protease type XIV (0.04 mg mL−1, Sigma). Following perfusion, the ventricles were cut into fragments (2–5 mm3) in digestion solution. The cell suspension was then filtered through a nylon sieve and centrifuged for 1 min at 300–400 g at room temperature. Cell pellets were resuspended in solution 1 containing 125 mg BSA and 500 μM CaCl2.

2.7 Cardiomyocyte shortening and relengthening

Cell contraction properties of cardiomyocytes were evaluated with a video-based sarcomere-spacing acquisition system (SarcLen, IonOptix, Milton, MA, USA), as previously described.11 Changes in sarcomere length were recorded and analysed using the IonWizard software (IonOptix, Milton, MA, USA). Sarcomere shortening was determined under basal conditions and during the peak response of isoproterenol (10 μM).

2.8 Cardiac structural analysis

Forty-eight hours after the end of the protocol, all rats were killed and their tissues were harvested. Cardiac chambers were then fixed by immersion in 4% buffered formalin and embedded in paraffin for routine histological processing. Sections (4 μm) were stained with Picrosirius red or Masson’s trichrome for the quantification of cardiac collagen content and myocardial infarct area, respectively. Cardiac collagen deposition was measured by scanning at least 15 fields per heart after serial sections at 1 mm intervals from apex to base. The analysis covered the whole LV viable area. For that we used a computer-assisted morphometric system (Leica Quantimet 500, Cambridge, UK), as previously described.12 The myocardial infarcted area was expressed as a percentage of total surface area of the LV. The observer was blinded to the treatment groups.
Cardiac function in myocardial infarction-induced heart failure model

Figure 1 Mitochondrial ALDH2 activation improves cardiac function in a rat model of post-myocardial infarction-induced HF. (A) Schematic panel. HF induction and treatment protocol. Twelve-week-old rats were subjected to myocardial infarction by LAD ligation. Four weeks after myocardial infarction induction, the rats were treated with Alda-1 (selective ALDH2 activator) or with vehicle solution (50% polyethylene glycol and 50% dimethyl sulfoxide by volume). Alda-1 treatment was continuous (for 6 weeks) using subcutaneous Alzet pump delivery at 10 mg/kg/day. (B) Ejection fraction, input: delta ejection fraction; (C) LVEDD and (D) left ventricle end-diastolic posterior wall thickness (LVEDPWth) before and after treatment periods in control (sham, white bars, n = 12), vehicle-treated HF (HF, grey bars, n = 12), and Alda-1-treated HF (HF + Alda-1, black bars, n = 14). (E) LVDP in isolated ex vivo perfused heart and (F) sarcomeric shortening in isolated ventricular cardiomyocytes from control (sham, white bars, n = 6), vehicle-treated HF (HF, grey bars, n = 6), and Alda-1-treated HF (HF + Alda-1, grey bars, n = 6). Both LVDP and sarcomeric shortening were determined under basal conditions and during the peak response of isoproterenol (10 μM) at 10 weeks after MI surgery. Data are means ± SEM. *P < 0.05 vs. control (sham) rats. †P < 0.05 vs. vehicle-treated HF rats. ‡P < 0.05 vs. before the experimental protocol. The observer was blinded to the treatment groups.
2.9 Mitochondrial isolation
Cardiac mitochondria were isolated as described elsewhere. Briefly, heart samples were minced and homogenized in isolation buffer (300 mM sucrose, 10 mM HEPES, 2 mM EGTA, pH 7.2, 4°C) containing 0.1 mg mL⁻¹ of Type I protease (bovine pancreas) to release mitochondria from within muscle fibres, and later washed in the same buffer in the presence of 1 mg mL⁻¹ of bovine serum albumin. The suspension was homogenized in a 40 mL tissue grinder and centrifuged at 950 g for 5 min. The resulting supernatant was centrifuged at 9500 g for 10 min. The mitochondrial pellet was washed, resuspended in isolation buffer, and submitted to a new centrifugation (9500 g for 10 min). The mitochondrial pellet was washed and the final pellet was resuspended in a minimal volume of isolation buffer.

2.10 Mitochondrial H₂O₂ release and O₂ consumption
Mitochondrial H₂O₂ release was determined by measuring the oxidation of Amplex Red in the presence of horseradish peroxidase using a spectrophotometer with 563 nm of excitation and 587 nm of emission. Mitochondrial O₂ consumption was monitored using a computer-interfaced Clark-type electrode (Hansatech Instruments) operating with continuous stirring at 37°C. Succinate, malate, and glutamate (2 mM of each) were used as substrates, and ADP (1 mM) was added to induce State 3 respiratory rate. A subsequent addition of oligomycin (1 μg mL⁻¹) was used to determine State 4 rate. Respiratory control ratio (RCR) was calculated by dividing State 3 by State 4 oxygen consumption rates.

2.11 Maximal mitochondrial calcium uptake
Extramitochondrial Ca²⁺ concentrations were measured in 0.125 mg protein mL⁻¹ of mitochondrial suspensions using the fluorescent probe Calcium Green (100 nM), as described elsewhere. The reactions were carried out under the same conditions as oxygen consumption measurements with continuous stirring at 37°C. For each experiment, consecutive additions of 50 μM CaCl₂ were made until the mitochondria failed to take up extramitochondrial Ca²⁺. We plotted a calibration curve that correlates fluorescence and Ca²⁺ concentration. Succinate, malate, and glutamate (2 mM of each) were used as substrates, and 100 μM EGTA was used to establish the baseline. Excitation/emission wavelength was 506/532 nm, respectively.

2.12 Enzymatic activity of ALDH2
Enzymatic activity of ALDH2 was determined by measuring the conversion of NAD⁺ to NADH, as described elsewhere. The assays were carried out at 25°C in 50 mM sodium pyrophosphate buffer (pH 9.5) in the presence of 10 mM acetaldehyde. Measurement of mitochondrial ALDH2 activity in the rat myocardium was determined by directly adding 400 mg of the mitochondrial fraction of the myocardium to the reaction mix and reading absorbance at 340 nm for 10 min.

2.13 Immunoblotting
Protein levels were evaluated by immunoblotting in cytosolic and mitochondrial extracts from the ventricular remote area. Briefly, samples were subjected to SDS–PAGE in polyacrylamide gels (6–15%) depending on protein molecular weight. After electrophoresis, proteins were electrotransferred onto nitrocellulose membranes (BioRad Biosciences, Piscataway, NJ, USA). Equal gel loading and transfer efficiency were monitored using 0.5% Ponceau S staining of blot membrane. A blotted membrane was then blocked [5% non-fat dry milk, 10 mM Tris–HCl (pH 7.6), 150 mM NaCl, and 0.1% Tween 20] for 2 h at room temperature and then incubated overnight at 4°C with specific antibodies. Binding of the primary antibody was detected with the use of peroxidase-conjugated secondary antibodies (rabbit or mouse, depending on the protein, for 2 h at room temperature) and developed using enhanced chemiluminescence (Amersham Biosciences, NJ, USA) detected by autoradiography. Quantification analysis of blots was performed with the use of Scion Image software (Scion based on NIH image). Samples were normalized to relative changes in housekeeping proteins and expressed as the percent of control.

2.14 Protein carbonyl levels
Protein carbonyl levels were determined as previously described. The carbonyl groups in the protein side chains were derivatized to 2,4-dinitrophenylhydrazone (DNPH) by reaction with 2,4-dinitrophenylhydrazine (DNPH). The DNPH-derivatized protein samples were separated by polyacrylamide gel electrophoresis followed by immunoblotting.

2.15 Cell culture
Cardiac fibroblasts were isolated from 1-day-old Sprague–Dawley rat litters, as described elsewhere. All rat litters were euthanized by decapitation.

2.16 Statistical analysis
Data are presented as means ± standard error of the mean (SEM). Data normality was assessed through Shapiro–Wilk’s test. One-way analysis of variance (ANOVA) was used to analyse data presented in Figures 1B (input), 2C–J, and 3–5. Two-way ANOVA for repeated measures was used to analyse data depicted in Figure 1B–D and Table 1. Two-way ANOVA was also used to analyse data depicted in Figure 1E and F. Whenever significant F-values were obtained, Duncan’s adjustment was used for multiple comparison purposes. Unpaired Student’s t-test was used to analyse data presented in Figure 2B. GraphPad Prism Statistics was used for the analysis, and statistical significance was considered achieved when the value of P was <0.05.

3. Results
3.1 ALDH2 activation improves cardiac function and reverses pathological ventricular remodelling in HF animals
ALDH2 plays a key role in protecting the heart against oxidative stress during acute ischaemic injuries, mainly through detoxification of reactive aldehydes, such as 4-hydroxynonenal (4-HNE). We therefore determined the effects of selective ALDH2 activation on the progression of myocardial infarction-induced HF in rats. Ten weeks after myocardial infarction surgery (Figure 1A), the rats exhibited signs of HF as demonstrated by left ventricular dysfunction and pathological cardiac remodelling (Figures 1 and 2, Table 1, as well as see Supplementary material online, Table S1). These rats displayed decreased cardiac ejection fraction, FS, and LVDP, as well as increased diastolic dilation compared with control animals under basal conditions (Figure 1B–E and Table 1). To determine whether the ventricular dysfunction observed in vivo was cardiomyocyte-specific, shortening parameters were characterized in isolated adult cardiomyocytes. Intracardiac shortening was significantly decreased in the HF group (Figure 1F). These differences seen at baseline in both isolated ex vivo perfused heart and isolated cardiomyocytes were exacerbated upon isoproterenol challenge (Figure 1E and F). Moreover, these rats had an increased heart weight/body weight (HW/ BW) ratio (see Supplementary material online, Table S1), increased both cardiomyocyte width and length (Figure 2A and G–I), and cardiac fibrosis (Figure 2A–F) compared with control rats. Cardiomyocyte width and length were measured in isolated cells using the Image J software (NIH, USA). Cardiac fibrosis was measured in the non-infarcted (remote) area. No changes in heart rate and blood pressure were observed in rats with HF (see Supplementary material online, Table S1).
Using subcutaneously implanted Alzet pumps, we delivered Alda-1 in a sustained fashion at 10 mg/kg/day.6 Delivery of Alda-1 from Weeks 4 to 10 after inducing myocardial infarction (Figure 1A) not only prevented the development of cardiac dysfunction, but also increased left ventricular ejection fraction by 34% (Figure 1B, input). Indeed, Alda-1 treatment improved sarcomeric shortening, cardiac FS, and LVDP under basal conditions (Table 1). Moreover, isolated hearts and cardiomyocytes from Alda-1-treated HF rats became more sensitive to isoproterenol-induced increased isotropism (Figure 1E and F). Finally, sustained Alda-1 treatment promoted a cardiac anti-remodelling effect by reducing LVEDD (Figure 1C, Table 1, and see Supplementary material online, Figure S1), posterior wall thickness (Figure 1D and Table 1), HW/BW (see Supplementary material online, Table S1), cardiac fibrosis (Figure 2A–F), and cardiomyocyte length (Figure 2H and I) relative to untreated HF rats. Of interest, prolonged ALDH2 activation by Alda-1 affected cardiac fibroblasts by decreasing the extent of collagen type I deposition and collagen type I/III ratio (Figure 2A and D–F), further contributing, at least in part, to a better myocardial compliance (Table 1). In order to evaluate the direct contribution of aldehydic load to fibrosis, we treated cardiac fibroblasts with 4-HNE (2 μM) over 96 h and observed that sustained aldehydic load increases fibroblast proliferation. Interestingly, co-administration of Alda-1 reduced fibroblast proliferation upon aldehydic load stress (Figure 2F). Finally, Alda-1 treatment did not significantly affect systolic blood pressure, heart rate, and myocardial infarct size in HF animals (see Supplementary material online, Table S1, and Figure 2A and B).

Since this is the first study evaluating the long-term effect of Alda-1 treatment, we performed some toxicity measurements in healthy (naive) rats treated with Alda-1 for 6 weeks (10 mg/kg per day). No changes in haemodynamic parameters, BW, organ weight, cardiac function, circulating aspartate aminotransferase and alanine aminotransferase activities, as well as serum uric acid and creatinine levels were observed in Alda-1-treated rats compared with untreated animals (see Supplementary material online, Tables S2 and S3), suggesting that sustained Alda-1 treatment is safe.

### 3.2 Increased detoxification activity of ALDH2 contributes to preventing 4-HNE accumulation in failing hearts

Excessive 4-HNE-protein adducts contribute towards cardiac ischaemic injuries.19 We have suggested that ALDH2 activation-mediated cardioprotection upon ischaemia/reperfusion injury occurs through preservation of protein function by reduction of the aldehydic load in the heart.3 ALDH2 metabolizes reactive aldehydes that accumulate under redox imbalance, and prevents the production of aldehydic adducts that inactivate key metabolic enzymes.20 HF rats already presented increased aldehydic overload at 4 weeks after myocardial infarction surgery. We therefore measured 4-HNE-protein adducts and total protein carbonyls in the heart (non-infarcted area) 10 weeks after myocardial infarction surgery. Placebo-treated HF rats with HF displayed a significant increase of cardiac 4-HNE-protein adducts (Figure 3A and E) and protein carbonyls (Figure 3B and E) compared with relative controls. Sustained Alda-1 treatment increased cardiac ALDH2 activity by 2.7-fold (Figure 3C) and significantly reduced 4-HNE-protein adducts and protein carbonyls compared with untreated failing hearts (Figure 3A–B and E). Finally, Alda-1 treatment significantly reduced 4-HNE-protein adducts and protein carbonyls compared with untreated failing hearts (Figure 3A–B and E). Finally, Alda-1 treatment significantly reduced 4-HNE-protein adducts and protein carbonyls. Of interest, sustained Alda-1 treatment significantly reduced 4-HNE-protein adducts and protein carbonyls compared with untreated failing hearts (Figure 3A–B and E). Finally, Alda-1 treatment significantly reduced 4-HNE-protein adducts and protein carbonyls.
3.3 Alda-1 treatment protects mitochondrial function and prevents increases in reactive oxygen species release

Since accumulation of aldehydic adducts inhibits mitochondrial respiration by reacting with and inactivating metabolic enzymes during acute ischaemic injuries, we set out to determine mitochondrial function in a scenario of chronic aldehydic overload in HF. To assess mitochondrial function, we measured oxygen consumption, absolute (H₂O₂) and relative (H₂O₂/O₂) reactive oxygen species (ROS) release in isolated mitochondria from myocardial infarction-induced HF rats, and age-matched control rats, 10 weeks after myocardial infarction.

Our results indicate that vehicle-treated HF rats displayed a significant decrease in the efficiency of mitochondrial oxidative phosphorylation compared with control (sham) rats, as measured by the RCR (State 3/State 4; Figure 3F). This response was mainly due to a reduction of State 3 respiratory rate (the oxygen consumption rate maximized by the addition of ADP) in failing hearts (Figure 4B), while respiration in the absence of oxidative phosphorylation (State 4) was not affected (Figure 4C). Of interest, sustained ALDH2 activation by treating with Alda-1 improved the efficiency of mitochondrial oxidative phosphorylation (Figure 3F, RCR: 2.6 ± 0.2 vs. 1.6 ± 0.2 for HF + Alda-1 and HF, respectively) by preserving State 3 respiratory rates. Moreover, oxygen consumption enhancement induced by the mitochondrial uncoupler, carbonyl cyanide m-chlorophenyl hydrazone, was attenuated in the placebo-treated HF rat, whereas sustained ALDH2 activation normalized it towards control (sham) values (data not shown). These results demonstrate that sustained pharmacological activation of mitochondrial ALDH2 with Alda-1 attenuates the prominent disruption of mitochondrial oxygen consumption rates observed in failing hearts.

Mitochondrial ROS release has been associated with reduction of oxygen consumption in HF, so we hypothesized that Alda-1 treatment-mediated improvements in mitochondrial oxygen consumption could be attributed to the prevention of excessive mitochondrial ROS release in failing hearts. Placebo-treated HF animals presented increased mitochondrial State 2 (basal), State 3, and State 4 H₂O₂ release when compared with control rats (Figure 4D–F). These changes were more pronounced when H₂O₂ release was normalized by oxygen consumption rates for each state (Figure 4G–I), demonstrating the tight interdependence between mitochondrial oxygen consumption and ROS release in HF. Mitochondria isolated from Alda-1-treated rats with HF did not present the increases in H₂O₂ and...
H$_2$O$_2$/O$_2$ release observed in vehicle-treated HF rat mitochondria (Figure 4D–I). These data demonstrate that activating ALDH2 in failing hearts using Alda-1 is sufficient to prevent excessive mitochondrial ROS release during HF.

Excessive mitochondrial ROS and aldehydes in the presence of Ca$^{2+}$ lead to non-selective inner mitochondrial membrane permeabilization known as mitochondrial permeability transition, a cause of cell death in the heart. To test the contribution of chronic aldehydic overload towards the occurrence of mitochondrial transition permeability in HF, we measured maximal Ca$^{2+}$ uptake in isolated mitochondria from vehicle- and Alda-1-treated failing hearts. Control rats presented significantly higher ability to accumulate Ca$^{2+}$ ions before undergoing mitochondrial permeability transition relative to vehicle-treated rats with HF (Figure 5A). Of interest, similar to our in vivo results, 4-HNE incubation affected mainly mitochondrial State 3 oxygen consumption rates in vitro, without changing State 4 oxygen consumption rates (Figure 5D). To confirm the benefits of Alda-1 on 4-HNE-induced mitochondrial dysfunction, isolated mitochondria were incubated with Alda-1 prior to 4-HNE (10 μM) treatment. Activation of ALDH2 protected against 4-HNE-mediated mitochondrial dysfunction, resulting in an improvement of RCRs and mitochondrial cytochrome c (Figure 5B and C). Alda1 treatment significantly reduced cytochrome c release in failing hearts.

### 3.4 Alda-1 attenuates in vitro 4-HNE-mediated mitochondrial dysfunction

Elevated ROS release has generally been implicated in cellular damage during pathological processes. The introduction of carbonyl functional groups into proteins by 4-HNE, a major product of ROS-mediated lipid oxidation, has been reported to induce protein inactivation during ischaemia/reperfusion injury. We thus tested whether excessive aldehydic load directly affects mitochondrial function. In vitro incubation of cardiac mitochondria with different concentrations of 4-HNE (0.5–10 μM) resulted in a dose-dependent reduction in the efficiency of mitochondrial oxidative phosphorylation (Figure 5D). Of interest, similar to our in vivo results, 4-HNE incubation affected mainly mitochondrial State 3 oxygen consumption rates in vitro, without changing State 4 oxygen consumption rates (Figure 5E and F). To confirm the benefits of Alda-1 on 4-HNE-induced mitochondrial dysfunction, isolated mitochondria were incubated with Alda-1 prior to 4-HNE (10 μM) treatment. Activation of ALDH2 protected against 4-HNE-mediated mitochondrial dysfunction, resulting in an improvement of RCRs and mitochondrial cytochrome c (Figure 5B and C). Alda1 treatment significantly reduced cytochrome c release in failing hearts.
4. Discussion

Over the past decades, rapid and substantial advances have been made in the understanding of intracellular processes involved in HF, positively contributing to drug development in this field.1 However, in spite of new therapies to improve clinical outcomes,26 myocardial infarction-induced HF remains the main cause of death worldwide.2 Thus, there is a compelling need for new pharmacological therapies that improve patient quality of life and survival once cardiac dysfunction occurs.

Due to its pivotal role in bioenergetics, redox homeostasis, ion handling, and cell death, mitochondrial dysfunction is considered a critical factor in the progression of HF.27,28 Mitochondrial ALDH2 has emerged as a key enzyme in cardioprotection, since it efficiently eliminates toxic aldehydes by catalyzing their oxidation to non-reactive acids.1 Experimental approaches using either pharmacological activation or genetic overexpression of ALDH2 have shown that improved detoxification of reactive aldehydes, such as 4-HNE, is protective against acute ischaemia/reperfusion injury,3 nitroglycerine tolerance,29 and alcoholic cardiomyopathy.30 More recently, mitochondrial ALDH2 has been associated with remote preconditioning in humans31 and metabolic remodelling-related cardioprotection in patients with congenital heart disease.32 However, the role of mitochondrial ALDH2 in HF has not been determined yet.

Using an unconscious in vivo model of post-myocardial infarction-induced HF, we demonstrated that sustained activation of mitochondrial ALDH2 with Alda-1 improves cardiac contractility and promotes a

**Figure 4** Selective ALDH2 activation restores mitochondrial oxygen consumption rates and ROS release in post-myocardial infarction-induced HF. State-dependent (A–C) oxygen consumption rates, (D–F) absolute H2O2 release, and (G–I) H2O2/O2 in heart samples from control (sham, white bars, n = 18), vehicle-treated HF (HF, grey bars, n = 9), and Alda1-treated HF (HF + Alda-1, grey bars, n = 12) rats. Measurements were performed at 10 weeks after MI surgery. Succinate, malate, and glutamate (2 mM of each) were used as substrates (State 2), and ADP (1 mM) was added to induce State 3 respiratory rate. Addition of oligomycin (1 μg mL−1) was used to determine State 4 respiratory rates. Data are means ± SEM. *P < 0.05 vs. control (sham) rats. †P < 0.05 vs. HF + Alda-1 rats.
ALDH2 decreases sensitivity to mitochondrial permeability transition in failing hearts and preserves in vitro mitochondrial function upon aldehydic overload (A) Maximum mitochondrial Ca\(^{2+}\) uptake (an index of mitochondrial permeability transition) and (B) cytosolic cytochrome c levels in heart samples from control (sham, white bars, \(n = 10\)), vehicle-treated HF (HF, grey bars, \(n = 10\)), and Alda-1-treated HF (HF + Alda-1, grey bars, \(n = 9\)). Measurements were performed at 10 weeks after MI surgery. Mitochondrial cytochrome c release into the cytosol is an index of mitochondrial-mediated activation of apoptosis. Cytosolic and mitochondrial values were normalized by troponin I and prohibitin, respectively, and expressed as % of controls. (C) Representative western blots showing the levels of cytosolic and mitochondrial cytochrome c in control and age-matched post-myocardial infarction HF rats treated with Alda-1 or vehicle. Troponin I and prohibitin were used as cytosolic and mitochondrial markers, respectively. No contamination was observed between cytosolic and mitochondrial fractions. Data are means ± SEM. *\(P < 0.05\) vs. control (sham) rats. †\(P < 0.05\) vs. HF + Alda-1 rats. (D) Mitochondrial respiratory rate. (E) State 3, and (F) State 4 oxygen consumption rates in isolated mitochondrial challenged with different concentrations of 4-HNE (0.5–10 \(\mu\)M) and Alda-1 (20 \(\mu\)M) (\(n = 5–6\) per experiment). Cardiac mitochondria were isolated from control animals and incubated with different concentrations of 4-HNE for 10 min (0.250 mg protein mL\(^{-1}\)) in buffer containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES, 2 mM inorganic phosphate, 2 mM MgCl\(_2\), 100 \(\mu\)M EGTA, and 0.01% bovine serum albumin, pH 7.2, with continuous stirring at 37°C. The benefits of Alda-1 were evaluated by treating isolated mitochondria with Alda-1 (20 \(\mu\)M) 5 min prior to 4-HNE (10 \(\mu\)M) incubation. Data are means ± SEM. *\(P < 0.05\) vs. control. †\(P < 0.05\) vs. 4-HNE (10 \(\mu\)M).

Figure 6 A proposed model for selective ALDH2 activation-mediated cardioprotection in myocardial infarction-induced HF.
cardiac anti-remodelling effect. This benefit of Alda-1 treatment can be obtained when treatment is initiated after cardiac dysfunction occurred, at 4 weeks after myocardial infarction. Alda-1-induced cardioprotection is evidenced by increased cardiac contractility, decreased LV dilation, reduced myocyte hypertrophy, and diminished cardiac fibrosis. Moreover, selective ALDH2 activation attenuated cardiac aldehydic load, mitochondrial dysfunction, and oxidative stress seen in HF. Finally, we showed that sustained treatment with Alda-1 is well tolerated in naive rats, suggesting that chronic activation of ALDH2 may be safe.

Most of the cardiac damage occurring during HF is due to exacerbated generation of ROS, which leads to excessive oxidation of polysaturated fatty acids presented in biological membranes and accumulation of reactive aldehydes (i.e. 4-HNE). 4-HNE can readily interact with cysteine, histidine, and lysine residues and inactivate key proteins via Michael addition or Schiff base reactions.19,33 Mak et al.34 demonstrated that 4-HNE levels are consistently elevated in the plasma of congestive HF patients and are inversely correlated with left ventricular contractility. Similar to findings in humans with failing hearts,36,37 in the present study we found a pronounced increase in aldehydic load in the myocardium from rats with HF, characterized by accumulation of cardiac 4-HNE-protein adducts and protein carbonyls. Furthermore, sustained Alda-1 treatment strikingly corrected these changes by activating cardiac ALDH2 by 2.7-fold. These data demonstrate that selective ALDH2 activation is sufficient to produce cardioprotection against HF.

Excessive aldehyde generation during lipid peroxidation negatively affects cardiac viability by disrupting mitochondrial metabolism during acute ischaemia/reperfusion injury.36,37 Here, we showed that aldehydic load contributes to mitochondrial dysfunction and ROS generation in chronic HF by demonstrating that selective ALDH2 activation restored mitochondrial permeability transition, improved bioenergetics, and reduced hydrogen peroxide release in failing hearts. Moreover, acute Alda-1 treatment protected isolated mitochondria from 4-HNE-mediated dysfunction in vitro. We believe that reactive aldehydes lie at the centre of a positive feedback loop in HF signalling, whereby ROS activates and decreases elevated oxidative stress in human failing myocardium. Circulation 2002;105:2867–2871.


Acknowledgements

We thank Katt C. Mattos, Marcel C. Coelho, and Camille C. Caldeira-da-Silva for technical assistance.

Conflict of interest: D.-R. and C.-H.C. are founders of ALDEA Pharmaceuticals. C.-H.C. is also a consultant to the company. Other authors have no disclosure.

Funding

This study was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo, São Paulo (FAPESP #2012/05765-2), Conselho Nacional de Pesquisa e Desenvolvimento—Brazil (CNPq #470880/2012-0) to J.C.B.F., and National Institutes of Health NIAAA 11147 to D.M.-R. K.M.S.G. holds master’s fellowship from FAPESP.

References


Supplementary material

Supplementary material is available at Cardiovascular Research online.


