Supplementation of Creatine and Ribose Prevents Apoptosis and Right Ventricle Hypertrophy in Hypoxic Hearts

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Abstract: Background/Aims. The simultaneous supplementation of creatine and D-ribose has been shown to reduce apoptosis in vitro in non-irreversibly injured cultured ischemic cardiomyocytes through down-regulation of the signaling mechanisms governing adenosine monophosphate-activated protein kinase (AMPK) and protein kinase B (Akt). Here, we test the hypothesis that an analogous mechanism exists in vivo when the challenge is chronic exposure to hypoxia.

Methods. Five week-old mice were exposed to an atmosphere containing 10% O2 for 10 days. Mice were gavaged daily with vehicle, creatine, D-ribose or creatine + D-ribose. After sacrifice, myocardial and pulmonary tissue were harvested for structural and biochemical analyses.

Results. Hypoxia induced right ventricle hypertrophy and left ventricle apoptosis. Both phenotypes were slightly reduced by either creatine or D-ribose, whereas the simultaneous administration of creatine + D-ribose almost completely reversed the effects of hypoxia. Furthermore, creatine + D-ribose diminished the hypoxia-induced increases in the activity of AMPK, Akt and JNK, but not of ERK. Finally, the hypoxia-induced pulmonary overexpression of endothelin-1 mRNA was markedly reduced by creatine + D-ribose.

Conclusions. The simultaneous administration of creatine + D-ribose confers additional cardiovascular protection with respect to that observed with either creatine or D-ribose. The mechanism stems from the AMPK and Akt signaling pathways. These findings may form the basis of a paradigm to re-energize non-irreversibly damaged cardiomyocytes, counteracting injury by triggering specific signaling pathways.

Keywords: Apoptosis, AMPK, Akt, creatine, ribose, hypoxia, right vs left ventricle, pulmonary endothelium.

INTRODUCTION

In comparison to the various organs of the body, mammalian myocardium operates at very high consumption of oxygen and oxidizable substrates [1]. In order to fuel its metabolic processes, the myocardium thus depends critically on adequate supply of blood. Whenever this supply becomes insufficient with respect to demand in a particular tissue, rapid ATP depletion triggers a chain of events leading to disruption of cell homeostasis and pathological outcomes. In such conditions, fast replenishment of the ATP pool has become a leading therapeutic strategy to re-energize non-irreversibly damaged cardiomyocytes. One effective countermeasure to ATP depletion is administration of creatine (Cr), an endogenous substance involved in muscle energy metabolism, known to reduce the extent of cardiac necrosis following coronary ligature [2]. Creatine has also been shown to attenuate metabolic stress [3] and improve contractile reserve during ischemia [4]. Another cardioprotective metabolite, D-ribose (Rib), the sugar moiety of ATP, has been shown to increase stress tolerance and to alleviate symptoms in chronic heart failure [5], coronary artery disease [6] and hypertension [7]. Because the mechanisms underlying the protective effects of these low-cost compounds are largely complementary, the simultaneous administration of Cr and Rib (CrRib) is expected to confer extra-advantage over that offered by administering Cr and Rib separately. The authors of the present report recently demonstrated that cultured cardiomyocytes undergoing simulated ischemia received anti-ischemic protection from CrRib via a mechanism that was absent when treating cardiomyocytes with either Cr or Rib [8]. The mechanisms of cardiomyocyte re-energization appear to involve attenuated signaling through adenosine monophosphate-activated protein kinase (AMPK). Also, the improvement in bioenergetic metabolism stimulated by CrRib is characterized by activation of protein kinase B (Akt) and mitogen-activated protein kinase (MAPK), leading to a cellular phenotype resistant to stress-induced apoptosis.

The present study evaluates the hypothesis that the outcomes of in vitro exposure of cardiomyocytes to creatine and ribose [8] are paralleled in vivo in hearts from animals exposed to a stress. Here, hypoxia was selected as a stressor leading to non-lethal damage: chronic exposure to 10% O2, equivalent to 5000 m altitude, has been shown to depress myocardial tolerance to ischemia/reperfusion [9] through complex mechanisms including the modulation of K+ATP channels [10], NO-cGMP pathway [11], MAPK [12-14] and apoptosis [15], but does not compromise survival. To assess the molecular bases of cardioprotection mediated by creatine and ribose, the authors characterized the phosphorylation of kinases that activate selected signaling pathways in myocardial tissue. Because pulmonary vasoconstriction is a known major side effect of hypoxia which must be minimized in countermeasures [16], the present investigation examined hypoxia-induced right ventricle hypertrophy. As hypoxia-induced right ventricle hypertrophy usually develops into pulmonary vasoconstriction, the authors evaluated the impact of CrRib on pulmonary endothelial function by assessing mRNA expression of endothelin-1 (ET1), a short-lived peptide which is known to play a role in development of pulmonary hypertension and heart disease [17]. The present report demonstrates that the protective effects mediated by CrRib in vitro are reflected in vivo outcomes. These results offer the possibility for co-administration of creatine and ribose in management of syn-
dromes involving systemic hypoxia, chronic pulmonary obstructive diseases, heart failure and various forms of anemia.

**MATERIALS AND METHODS**

**Strategy.** The present report describes a 2-part investigation. Part 1 focused on establishing whether co-administration of creatine and ribose provided superior outcomes to treatment with each compound independently. Part 2 focused on characterization of molecular mechanisms modulated by CrRib. These experiments were undertaken to begin to identify the primary cell signaling pathways contributing to the observed outcomes.

**Animals.** Five-week-old male ICR/CD-1 mice (Harlan Laboratories, Italy, n=26), weighing 25–30 g at the entry into the study, were cared in accordance to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85–23, revised 1996), and the protocol was approved by the Ethics Committee of the University of Milan (sentence 16.03.2010). Mice had free access to water and conventional laboratory diet until 24 h before sacrifice. A 12/12 h light/dark cycle was maintained. All animal operations were performed under sterile laminar flow hoods.

**Materials.** Creatine monohydrate (Cr) and D-(-)ribose (Rib) from Gieellepi Chemicals (Milano, Italy) were daily dissolved in sterilized water (16.6 mg/ml and 83.3 mg/ml, respectively) and filtered (0.22 µm pore diameter, Nalgene) for immediate administration to animals by gavage (100 µl/mice, corresponding to total administration of 16.6 and 83.3 mg Cr and Rib over the whole observation window, respectively). Control mice were gavaged with an equal amount of sterilized water.

**Treatments.** Mice were divided into five groups: control (exposed to room air at 21% O₂ and gavaged with water), hypoxic vehicle or Hv (exposed to hypoxia and gavaged with water), hypoxic creatine or HCr (exposed to hypoxia and gavaged with Cr), hypoxic ribose or HRRib (exposed to hypoxia and gavaged with Rib) and hypoxic creatine + ribose or HCRRib (exposed to hypoxia and gavaged with both Cr and Rib). All treatments began concurrently with hypoxia and lasted for 10 days. Hypoxic mice were maintained continuously in an atmosphere containing 10% O₂ in specially designed chambers [18] until sacrifice, performed as described below. During the course of hypoxia regimens, hypoxic mice were anaerobically transferred one animal at a time into the compensation chamber and treated by oral gavage as described below. Mice included as non-hypoxic controls were maintained in chambers ventilated with normal room air.

**Sacrifice.** At day ten, hypoxic mice were transferred one at a time into the compensation chamber, anesthetized with Na-thiopental (10 mg/100 g body weight) plus heparin (500 U) i.p., then euthanized by cervical dislocation. Following sacrifice, blood samples were collected into a heparinized syringe by left ventricular puncture, concurrent with harvest of tissue from the right lobe of each lung. These activities were typically accomplished during a timeframe of less than 1 minute beginning with euthanasia. Hearts designated for biochemical analyses were immediately frozen in liquid nitrogen and stored at −80°C, with remaining hearts designated for morphologic and histological measurements.

**Morphologic measurements.** Excess water was absorbed on tissue paper, atria, vessels as well as connective tissue were excised and the heart mass was weighed. The free walls of the right (RV) and left (LV) ventricles were dissected free and weighed. RV hypertrophy was assessed from the weight ratio RV/(LV + septum).

**Hemoglobin.** Hemoglobin (Hb) concentration was measured in blood by diluting 10 µl of well-stirred blood to 1 ml of Drabkin reagent, followed by incubation for 30 min at room temperature and absorbance reading at λ=540 nm. The concentration was calculated assuming ε=11.05 cm⁻¹ mM⁻¹.

**In Situ TdT assay for detection of apoptosis.** Frozen specimens were analyzed for apoptosis via the Terminal deoxynucleotidyl transferase (TdT) assay [19]. Details and sample microphotographs are shown in the Supplementary (Fig. S2). To obtain the number of TdT-labelled nuclei, 8-10 random fields were counted in a blinded procedure and are expressed as number of TdT-labelled nuclei per unit area (0.037 mm²). To estimate the percent of TdT-positive nuclei, observations were made in three areas in both ventricle and pulmonary tissue. In each area, merged TdT-positive (red) and total (blue) nuclei were counted in 3-to-5 fields and the values averaged. The measure of apoptosis is reported as the average ratio of TdT-positive/total nuclei.

**Western Blot.** Cytosolic extracts from whole myocardium tissue were prepared as described [19] and the following proteins were analyzed by Western blot technique as detailed in the Supplementary section S3: Akt, p-Akt (Ser^{472}), AMPKα, p-AMPK (Thr^{172}), ERK1, p-ERK1/2 (Tyr^{185}), JNK, p-JNK (Thr^{183} and Thr^{183}), tubulin. The protein signals were detected by incubating the membrane for 1 min with LiteAblot Chemiluminescent substrate (Lite Ablot, EuroClone, EMPO10004) followed by x-ray film exposure (Kodak X-Omat Blue XB-1 Film, Eastman Kodak Company, Rochester, NY). The resulting image was quantified using the Gel Doc system (Bio-Rad quantitation software Quantity One).

**Pulmonary RNA preparation and cDNA synthesis.** Lung tissue was reduced to powder in liquid N₂, then RNA was prepared as described in [20] by extracting total RNA with TRIZOL® Reagent (Invitrogen, Milan, Italy) according to the manufacturer’s instructions. For each sample, after a denaturing electrophoresis gel, RNA quality was assessed by evaluating the 18S and 28S band sharpness. Two samples belonging to the Hv and HCRib groups showed RNA degradation and were not evaluated. RNA purity and concentration was measured spectrophotometrically (Ultraspres 3000, Pharmacia Biotech, Cambridge, UK). An equal amount of total RNA was reverse transcribed using the Omniscript Reverse Transcription Kit (Qiagen GmbH, Hilden, Germany) following the manufacturer’s instructions.

**Quantitative Real Time PCR.** Specific primer sequences for ET1, as well as three housekeeping genes (β-actin, Hprt and Rplp0) were designed with the help of two freely-distributed software tools (Primer3 and Amplify). Primer sequences (Table 1), designed to span an exon-exon junction, were purchased from Sigma-GENOSYS (Sigma-Aldrich). Primer specificity was assessed by both electrophoresis gel and melting curve analysis. For each primer pair, PCR efficiency was evaluated using a cDNA pool constituted by the same amount of cDNA of two samples representative of each group. Primer efficiency was in the range 95-105%.

Quantitative Real Time PCR (qRT-PCR) was performed in a BioRad CFX96 real-time thermal cycler using the SsoFast™ Exaq-Gen® Supermix (Bio-Rad Laboratories, Hercules, CA) as follows: a denaturing stage at 95°C for 30 s, then 45 cycles at 95°C for 5 s, followed by 60°C for 5 s. Negative controls were used to check for contamination and unspecific amplifications. The selected housekeeping genes showed a great inter-group stability and their expression was not influenced by the different treatments. The use of an internal calibrator allowed the inter-pair comparison. Gene expression analysis was based on the 2^-ΔΔCT method taking into account the different primer sequence efficiencies [21]. Target gene expression was normalized for the housekeeping genes and expressed relative to the average of control samples.

**Statistics.** All data are mean ± SEM. To assess the significance of the differences among the various treatments, one-way ANOVA was performed. If significant, the Bonferroni multiple comparisons test was used to evaluate the differences between selected pairs of data (significance level set to P=0.05).
RESULTS

Performance of CrRib, Cr and Rib

All animals survived hypoxia and were admitted to statistical analysis. Body weight (Table 2) increased during the observation window in control animals, while decreasing in hypoxic animals in all the groups, irrespective of treatments. Hypoxia increased blood hemoglobin, whereas the other treatments did not affect this parameter.

Whole heart myocardial hypertrophy was not detected under any condition (data not shown), but hypoxia led to RV hypertrophy (Fig. 1). Treatment with either Cr or Rib did not reverse RV hypertrophy, but the combined (CrRib) treatment reduced RV hypertrophy to a value close to that of control animals subjected to hypoxia, but not receiving CrRib. Supplementary (Fig. S1) shows some histological samples indicating slight enlargement of cardiomyocytes.

To evaluate apoptosis in the myocardium, we first performed TUNEL analysis to identify and quantify DNA strand breaks. DNA strands were labeled with TdT (Rhodamine) and counted (Fig. 2). Hypoxia markedly increased TdT positivity, whereas treatment with either Cr or Rib reduced such increase by similar extents to a value less than that measured in hypoxia, but statistically higher than control. The combined CrRib treatment further reduced TdT positivity to a value close to control. Thus, data clearly show that the combined CrRib treatment confers greater advantage than that associated to the treatment with either Cr or Rib alone.

Cell Signaling Elicited by CrRib

To further assess the mechanisms underlying the molecular effects led by CrRib, the dependence of specificity of TdT positivity upon the number of apoptotic nuclei and their RV/LV distribution was assessed via the procedure described in detail in the Supplementary section S2 to examine co-localization of the red (TdT-positivity) and blue (nuclei). The purple signals resulting from the merge were counted and averaged in the two ventricles. Fig. (3) shows that exposure to hypoxia affected mainly LV while leaving

Table 1. Primer Sequences and Amplicon Length Used for the qRT-PCR Evaluation of mRNA Abundance. β-Actin, Hprt and Rplp0 were Used as Housekeeping Genes for Normalization Purposes.

<table>
<thead>
<tr>
<th>Unigene accession no.</th>
<th>Gene</th>
<th>Left Primer</th>
<th>Right Primer</th>
<th>Amplicon length, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mm.391967</td>
<td>β-actin</td>
<td>TTGCTGACAGGATGCAGAAG</td>
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<tr>
<td>Mm.299381</td>
<td>Hprt</td>
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<tr>
<td>Mm.371545</td>
<td>Rplp0</td>
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<td>GACCAATCCCATATCCTCA</td>
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<tr>
<td>Mm.1453</td>
<td>ET-1</td>
<td>CTGCAACGAGGAAAAAAGAC</td>
<td>TTTGCAACAGGAAAAAGAC</td>
<td>186</td>
</tr>
</tbody>
</table>

Table 2. Animal Data. One-Way, Two-Tailed ANOVA P is Reported. #, P<0.05 with Respect to Control (Bonferroni Post Test).

<table>
<thead>
<tr>
<th>n</th>
<th>Body weight change, g</th>
<th>Hemoglobin, g/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>+5.70±0.91</td>
</tr>
<tr>
<td>Hypoxia vehicle (Hv)</td>
<td>5</td>
<td>-3.60±0.25*</td>
</tr>
<tr>
<td>Hypoxia + creatine</td>
<td>5</td>
<td>-2.58±0.42*</td>
</tr>
<tr>
<td>Hypoxia + D-ribose</td>
<td>5</td>
<td>-2.30±0.65*</td>
</tr>
<tr>
<td>Hypoxia + creatine + D-ribose</td>
<td>5</td>
<td>-2.40±0.29*</td>
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<td>ANOVA P</td>
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<td>0.0005</td>
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</table>
RV almost undisturbed. Likewise, the protective effect of CrRib was almost exclusively directed to LV. The same figure also shows the effect of hypoxia and CrRib on pulmonary tissue apoptosis. Whereas hypoxia markedly increased the number of apoptotic nuclei, CrRib reduced this increase.

We then performed Western blotting analyses (see Supplementary section S3) to assess the impact of specific signalling paths. To evaluate the imbalance of myocardial bioenergetics, the levels of AMPK expression in whole myocardium tissue and its phosphorylation at Thr\(^{172}\) (p-AMPK) were assessed. Whereas AMPK expression remained unchanged, the p-AMPK/AMPK ratio increased in Hv with respect to control by approximately 70%, and CrRib returned this value to near control level (Fig. 4). As this ratio is considered a marker of the intracellular energy level reflecting impaired AMP-to-ATP conversion, it appears that CrRib corrects the bioenergetic derangement caused by hypoxia.

To define the death pathways potentially modulated by CrRib, Akt, JNK and ERK were assessed in whole myocardium tissue. Although the expression of these proteins remained unaffected under the selected experimental conditions, their phosphorylation or activation was different. The p-Akt/Akt ratio almost doubled in Hv with respect to control, but returned to control value following CrRib. Likewise, the p-JNK/JNK ratio showed a similar trend, increasing by 55% in Hv vs control and returning to baseline levels in CrRib. By contrast, the p-ERK/ERK ratio remained unaffected by either hypoxia or CrRib.

Finally, in a measure of the effects of CrRib on the function of hypoxic pulmonary endothelium, qRT-PCR study in pulmonary tissue (Fig. 5) showed a marked 4-5-fold up-regulation in ET1 expression, accompanied by significant depression following CrRib treatment.

DISCUSSION

In a previous work [8], the authors showed that CrRib prevents apoptosis in H9c2 cardiomyocytes subjected to simulated ischemia in vitro. The underlying mechanism originates from improved bioenergetics, which reflects a complex interplay between AMPK and the activation of Akt. The present study demonstrates that CrRib is also effective in an in vivo model of hypoxia where mice were exposed to 10% \(O_2\) for 10 days. Without being lethal, this degree of hypoxia nevertheless causes an array of dysfunctions that involve specific signaling pathways [9, 13, 14], resulting in derangements of myocardial morphology [22] and depressed cardioprotection [11]. However, when hypoxic mice were treated daily by oral gavage with CrRib, myocardial apoptosis, pulmonary derangement and right ventricle hypertrophy were almost completely reversed.

The existence of subtle differences between RV and LV responses to hypoxia is now beginning to be better understood. Indeed, exposure of pulmonary arterial smooth muscle cells to a hypoxic environment and the consequent inhibition of mitochondrial oxidative phosphorylation may be related to intracellular \(Ca^{++}\) accumulation by one or more of the following mechanisms: activation of L-type \(Ca^{++}\) channels, generation of reactive \(O_2\) species with multiple potential targets, and AMPK activation with consequent \(Ca^{++}\) release from sarcoplasmic reticulum ryanodine receptors [16]. The subsequent hypoxic pulmonary vasoconstriction, likely mediated by ET1 overexpression in pulmonary tissue [17] (see also Fig. 5), leads to RV pressure overload, and different transcriptional profiles are thus expected in the right and left ventricles. It has been shown that chronic hypoxia induces RV hypertrophy without fibrosis, but is accompanied by downregulated fatty acid metabolism, increased glucose metabolism, and decreased sarcoendoplasmic...
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Of interest, the development of RV hypertrophy is associated with LV apoptosis [24], as reported here (Fig. 3). Here we show that the protective effect by CrRib is double faced: it prevents apoptosis in LV and hypertrophy in RV.

The increase in AMPK observed during hypoxia is an expected finding resulting from inadequate O2 supply. Due to its strict dependence on energy-yielding processes, the myocardium is extremely sensitive to imbalances in bioenergetics that translate into ATP depletion, increased AMP, elevated AMP/ATP ratio and hence activation of AMPK, which serves as an integrator of metabolic responses to changes in energy availability [25]. An increase in the p-AMPK/AMPK ratio is therefore a consequence of both acute and chronic hypoxia [26]. Impaired AMPK activation induced by CrRib, a hallmark in cardiomyocytes exposed to simulated ischemia [8], reflects improved bioenergetic recovery. Whereas Cr improves bioenergetics by recycling ADP into ATP via its shuttle property [27], Rib protects ischemic cardiac tissue by replenishing building blocks for ATP synthesis [28].

We assessed the activation of the Akt pathway by observing its phosphorylation at Ser473, which is more crucial than that of Thr308 [29]. Despite the observation that one of the paths activated by Akt prevents AMPK phosphorylation through LKB1, an AMP kinase abundantly expressed in cardiomyocytes [30], AMPK appears to lie upstream from Akt in several signaling pathways [31-33]. In the myocardium, Akt is associated with several protective and tissue remodeling pathways [34]. Whereas p-Akt mediates cardiac protection against hypoxia and reoxygenation injury [35] and delays the onset of apoptosis by inducing ERK and inhibiting JNK and p38 [36], it is also recognized as a pivotal participant in hypertrophic signaling [37]. Indeed, Akt regulates cardiac hypertrophy mainly by increasing cardiomyocyte size rather than cardiomyocyte proliferation [38, 39]. By preventing the activation of AMPK and Akt, administration of CrRib should lead to the reduction of hypertrophy and, at the same time, the increase of apoptosis. Whereas the first prediction was met in the experiments reported here, the second was not. It remains to be investigated whether this discrepancy was due to a small effect of CrRib on the Akt pathway leading to apoptosis control, due perhaps to the need for other as yet unidentified factors. Alternatively, the stimulus represented by hypoxia at 10% O2 may be too mild to trigger an efficient direct effect of Akt on the paths leading to the reduction of apoptosis.

To further clarify this issue, the role of JNK activation was assessed. JNK is a factor that promotes apoptosis by inactivating Bcl2 [40, 41], favoring the mitochondrial release of cytochrome c [42] and facilitating caspase-8 phosphorylation [43] via both transcription-dependent and transcription-independent mechanisms [44]. Whereas hypoxia activates JNK, likely through p-AMPK [45], CrRib downregulates it, possibly favoring cardioprotection. In support of this, JNK activation was found protective in mice subjected to brief, rather than extended ischemia [46], in agreement with the results of the present study, for which the duration of hypoxia was 10 days.

The observation that hypoxia does not alter ERK expression or phosphorylation is in agreement with our previous results obtained in hypoxic hearts [13], but in contrast with data obtained in vitro [47] and in neonatal rat brains [48]. Fig. (6) reports a possible mechanism to explain the features observed in this study.

CONCLUSION

CrRib treatment is effective in reducing myocardial cell death in the left ventricle and hypertrophy in the right ventricle of mice exposed to chronic hypoxia. AMPK acts as the main effector driving the protection conferred by CrRib. The inhibition of AMPK-signaling upon CrRib treatment correlates with p-Akt and p-JNK down-regulation, but ERK expression and phosphorylation are not affected. Furthermore, pulmonary ET1 mRNA overexpression correlates positively with CrRib. We conclude that in vivo CrRib treatment is effective in re-energizing the myocardium when challenged by hypoxia and blunting the pathways originated from AMPK.

LIST OF ABBREVIATION

Akt = Protein kinase B
AMP = Adenosine-monophosphate

Myocardium

Creatine + D-Ribose

Hypoxia

Energy lack

ATP

PCr

AMPK

↑

JNK

Left ventricle apoptosis

Akt

Right ventricle hypertrophy

Ca++

smooth muscle

Pulmonary vasoconstriction

ET1 gene expression

Pulmonary apoptosis

Lung

Fig. (6). Scheme of the interactions between hypoxia and CrRib in myocardium and lung.
AMPK = AMP-activated kinase
ATP = Adenosine triphosphate
cGMP = Cyclic guanosine monophosphate
Cr = Creatine
CrRib = Combination Creatine + D-Ribose
ERK = Extracellular-regulated kinase
ET1 = Endothelin-1
HCr = Hypoxia + Cr treatment
HCrRib = Hypoxia + CrRib treatment
HRib = Hypoxia + Rib treatment
Hv = Hypoxia + vehicle treatment
JNK = c-Jun N-terminal kinases
LV = Left ventricle
MAPK = Mitogen-activated protein kinase
PBS = Phosphate-buffered saline
PCr = Phosphocreatine
Rib = D-ribose
RV = Right ventricle
SEM = Standard error of the mean
TdT = Terminal deoxynucleotidyl transferase.

CONFLICT OF INTEREST
CT and FL are employees at Giellepi Chemicals, Milan, Italy, who supported in part this work. No other conflicts of interest are declared. Contract grant sponsor: Italian Ministry of University and Research (PRIN 2007), contract grant number: 12-1-5201001-211. AC performed part of her work as a fellow on the Istituto Nazionale delle Ricerche Cardiovascolari.

ACKNOWLEDGEMENTS
We gratefully acknowledge the great technical help provided by Ms.Nadia Toppi. We also acknowledge the critical revision of the manuscript by Dr. David J. Muehsam.

REFERENCES


Supplementary Material

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S1 - Histological Measurements

Myocardium was fixed in 4% buffered formaldehyde, two adjacent slices were taken perpendicular to the long axis of the heart, dehydrated and embedded in paraffin. Sections 4 μm thick were cut on a microtome and stained with hematoxylin-eosin and periodic acid-Schiff to emphasize the basal membrane and facilitate cardiomyocyte visualization.

Fig. (S1). Hematoxylin-eosin staining in right ventricle of control, Hv and HCrRib (three random samples per each group). Magnification 40x.

S2 – Apoptosis

Frozen specimen were embedded in Optimum Cutting Temperature (OCT-Compound, Leica Instruments, Nussloch, Germany), serial 5-μm thick sections were obtained in a cryomicrotome (Leica CM1510) and placed on silanized glass slides. The sections were dried at room temperature for 3 min, fixed in 4% buffered formalin for 45 min at 4°C, rinsed twice for 5 min in PBS, post-fixed with ethanol-acetic acid 2:1 (v/v) at −20°C for 5 min, rinsed twice for 5 min in PBS, boiled in 10 mM citrate buffer at pH 6.0 for 10 min, washed once in distilled water and three times in PBS, and finally used for DNA fragmentation staining. The degree of apoptosis was determined by the TdT assay (In situ Cell Death Detection Kit, TMR Roche Diagnostics, Mannheim, Germany) in an inverted fluorescence microscope (40x magnification) Axiovert 25 CFL (Zeiss, Göttingen, Germany) equipped for the detection of rhodamine (filter set 15, excitation band pass 546 nm, emission low-pass 590 nm). Images were acquired by a digital camera (DS-5M; Nikon, Tokyo, Japan).

To estimate the percent of TdT-positive nuclei, additional slices were taken and nuclei were stained with the karyophilic dye Hoechst 33258 (250 ng/ml, Sigma, St. Louis, Mo) for 3 min at room temperature in the dark, followed by rinsing twice in PBS and coverslipping. Three areas were considered in either ventricle and pulmonary tissue and then counted for both total nuclei (blue) and TdT-positive nuclei (pink).
**S3 – Western Blot**

Protein cytosolic extracts were prepared at 4°C. Frozen hearts (60–80 mg) were homogenized in a glass potter in a 1:3 ratio (w/v) with 10 mM HEPES, 1.5 mM MgCl₂, 0.5 mM DTT, 0.2 mM PMSF, 10 mM KCl and 10% Protease Inhibitor Cocktail (Complete Protease Inhibitor Cocktail Tables, EDTA-free, Roche Diagnostics GmbH, Mannheim, Germany), pH 7.9. The homogenate was centrifuged for 20 min at 14,000 rpm, the pellet was resuspended and centrifuged again for 10 min at 14,000 rpm. The cytosolic extract was obtained by pooling the supernatant fractions from both centrifugations. To obtain the nuclear extract, the pellet was resuspended in 20 mM Heps, 1.5 MgCl₂, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMFS, 25% glycerol, 10% Protease Inhibitor Cocktail, pH 7.9, kept in ice for 20 min and centrifuged for 20 min at 14,000 rpm.

Extracts were separated by SDS-PAGE on 8% acrylamide gels. The protein concentration was measured by the Coomassie Plus Protein Assay reagent Kit (Pierce, Rockford, IL) and 40 µg protein was loaded per each lane. After separation, proteins were blotted onto a nitrocellulose membrane (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) and blocked with 5% nonfat dry milk in TRIS-buffered saline containing 0.1% Tween (1 h, room temperature). Membranes incubation overnight at 4°C with the primary antibody was followed by incubation with horseradish peroxidase-conjugated secondary antibody (1 h, room temperature). The following primary antibodies and dilutions were used: rabbit polyclonal anti-Akt (Cell Signaling Technology, 1:1000) and rabbit polyclonal anti-p-Akt (Ser473) (Cell Signaling Technology, 1:1000), rabbit anti-AMPKα1/2 (Santa Cruz, 1:1000) and anti-p-AMPK (Thr172) (Santa Cruz 1:500), mouse monoclonal anti-p-ERK1/2 (Tyr204) (Santa Cruz 1:250), rabbit polyclonal anti-ERK1 (Santa Cruz, 1:500), mouse monoclonal anti-p-JNK (Thr183 and Thr185) (Santa Cruz, 1:250), mouse monoclonal anti-JNK (Santa Cruz, 1:500). Rabbit anti-tubulin (Santa Cruz, 1:200) was used as loading control for cytosolic extracts. The secondary antibodies included horseradish peroxidase conjugated anti-mouse IgG (Jackson Immuno Research, West Grove, PA, 1:10000), anti-rabbit IgG (Jackson Immuno Research, West Grove, PA, 1:10000). The chemiluminescent signal was detected by incubating for 1 min the membrane with LiteAblot Chemiluminescent substrate (Lite Ablot, EuroClone, EMPO10004) followed by x-ray film exposure (Kodak X-Omat Blue XB-1 Film, Eastman Kodak Company, Rochester, NY). The resulting image was acquired and quantified using the Gel Doc system (Bio-Rad quantitation software Quantity One).
Fig. (S3). Sample Western blot obtained in one myocardial tissue per group. The lanes represent a typical run for (from left to right) molecular weight marker, Control, Hypoxic vehicle and Hypoxic treated with CrRib.