Original Paper

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2010;26:831-838

Accepted: November 04, 2010

# Supplementation of Creatine and Ribose Prevents Apoptosis in Ischemic Cardiomyocytes

Anna Caretti*, Paola Bianciardi*, Giusy Sala*, Carlo Terruzzi#, Franco Lucchina# and Michele Samaja*

*Department of Medicine, Surgery and Dentistry, University of Milan, San Paolo Hospital, and #Giellepi Chemicals, Milan

**Key Words**
AMPK • Akt • Creatine • Ribose • Cardiomyocytes • Ischemia

**Abstract**
Background/Aims. To alleviate ischemia-induced injury in the myocardium, a tissue that depends critically on energy-yielding processes, creatine may be used to enhance energy metabolism, whereas D-ribose may provide building blocks for ATP synthesis. We test the hypothesis that simultaneous supplementation of creatine+D-ribose protects non-irreversibly injured ischemic cardiomyocytes by reducing apoptosis. Results. When H9c2 cardiomyocytes were exposed to 24-h ischemia (1% $O_2$ with glucose deprivation), viability was severely compromised, but administration of 2.5 mM creatine + 5 mM D-ribose alleviated the fall in viability, whereas 2.5 mM creatine or 5 mM D-ribose did not. These findings correlated with up-regulation of protein kinase B (Akt) phosphorylation. Creatine+D-ribose also blunted adenosine monophosphate-activated protein kinase (AMPK) and down-regulated apoptosis by reducing caspase-3 activation and poly (ADP-ribose) polymerase (PARP) cleavage. Conclusions. Simultaneous administration of creatine+D-ribose confers anti-ischemic protection that was absent when treating cardiomyocytes with either creatine or D-ribose. The involved mechanisms stem from the Akt and AMPK signaling pathways. These findings may form the basis of a paradigm whereby re-energization of non-irreversibly damaged cardiomyocytes is a critical step to counteract apoptosis.



## Introduction

To fuel its metabolic processes, the myocardium depends critically on continuous supply of blood, oxygen and oxidizable substrates. Whenever this supply becomes insufficient with respect to needs, the rapid depletion of ATP leads to intracellular $Ca^{++}$ overload, failure and necrosis. Re-energization of non-irreversibly damaged cardiomyocytes depends on fast replenishment of the ATP pool. Administration of creatine (Cr), an endogenous substance involved in muscle energetic metabolism, has been shown to reduce the necrotic zone after coronary ligature [1], attenuate the metabolic stress [2] and improve the contractile reserve during ischemia [3]. On the other

KARGER
Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com


1015-8987/10/0266-0831$26.00/0

Accessible online at:
www.karger.com/cpb

Michele Samaja
University of Milan - San Paolo, Department of Medicine, Surgery and Dentistry, via di Rudinì 8, I-20142 Milan (Italy)
Tel. +39-0250323264, Fax +39-0250323245
E-Mail michele.samaja@unimi.it

hand, D-ribose (Rib), the sugar moiety of ATP, increases stress tolerance and alleviates symptoms in chronic heart failure [4], coronary artery disease [5] and hypertension [6].

As the mechanisms underlying the effects of these low-cost compounds are largely complementary, the simultaneous administration of Cr and Rib (Cr+Rib) is expected to confer additional advantages over those offered by Cr and Rib separately. However, despite the availability of commercial products, to our knowledge there is no mechanistic support for the potential advantages of such combination. Here, we test the hypothesis that supplementing ischemic cardiomyocytes with Cr+Rib interferes positively with the cell signaling pathways that enable protection against stress. To test such role, we focused into viability and apoptosis that are impaired by ischemia in cardiomyocytes. To provide mechanistic support, we will assess the impact of Cr+Rib on the signaling pathways and proteins that modulate those paradigms, including protein kinase B (Akt), mitogen-activated protein (MAP) kinases and adenosine monophosphate-activated protein kinase (AMPK).

## Materials and Methods

*Materials*

Creatine monohydrate and D-(-)ribose (Giellepi Chemicals, Milano, Italy) were dissolved in the cell culture medium, filtered (0.22 μm pore diameter, Nalgene) and stored at 4°C. All other reagents were of the maximal available purity degree. Mycoplasma-free H9c2 cardiomyocytes were obtained from American Type Culture Collection (Rockville, MD), maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin under a 5% $CO_2$ humidified atmosphere at 37°C. Cells were sub-cultured at a 1:3 ratio every 3 days in 25 $cm^2$ flasks. After reaching 80% confluence, sub-cultured cells underwent the various treatments. Four-to-six pools were used for each treatment. To mimic ischemia, cells were incubated for 24 h in glucose-free medium (Gibco, Invitrogen, #11966) under a 1% $O_2$, 5% $CO_2$ atmosphere.

*Viability tests*

Cells were seeded at $1.8x10^3$ cells per well in 96-well plates, incubated at 37°C for 24 h and then for additional 24 h with either saline or 0.5 μM doxorubicin (Sigma, St Louis, Mo) in the presence of varying concentrations of Cr and/or Rib. Viability was assessed by the 2-(4,5-dimethyltriazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma) assay (five replicates). Alternatively, trypsinized cells were mixed with trypan blue and counted under the light microscope to determine the percent of viable vs. control cells.

*Immunoblotting*

Cells were washed twice with ice-cold PBS and incubated for 30 min with lysis buffer (50 mM TRIS-HCl pH 7.4, 250 mM NaCl, 1% Triton X-100, 50 mM β glycerol-phosphate, 1 mM $Na_3VO_4$, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, Sigma, St. Louis, Mo) and 2% Protease inhibitor cocktail (Complete EDTA-free, Roche Diagnostics, Germany). Cells were handled in a hypoxic chamber [7]. The lysate was frozen at –20°C for 20 min, thawed at room temperature, centrifuged (14000 rpm for 15 min at 4°C) and the supernatant recovered. Protein concentration was determined by the Coomassie Plus Protein Assay (Pierce). Forty 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 for 1 h with 5% nonfat dry milk in TRIS-buffered saline containing 0.1% Tween. The membranes were incubated overnight at 4°C with the primary antibody followed by horseradish peroxidase-conjugated secondary antibody (1 h, room temperature). The following antibodies and dilutions were used: rabbit polyclonal anti-phospho-Akt ($Ser^{473}$, Cell Signaling, 1:1000), rabbit polyclonal anti-Akt (Cell Signaling, 1:1000), rabbit polyclonal anti-β-actin (Cell Signaling, 1:1000), rabbit anti-AMPKα1/2 (Santa Cruz, 1:1000) and anti-phospho-AMPK ($Thr^{172}$, Santa Cruz 1:500), rabbit polyclonal anti-caspase-3 (Cell Signaling, 1:1000), rabbit polyclonal anti-PARP (Santa Cruz, 1:500), mouse monoclonal anti-phospho-ERK1/2 (Santa Cruz, 1:250), rabbit polyclonal anti-ERK1 (Santa Cruz, 1:500), mouse monoclonal anti-phospho-JNK (Santa Cruz, 1:250), mouse monoclonal anti-JNK (Santa Cruz, 1:250), mouse monoclonal anti-phospho-p38 (Santa Cruz, 1:250), mouse monoclonal anti-p38 (Santa Cruz, 1:250). 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). Actin was used as loading control. 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).

*In Situ TdT assay for detection of apoptosis*

Trypsinized cells were washed in 2 ml cold PBS and centrifuged 5 min at 1000 rpm at 4° C. The pellet was resuspended in 500 μl of 4% cold buffered formalin for 30 min, washed in PBS, centrifuged (1000 rpm, 5 min, 4°C), resuspended in 50 μl PBS and stratified on slides (Superfrost Plus-Menzel Gmbh and CoKG, Braunschweg, Germany). After drying at room temperature, slides were maintained at –20°C till use. 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). Nuclei were stained with the karyophilic dye Hoechst 33258 (250 ng/ml, Sigma, St. Louis, Mo) for 3 min at

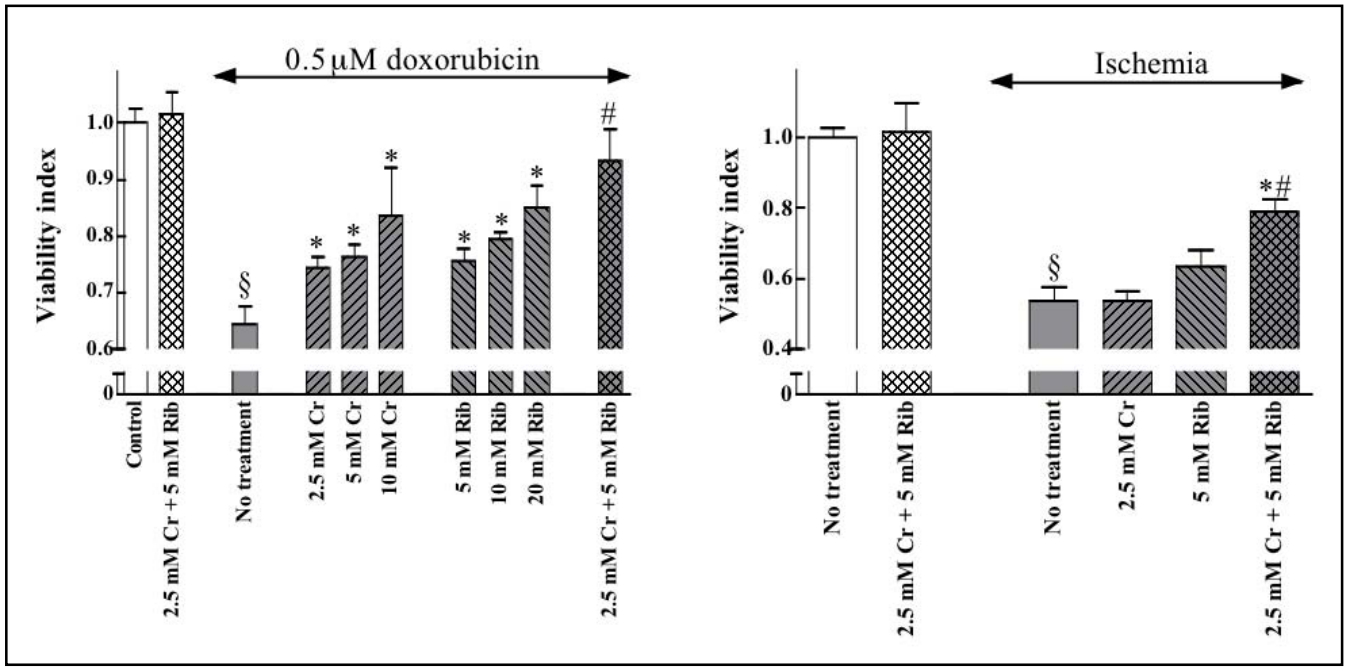


**Fig. 1.** Cell viability in challenged H9c2 cardiomyocytes treated with creatine, ribose or creatine+ribose. *(A)* Viability (MTT assay) of H9c2 cardiomyocytes challenged with 0.5 µM doxorubicin at varying creatine (Cr), ribose (Rib) and Cr+Rib. Data normalized with respect to untreated non-challenged cardiomyocytes at time zero and expressed as mean ± SEM (n=6). ANOVA P<0.0001. §, P<0.001 vs. control; *, P<0.01 vs. doxorubicin with no treatment; #, P<0.05 vs. doxorubicin-challenged cells treated with either 2.5 mM Cr or 5 mM Rib (Bonferroni post test). *(B)* Viability (Trypan blue test) in ischemic cardiomyocytes with no treatment, 2.5 mM Cr, 5 mM Rib, or both. Data normalized with respect to untreated non-challenged cardiomyocytes at time zero and expressed as mean ± SEM (n=6). ANOVA P<0.0001. §, P<0.001 vs. control; *, P<0.01 vs. ischemia with no treatment; #, P<0.05 vs. ischemia-challenged cells treated with either 2.5 mM Cr or 5 mM Rib (Bonferroni post test).

room temperature in the dark, followed by rinsing twice in PBS and coverslipping. Slides were examined using a filter for Hoechst staining (filter set 02, excitation band pass 365 nm, emission low-pass 420 nm). Images were acquired by a digital camera (DS-2MV; Nikon, Tokyo, Japan) and the number of TdT-labeled nuclei counted (8-10 random fields in a blinded procedure). Results are expressed as number of TdT-labeled nuclei/total nuclei.

*Statistics*

All data are mean ± SEM (n=4-6 for each treatment). 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

*Cr+Rib (but not Cr or Rib), improves viability in ischemic H9c2 cardiomyocytes*

The optimal dose of Cr and Rib was set by challenging H9c2 cardiomyocytes for 24 h with 0.5 µM doxorubicin and measuring cell viability (Fig. 1, left panel). Doxorubicin reduced viability by 35% (P<0.001). Administration of either Cr or Rib improved it in a dose-dependent manner, but when 2.5 mM Cr and 5 mM Rib were administered together (Cr+Rib), viability was greater than with either 2.5 mM Cr or 5 mM Rib, without effects in non-challenged cardiomyocytes.

Ischemia was induced by incubation for 24 h under 1% $O_2$ in a glucose-free medium. The viability of ischemic H9c2 cardiomyocytes (Trypan blue staining test) decreased by 50±4% with respect to control (Fig. 1, right panel). Whereas administration of either 2.5 mM Cr or 5 mM Rib did not improve viability (P=NS), administration of 2.5 mM Cr + 5 mM Rib (Cr+Rib) improved viability by 30±3% (P<0.01). When non-ischemic cardiomyocytes were treated with Cr+Rib, no change was observed (P=NS).

*Cr+Rib reduces ischemia-induced apoptosis*

The apoptotic potential was evaluated by merging the immunofluorescence images obtained after labeling DNA strands with TdT (red) with those obtained after labeling nuclei with Hoechst 33258 (blue). Co-localization of the two dyes yields a purple signal that enables

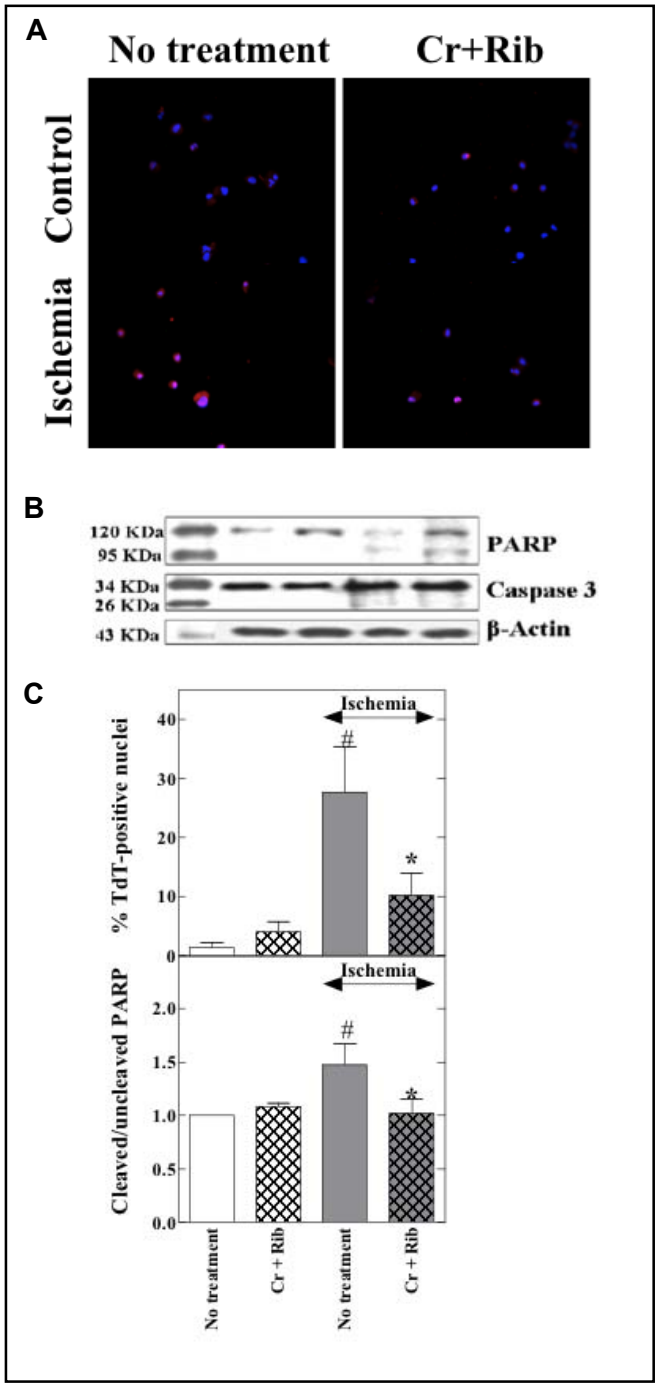


**Fig. 2.** Apoptosis in ischemic H9c2 cardiomyocytes treated with Cr+Rib. *(A)* Representative images showing TdT-positive nuclei labeled with rhodamine (red). Nuclei were counterstained with the karyophilic dye Hoechst 33258 (blue). *(B)* Western blots showing activation of caspase-3 and cleavage of PARP. *(C)* Average number of TdT-positive nuclei expressed as percentage of all nuclei present (top panel). ANOVA $P<0.01$; *, $P<0.05$ vs. untreated ischemic cells; #, $P<0.01$ vs. untreated non-ischemic cells (Bonferroni post test). Densitometry ratio of cleaved/uncleaved PARP (bottom panel). ANOVA $P<0.05$; *, $P<0.05$ vs. untreated ischemic cells; #, $P<0.05$ vs. untreated non-ischemic cells (Bonferroni post test).

identifying apoptotic nuclei. Virtually nil in non-ischemic cells regardless of Cr+Rib (Fig. 2A), TdT-positive nuclei increased to 27±7% ($P<0.001$) of total nuclei during ischemia. Cr+Rib reduced this number to 11±4% ($P<0.05$), indicating a marked anti-ischemic effect.

In support of TdT data, we measured caspase-3 cleavage. It was absent in treated and untreated non-ischemic cells, but became evident in ischemic cells (Fig. 2B), with Cr+Rib reducing consistently cleavage. Likewise, poly (ADP-ribose) polymerase (PARP) cleavage was absent in treated and untreated non-ischemic cells, but became evident in ischemic cells, with Cr+Rib reducing consistently such cleavage. The ratio between the 85 kDa and the 116 kDa fragments was unaffected by Cr+Rib in non-ischemicg cells, but was markedly reduced ($P<0.05$) in ischemic cells.

*Cr+Rib increases Akt phosphorylation but blunts the ischemia-induced increase in phosphorylated AMPK*

As the Akt pathway might modulate negatively apoptosis, we examined $Ser^{473}$ phosphorylation of Akt (Fig. 3). Ischemia alone affected neither Akt phosphorylation, nor Akt expression, but Cr+Rib increased Akt phosphorylation, at constant Akt protein expression, thereby increasing the phospho-Akt/Akt ratio ($P<0.05$). This effect was absent in non-ischemic cardiomyocytes.

To assess the role for improved bioenergetics, we measured the level of $Thr^{172}$ phospho-AMPK, a marker of the intracellular energy level that reflects impaired AMP-to-ATP conversion rate (Fig. 3). Ischemia increased the phospho-AMPK/AMPK ratio ($P<0.05$). Cr+Rib decreased this ratio to a value similar to that found in control non-ischemic cardiomyocytes ($P<0.05$). The phospho-AMPK/AMPK ratio was unaffected by Cr+Rib in non-ischemic cardiomyocytes (P=NS).

*Cr+Rib differentially modulates MAP kinases signaling*

MAP kinases play key roles in the control of apoptosis. Fig. 4 shows typical immunoblots of JNK, ERK and p38 proteins in both the phosphorylated and total protein forms. Because the total protein isoform of all MAP kinases was unaffected by the treatments, the changes in the phosphorylation rate reflect into altered phosphorylated/total protein ratios. Although slightly decreased (P=NS) for the phospho-JNK/JNK ratio, the

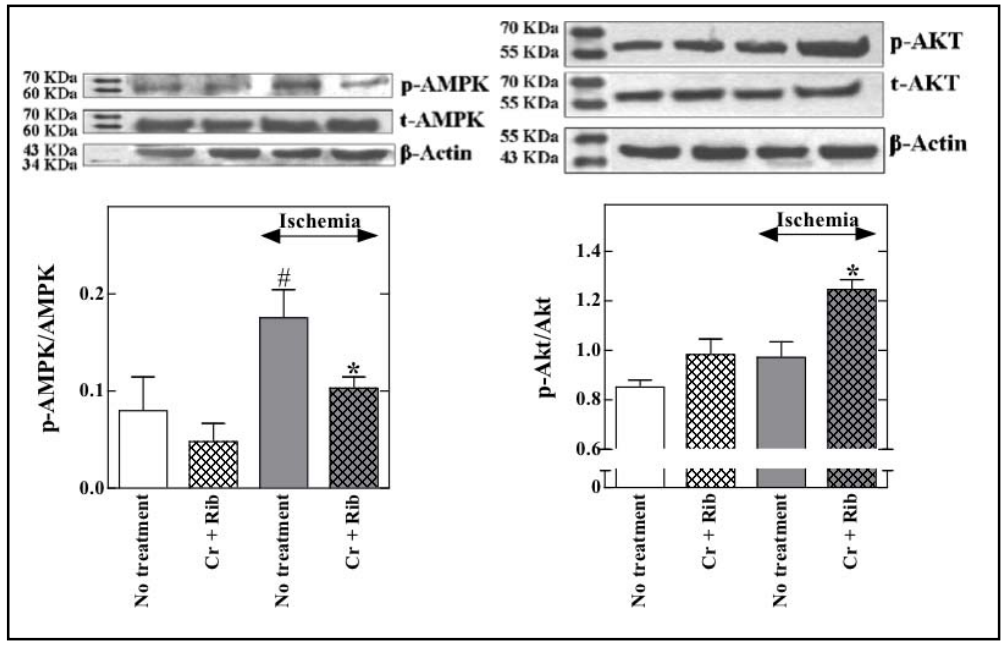


**Fig. 3.** AMPK and Akt in ischemic H9c2 cardiomyocytes treated with Cr+Rib. Representative Western blots with the expression of phosphorylated (p-)AMPK, total (t-)AMPK and β-actin (left), and p-Akt, t-Akt and β-actin (right). The plots report the quantification of the p-Akt/Akt and p-AMPK/AMPK ratio (mean±SEM, n=4) determined by blot densitometry. ANOVA P=0.002 and P=0.02, respectively. *, P<0.05 vs. untreated ischemic cells; #, P<0.05 vs. untreated non-ischemic cells (Bonferroni post test).

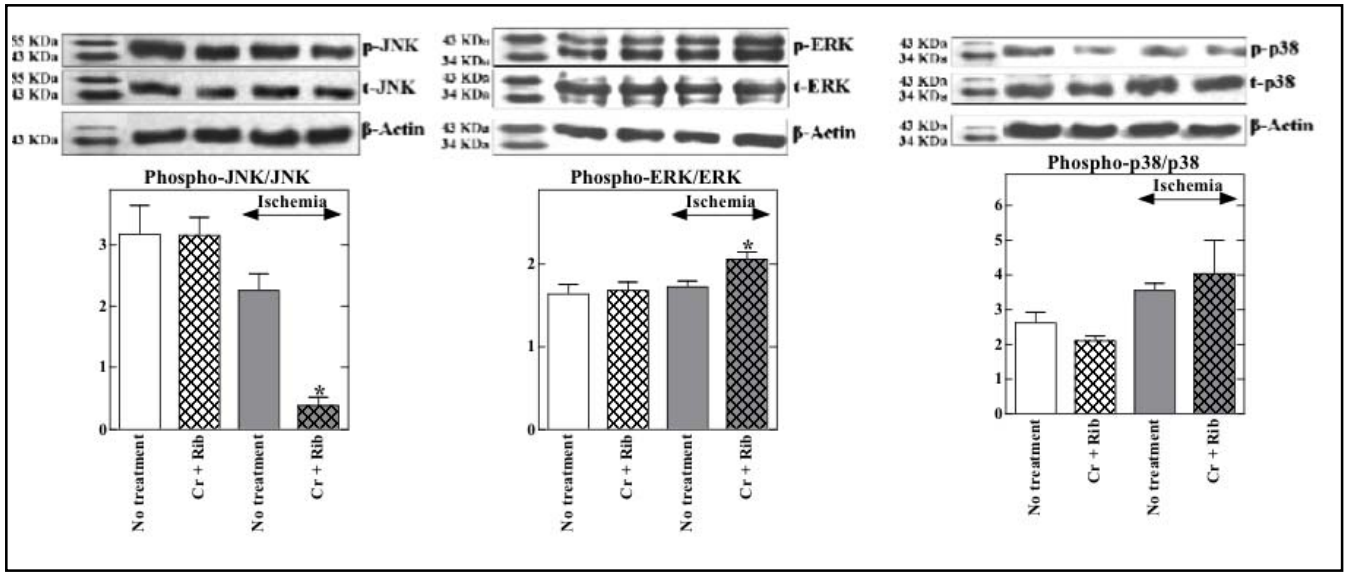


**Fig. 4.** Mitogen-activated kinases in ischemic H9c2 cardiomyocytes treated with Cr+Rib. Representative Western blots with the expression of the phosphorylated and total isoforms of JNK, ERK and p38, as well as β-actin as loading control. The plots report the quantification of the phosphorylated/total protein isoforms ratio (mean±SEM, n=4) determined by blot densitometry. ANOVA P<0.05, 0.0005 for ERK and JNK, respectively. *, P<0.05 vs. untreated ischemic cell.

phosphorylation rate of MAP kinases was not affected by ischemia. However, Cr+Rib markedly decreased the phospho-JNK/JNK ratio (P<0.01) during ischemia, whereas the phospho-ERK/ERK ratio was increased (P<0.05). The phospho-p38/p38 ratio remained unaffected (P=NS).

## Discussion

Ischemia (reduced $O_2$ and glucose supply) challenged the viability of H9c2 cardiomyocytes and accelerated apoptosis. Administration of Cr+Rib, but not either Cr or Rib, counteracted apoptosis and contributed

to restore cell viability in ischemic cardiomyocytes. The underlying mechanisms involve fine-tuning of the MAP kinases. A common denominator in these pathways, Cr+Rib-dependent activation of Akt and down-regulation of AMPK orchestrated this complex array of protective mechanisms.

*Creatine and ribose*

The favorable metabolic effects of either Cr or Rib have been studied extensively. Cr is quickly accumulated into cardiomyocytes through an insulin-dependent $Na^+$-Cr co-transporter [8]. In the cell, Cr equilibrates with ATP, forming phospho-creatine and ADP within its dual function of energy store and energy shuttle [9]. Presently under consideration to increase muscle fiber size, strength and performance [10, 11], Cr has anti-ischemic effects as well. For example, Cr inhibits apoptosis by reducing caspase-3 activation and cytochrome *c* release [12] and lowers cell $Ca^{++}$ by stimulating sarcoplasmic reticulum $Ca^{++}$-ATPase [13]. On the other hand, Rib does not affect muscle strength and performance [14], yet it accelerates ATP repletion in energy-depleted cardiomyocytes [15] and in muscle subjected to intense contraction [16, 17]. Furthermore, Rib pre-treatment before ischemia elevates the energy stores and delays the onset of irreversible injury [6]. The underlying mechanism involves enhanced nucleotide synthesis [18] and increased intracellular concentration of 5-phosphoribosyl-1-pyrophosphate, an essential precursor of ATP synthesis [19]. Thus, whereas Cr improves bioenergetics, Rib replenishes the building blocks for ATP synthesis. The superiority of Cr+Rib over either Cr or Rib is thus attributable to the complementarity of their effects. The dose selected for this study compares with 5 mM Cr, effective for neuron survival [20, 21] and 10 mM Rib, effective for energy repletion in colon carcinoma cells [22]. This dose reflects into decreased AMPK phosphorylation, an early marker of cell bioenergetics that is rapidly activated by $O_2$ and glucose deprivation [23].

*Apoptosis*

Apoptosis is considered a hallmark of ischemia-induced injury. Here, ischemia markedly increased the number of TdT-labelled nuclei as well as various markers of apoptosis, as caspase-3 activation and PARP cleavage [24]. All these parameters tended to return to control values in ischemic cardiomyocytes treated with Cr+Rib. Although the three major MAP kinases are believed to play significant roles in the regulation of apoptosis in cardiomyocytes, the role of JNK activity appeared preponderant here. The marked reduction of phospho-JNK by Cr+Rib is consistent with the protective effects of JNK inhibition observed in an *in vivo* model of ischemia/reperfusion [25]. In neonatal rat cardiomyocytes, ERK, JNK and p38 pathways are activated rapidly but transiently by ischemia, and their protein expression level return to basal levels within 1.5 h [26].

*The AMPK-Akt pathway*

ATP depletion is associated with an increase in AMP, elevated AMP/ATP ratio and AMPK activation, which serves as an integrator of metabolic responses to changes in energy availability [27], responding to glucose deprivation and ischemia in cardiomyocytes [28]. Although controversial [29], AMPK seems to lie upstream Akt in several signaling pathways [30-32], thus favoring the hypothesis that enhanced bioenergetics benefits the onset of protective mechanisms. For example, Akt stimulation triggers anti-apoptotic and survival pathways in pancreatic β cells challenged by glucose depletion and AMPK activation [33], a situation similar to that observed here. This is complicated by the observation that one of the paths activated by Akt includes prevention of AMPK phosphorylation through LKB1, an AMPK kinase abundantly expressed in cardiomyocytes [34]. Irrespectively of the mechanism, Akt phosphorylation at $Ser^{473}$, which is more crucial than that of $Thr^{308}$ for activation [35], might represent a feature for anti-apoptotic protection. A recognized mediator of cardiac protection against hypoxia and reoxygenation injury [36], phospho-Akt delays the onset of apoptosis by inducing ERK and inhibiting JNK and p38 [37], in agreement with the observations reported in this study. Sustained JNK activation promotes apoptosis by transcription-dependent and –independent mechanisms that are now being elucidated [38].

*Conclusion*

The treatment of ischemic cardiomyocytes with Cr+Rib confers additional advantages over either Cr or Rib in contrasting ischemia-induced apoptosis. The bioenergetic improvement led by Cr+Rib increases the cell signaling originating from Akt activation and involving MAP kinases. Further studies on this issue are warranted to assess the effectiveness of low-cost substances in the prevention of the symptoms associated to high-cost diseases as myocardial ischemia.

## Acknowledgements

We thank Dr. Roberta Maffei for her invaluable help in the setting of our experimental protocols. Contract grant sponsor: Italian Ministry of University and Research (PRIN 2007). Contract grant number: 12-1-5201001-211. This work was also supported in part by Giellepi Chemicals, Milan, Italy. AC performed part of her work as a fellow of the Istituto Nazionale delle Ricerche Cardiovascolari.

## References

1 Sharov V, Saks V, Kupriyanov V, Lakomkin V, Kapelko V, Steinschneider A, Javadov S: Protection of ischemic myiocardium by exogenus phosphocreatine. J Thorac Cardiovasc Surg 1987;94:749-761.

2 Constantin-Teodosiu D, Greenhaff PL, Gardiner SM, Randall MD, March JE, Bennett T: Attenuation by creatine of myocardial metabolic stress in brattleboro rats caused by chronic inhibition of nitric oxide synthase. Br J Pharmacol 1995;116:3288-3292.

3 Hamman B, Bittl J, Jacobus W, Allen P, Spencer R, Tian R, Ingwall J: Inhibition of the creatine kinase reaction decreases the contractile reserve of isolated rat hearts. Am J Physiol 1995;269:H1030-H1036.

4 Omran H, Illien S, MacCarter D, St Cyr J, Luderitz B: D-ribose improves diastolic function and quality of life in congestive heart failure patients: A prospective feasibility study. Eur J Heart Fail 2003;5:615-619.

5 Pliml W, von Arnim T, Stablein A, Hofmann H, Zimmer HG, Erdmann E: Effects of ribose on exercise-induced ischaemia in stable coronary artery disease. Lancet 1992;340:507-510.

6 Wallen WJ, Belanger MP, Wittnich C: Preischemic administration of ribose to delay the onset of irreversible ischemic injury and improve function: Studies in normal and hypertrophied hearts. Can J Physiol Pharmacol 2003;81:40-47.

7 Corno AF, Milano G, Samaja M, Tozzi P, von Segesser LK: Chronic hypoxia: A model for cyanotic congenital heart defects. J Thorac Cardiovasc Surg 2002;124:105-112.

8 Wallis J, Lygate CA, Fischer A, ten Hove M, Schneider JE, Sebag-Montefiore L, Dawson D, Hulbert K, Zhang W, Zhang MH, Watkins H, Clarke K, Neubauer S: Supranormal myocardial creatine and phosphocreatine concentrations lead to cardiac hypertrophy and heart failure: Insights from creatine transporter-overexpressing transgenic mice. Circulation 2005;112:3131-3139.

9 Kammermeier H: Why do cells need phosphocreatine and a phosphocreatine shuttle. J Mol Cell Cardiol 1987;19:115-118.

10 Andrews R, Greenhaff P, Curtis S, Perry A, Cowley AJ: The effect of dietary creatine supplementation on skeletal muscle metabolism in congestive heart failure. Eur Heart J 1998;19:617-622.

11 Gordon A, Hultman E, Kaijser L, Kristjansson S, Rolf C, Nyquist O, Sylven C: Creatine supplementation in chronic heart failure increases skeletal muscle creatine phosphate and muscle performance. Cardiovasc Res 1995;30:413-418.

12 Zhu S, Li M, Figueroa BE, Liu A, Stavrovskaya IG, Pasinelli P, Beal MF, Brown RH Jr, Kristal BS, Ferrante RJ, Friedlander RM: Prophylactic creatine administration mediates neuroprotection in cerebral ischemia in mice. J Neurosci 2004;24:5909-5912.

13 Pulido SM, Passaquin AC, Leijendekker WJ, Challet C, Wallimann T, Ruegg UT: Creatine supplementation improves intracellular $Ca^{2+}$ handling and survival in mdx skeletal muscle cells. FEBS Lett 1998;439:357-362.

14 Op't Eijnde B, Van Leemputte M, Brouns F, Van Der Vusse GJ, Labarque V, Ramaekers M, Van Schuylenberg R, Verbessem P, Wijnen H, Hespel P: No effects of oral ribose supplementation on repeated maximal exercise and *de novo* atp resynthesis. J Appl Physiol 2001;91:2275-2281.

15 Smolenski RT, Kalsi KK, Zych M, Kochan Z, Yacoub MH: Adenine/ribose supply increases adenosine production and protects atp pool in adenosine kinase-inhibited cardiac cells. J Mol Cell Cardiol 1998;30:673-683.

16 Zarzeczny R, Brault JJ, Abraham KA, Hancock CR, Terjung RL: Influence of ribose on adenine salvage after intense muscle contractions. J Appl Physiol 2001;91:1775-1781.

17 Hellsten Y, Skadhauge L, Bangsbo J: Effect of ribose supplementation on resynthesis of adenine nucleotides after intense intermittent training in humans. Am J Physiol Regul Integr Comp Physiol 2004;286:R182-188.

18 Brault JJ, Terjung RL: Purine salvage to adenine nucleotides in different skeletal muscle fiber types. J Appl Physiol 2001;91:231-238.

19 Zimmer HG: Significance of the 5-phosphoribosyl-1-pyrophosphate pool for cardiac purine and pyrimidine nucleotide synthesis: Studies with ribose, adenine, inosine, and orotic acid in rats. Cardiovasc Drugs Ther 1998;12:179-187.

20 Andres RH, Ducray AD, Perez-Bouza A, Schlattner U, Huber AW, Krebs SH, Seiler RW, Wallimann T, Widmer HR: Creatine supplementation improves dopaminergic cell survival and protects against $mpp^+$ toxicity in an organotypic tissue culture system. Cell Transplant 2005;14:537-550.
21 Ducray A, Kipfer S, Huber AW, Andres RH, Seiler RW, Schlattner U, Wallimann T, Widmer HR: Creatine and neurotrophin-4/5 promote survival of nitric oxide synthase-expressing interneurons in striatal cultures. Neurosci Lett 2006;395:57-62.
22 Giannecchini M, Matteucci M, Pesi R, Sgarrella F, Tozzi MG, Camici M: Uptake and utilization of nucleosides for energy repletion. Int J Biochem Cell Biol 2005;37:797-808.
23 Laderoute KR, Amin K, Calaoagan JM, Knapp M, Le T, Orduna J, Foretz M, Viollet B: 5'-amp-activated protein kinase (ampk) is induced by low-oxygen and glucose deprivation conditions found in solid-tumor microenvironments. Mol Cell Biol 2006;26:5336-5347.
24 Takemura G, Fujiwara H: Morphological aspects of apoptosis in heart diseases. J Cell Mol Med 2006;10:56-75.
25 Milano G, Morel S, Bonny C, Samaja M, von Segesser L, Nicod P, Vassalli G: A peptide inhibitor of c-jun nh2-terminal kinase (jnk) reduces myocardial ischemia/reperfusion injury and infarct size *in vivo*. Am J Physiol 2007;292:H1828-H1835.
26 Yue TL, Wang C, Gu JL, Ma XL, Kumar S, Lee JC, Feuerstein GZ, Thomas H, Maleeff B, Ohlstein EH: Inhibition of extracellular signal-regulated kinase enhances ischemia/reoxygenation-induced apoptosis in cultured cardiac myocytes and exaggerates reperfusion injury in isolated perfused heart. Circ Res 2000;86:692-699.
27 Gurusamy N, Das DK: Autophagy, redox signaling, and ventricular remodeling. Antioxid Redox Signal 2009;11:1975-1988.
28 Matsui Y, Takagi H, Qu X, Abdellatif M, Sakoda H, Asano T, Levine B, Sadoshima J: Distinct roles of autophagy in the heart during ischemia and reperfusion: Roles of amp-activated protein kinase and beclin 1 in mediating autophagy. Circ Res 2007;100:914-922.
29 Morrow VA, Foufelle F, Connell JM, Petrie JR, Gould GW, Salt IP: Direct activation of amp-activated protein kinase stimulates nitric-oxide synthesis in human aortic endothelial cells. J Biol Chem 2003;278:31629-31639.
30 Levine YC, Li GK, Michel T: Agonist-modulated regulation of amp-activated protein kinase (ampk) in endothelial cells. Evidence for an ampk -> rac1 -> akt -> endothelial nitric-oxide synthase pathway. J Biol Chem 2007;282:20351-20364.
31 Nagata D, Mogi M, Walsh K: Amp-activated protein kinase (ampk) signaling in endothelial cells is essential for angiogenesis in response to hypoxic stress. J Biol Chem 2003;278:31000-31006.
32 Ouchi N, Kobayashi H, Kihara S, Kumada M, Sato K, Inoue T, Funahashi T, Walsh K: Adiponectin stimulates angiogenesis by promoting cross-talk between amp-activated protein kinase and akt signaling in endothelial cells. J Biol Chem 2004;279:1304-1309.
33 Cai Y, Wang Q, Ling Z, Pipeleers D, McDermott P, Pende M, Heimberg H, Van de Casteele M: Akt activation protects pancreatic beta cells from ampk-mediated death through stimulation of mtor. Biochem Pharmacol 2008;75:1981-1993.
34 Soltys CL, Kovacic S, Dyck JR: Activation of cardiac amp-activated protein kinase by lkb1 expression or chemical hypoxia is blunted by increased akt activity. Am J Physiol Heart Circ Physiol 2006;290:H2472-H2479.
35 Hanada M, Feng J, Hemmings BA: Structure, regulation and function of pkb/akt—a major therapeutic target. Biochim Biophys Acta 2004;1697:3-16.
36 Pachori AS, Smith A, McDonald P, Zhang L, Dzau VJ, Melo LG: Heme-oxygenase-1-induced protection against hypoxia/reoxygenation is dependent on biliverdin reductase and its interaction with pi3k/akt pathway. J Mol Cell Cardiol 2007;43:580-592.
37 Mukherjee S, Lekli I, Das M, Azzi A, Das DK: Cardioprotection with alpha-tocopheryl phosphate: Amelioration of myocardial ischemia reperfusion injury is linked with its ability to generate a survival signal through akt activation. Biochim Biophys Acta 2008;1782:498-503.
38 Aikin R, Maysinger D, Rosenberg L: Cross-talk between phosphatidylinositol 3-kinase/akt and c-jun nh2-terminal kinase mediates survival of isolated human islets. Endocrinology 2004;145:4522-4531.