Insulin protects cardiomyocytes against reoxygenation-induced hypercontracture by a survival pathway targeting SR Ca\textsuperscript{2+} storage

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Abstract

Objective: Experimental and clinical studies have shown that administration of insulin during reperfusion is cardioprotective, but the underlying mechanisms are still unknown. In this study, we investigated in isolated rat cardiomyocytes subjected to hypoxia and reoxygenation whether administration of insulin during reoxygenation reduces reoxygenation-induced hypercontracture, a hallmark of acute reperfusion injury. The effects of insulin on potential pro-survival kinases, i.e., PI 3-kinase, NO synthase (eNOS), and cGMP-dependent protein kinase (PKG), and on cytosolic Ca\textsuperscript{2+} control in reoxygenated cardiomyocytes were investigated.

Results: Administration of insulin (10mU/L) during reoxygenation protected cardiomyocytes against hypercontracture development (cell length as % of end-hypoxic length: control 61.6±3.2; insulin 76.3±2.9*; n=26; *p<0.05 vs. control). Cytosolic [Ca\textsuperscript{2+}] recovery during the first 2min of reoxygenation was accelerated (fura-2 ratio after 2min of reoxygenation; control 1.01±0.05; insulin 0.79±0.04*; n=26; *p<0.05 vs. control). The beneficial effects of insulin on cytosolic [Ca\textsuperscript{2+}] recovery and hypercontracture were suppressed in the presence of inhibitors of PI 3-kinase (LY294002, 1\textmu M), eNOS (L-NMMA, 100\textmu M), PKG (KT 5823, 1\textmu M), or sarcoplasmic reticulum Ca\textsuperscript{2+} pump (SERCA) (thapsigargin, 150nM). Insulin increased phosphorylation and activity of eNOS and augmented phospholamban phosphorylation in reoxygenated cardiomyocytes. Correlated with phospholamban phosphorylation, insulin also augmented SR Ca\textsuperscript{2+} load.

Conclusions: Insulin protects cardiomyocytes against reoxygenation-induced hypercontracture. This is due to acceleration of cytosolic [Ca\textsuperscript{2+}] recovery by enhanced Ca\textsuperscript{2+} sequestration into the sarcoplasmic reticulum via SERCA activation. This protective mechanism is activated through the survival pathway consisting of PI 3-kinase, eNOS, and PKG.

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1. Introduction

Even though early reperfusion of ischemic myocardium presents the most effective intervention to restore cardiac function in case of acute myocardial infarction, reperfusion per se can also contribute to development of lethal myocardial cell damage, a phenomenon termed “reperfusion injury” [1,2]. Reperfusion injury of the cardiomyocytes is initiated within the first minutes of reperfusion. Therefore, any intervention aiming at protection against this form of injury must be applied immediately upon the onset of reperfusion. Several experimental and clinical studies have investigated if insulin administered at the time of reperfusion protects against myocardial reperfusion injury. This protective strategy goes back to a treatment scheme for acute myocardial infarction of the 1960s [3]. It was previously found that patients with acute myocardial infarction have a

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reduced in-hospital mortality risk when treated early with a Glucose–Insulin–Potassium scheme (GIK) followed by effective primary angioplasty [4]. Another recent clinical trial, however, found only a “neutral” effect of GIK treatment on mortality, cardiac arrest, and cardiogenic shock in patients with acute ST-segment elevation myocardial infarction [5]. The differences among the reported clinical effects are yet unexplained.

The underlying mechanism of this potentially beneficial therapy cannot be elucidated from the clinical studies carried out to date. From experimental studies, there is accumulating evidence, however, that insulin provides myocardial protection by mechanisms acting at the very beginning of reperfusion [6], i.e., that it is protective against acute reperfusion injury. Experimental results indicate that the achieved protection is not dependent on a systemic metabolic effect since administration of insulin alone reduces cell death in isolated ischemic/reperfused hearts or in isolated cardiomyocytes [7,8]. The insulin-mediated protection in reperfused myocardium was found to be mediated through a cellular signalling mechanism involving the activation of PI 3-kinase, Akt, and NO synthase [7], a signalling pathway also identified for protection achieved by postconditioning, i.e., application of repetitive, brief periods of ischemia within the early phase of reperfusion [9]. At present, the mechanism by which activation of this pathway provides protection is unclear. Some studies have suggested that myocardial protection by insulin occurs by an anti-apoptotic mode of action [7,8].

The development of hypercontracture of cardiomyocytes is an acute cause of reperfusion injury, resulting in the characteristic histological pattern of contraction band necrosis. The importance of this pathological mechanism in vivo was demonstrated by the finding that temporary inhibition of the contractile apparatus in reperfused pig hearts with 2,3-butanedione monoxime reduces infarct size and occurrence of contraction band necrosis [10]. The underlying mechanisms of this form of injury have been identified predominantly on the cellular level [11–14]. In model studies with isolated adult cardiac myocytes, it was shown that hypercontracture developing in the early minutes of reoxygenation after preceding hypoxia is due to an excessive and abrupt activation of the contractile machinery. This hypercontracture results from resumption of ATP synthesis at still elevated cytosolic calcium levels.

Essential triggers for reperfusion injury can be studied in great detail in isolated cells when the cells are first subjected to hypoxia with medium acidosis and then reoxygenated at physiological pH. We have recently shown on the cellular level that a rapid clearing of calcium from the cytosol at the beginning of reoxygenation can effectively reduce the development of hypercontracture [12]. If this does not occur, the excessive cytosolic calcium is rapidly shifted within the re-energized myocardial cells between cytosol and sarcoplasmic reticulum (SR). This leads to repetitive, high cytosolic calcium peaks that cause an uncontrolled contractile activation of the myofibrils and, hence, hypercontracture [11,14]. Several studies have demonstrated that interventions that attenuate these oscillatory Ca\(^{2+}\) elevations or reduce the Ca\(^{2+}\) sensitivity of the myofibrils in the first minutes of reoxygenation reduce hypercontracture development in reoxygenated isolated cells and infarct size in reperfused hearts in vivo [10,14].

The aim of the present study was to investigate on the cellular level in isolated cardiomyocytes whether administration of insulin at the time of reperfusion interferes with the described triggering mechanism of reoxygenation-induced hypercontracture, and if so, whether this is due to the described signalling mechanism that involves PI 3-kinase and NO synthase. Since activation of cGMP-dependent protein kinase (PKG) represents a downstream signal of NO synthase and since we had shown recently that activation of the PKG pathway confers protection against reperfusion-induced hypercontracture by a mechanism targeting the sequestration of cytosolic Ca\(^{2+}\) into the SR through an activation of the Ca\(^{2+}\)-ATPase (SERCA), we tested the hypothesis that the same effector mechanism is also responsible for a protective effect of insulin against reperfusion-induced hypercontracture [12].

2. Material and methods

2.1. Isolation of cardiomyocytes

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23 1985). Ventricular cardiomyocytes were isolated from 200–250 g adult male Wistar rats as described in detail previously [15].

2.2. Experimental protocols

2.2.1. Media

The normoxic buffer contained (mmol/L): 125.0NaCl, 2.6KCl, 1.2KH\(_2\)PO\(_4\), 1.2MgSO\(_4\), 1.0CaCl\(_2\), 25.0 HEPES and 2.5 glucose. Normoxic buffer was adjusted to pH\(_0\) 7.4. For hypoxia, the buffer was adjusted to pH\(_0\) 6.4; glucose was omitted and the buffer was gassed with 100% N\(_2\). The p\(_{O_2}\) value in the hypoxic medium was less than 22μPa.

2.2.2. Hypoxia–reoxygenation experiments

Cover slips with cardiomyocytes were introduced into a gas-tight, temperature-controlled (37°C), transparent perfusion chamber positioned in the light path of an inverted microscope. For each experiment and condition, a field containing 4–6rod-shaped cells was chosen for the whole set of hypoxia–reoxygenation experiments. Cell length, cytosolic [Ca\(^{2+}\)], or NO production of these cells were measured simultaneously during the whole experiment. After a 10-min perfusion with normoxic buffer, cardiomyo-
cytes were superfused with the hypoxic buffer (0.5 ml/min) for 60 min and then reperfused for 15 min with normoxic buffer in the presence of insulin (10 mU/L), L-NMMA (100 μmol/L), LY 294002 (1 μmol/L), thapsigargin (150 nmol/L), or KT 5823 (1 μmol/L). Caffeine (10 mM) was applied after 20 min reoxygenation.

2.2.3. Ca2+, NO, and cell length measurements

To measure cytosolic [Ca2+], cardiomyocytes were loaded at 37°C with 5 μM Fura-2 AM for 30 min and then washed for further 30 min as described in our previous studies [11–14]. To detect cytosolic NO, cells were loaded at 37°C with 5 μM DAF-FM diacetate for 45 min and then washed for 45 min. Alternating excitation of the fluorescent dye at wavelengths of 340/380 nm for Fura-2 and 470 nm for DAF was performed with a Video-Imaging-System (Till Photonics) adapted to the microscope.

2.2.4. Determination of phospholamban phosphorylation

Phospholamban phosphorylation was determined by Western blotting. Briefly, cardiomyocytes were treated with lysis buffer under normoxic conditions, at the end of 60 min hypoxia, and during 5 min reoxygenation under control conditions (R) in the presence of insulin (10 mU/L), insulin + LY 294002 (1 μM), insulin + L-NMMA (100 μM), or insulin + KT 5823 (1 μM).

Protein extracts from cardiomyocytes were prepared and loaded (30 μg) onto a 18.5% (wt/vol) SDS–polyacrylamide gel. After electrophoresis and blotting, sheets were incubated with a phospho-specific phospholamban antibody (anti-pPLB, alkaline phosphatase-labeled anti-rabbit IgG antibody, incubated with a phospholamban antibody (anti-PLB) or a phospho-specific phospholamban antibody (anti-pPLB, Ser16) (0.5 μg/ml) and then incubated for 2 h with an alkaline phosphatase-labeled anti-rabbit IgG antibody (0.5 μg/ml). Bands were visualized by alkaline phosphatase reactivity. Total phospholamban and phosphorylated phospholamban were quantified densitometrically.

2.2.5. Determination of eNOS phosphorylation by immunocytochemistry

At a given time-point of hypoxia/reoxygenation, cells were fixed with ice-cold methanol for 20 min, subsequently washed with phosphate-buffered saline (PBS), and then incubated for 1 h with a phospho-specific anti-eNOS antibody (rabbit, Ser1177). After incubation, cells were washed with PBS and incubated with a biotinylated anti-rabbit antibody for 30 min followed by extensive wash steps with PBS. Subsequently, cells were incubated overnight with Texas-red conjugated streptavidin (dilution 1:800). To quantify the fluorescence intensity, cells were imaged with a confocal laser scanning microscope (Zeiss).

3. Materials

The antibodies for p-eNOS (Ser1177), p-phospholamban (Ser16) and the second antibody (anti-rabbit IgG, biotin conjugated) were from Santa Cruz. Texas-red was from Rockland Immunochemical. All other chemicals were from Calbiochem or Sigma Aldrich.

3.1. Statistics

Data are given as mean values ± S.E.M. from n individual cells investigated in separate experiments. Statistical comparisons were performed by one-way ANOVA and use of the Student–Newman–Keuls test for post hoc analysis. Differences with p < 0.05 were regarded as statistically significant.

4. Results

4.1. Effect of insulin on cytosolic Ca2+ and cell length during reoxygenation

During simulated ischemia, cardiomyocytes shortened to about 70% of their initial length, due to a rigor-type mechanism caused by ATP depletion [16], and developed cytosolic Ca2+ overload. The fura-2 ratio increased from a pre-hypoxic value of 0.33 ± 0.01 to an end-hypoxic value of 1.29 ± 0.03 (n = 25), corresponding to intracellular Ca2+ concentrations of pre-hypoxic 85 ± 20 nmol/L and end-hypoxic 1.62 ± 0.09 μmol/L. During 15 min reoxygenation, cytosolic [Ca2+] recovered to the pre-hypoxic value. Cytosolic Ca2+ oscillations occurred concomitantly with the Ca2+ recovery, indicating temporary cytosolic Ca2+ overload. Fig. 1A shows a single-cell recording of the fura-2 ratio during hypoxia and reoxygenation under control conditions and in the presence of insulin (10 mU/L). Insulin accelerated the recovery of cytosolic Ca2+ and reduced the amplitude of Ca2+ oscillations during the first minutes of reoxygenation. The roles of PI 3-kinase, NO synthase and PKG were investigated as signalling steps potentially involved in insulin-mediated protection. Cytosolic Ca2+ recovery and cell length were analysed in the presence of appropriate inhibitors. Fig. 1B shows the fura-2 ratio at the second minute of reoxygenation after application of insulin in the presence or the absence of LY 294002 (1 μmol/L), L-NMMA (100 μmol/L), or KT 5823 (1 μmol/L). These inhibitors of PI 3-kinase, NO synthase or PKG abolished the acceleration of Ca2+ recovery achieved by insulin alone.

During the first minutes of reoxygenation, cells shortened to about 60% of their end-hypoxic length, i.e., they developed hypercontracture. Reoxygenation-induced hypercontracture was significantly attenuated when insulin was present during this period. Fig. 2 presents the cell length in % of end-hypoxic length after 10 min of reoxygenation under the same experimental conditions as investigated for Ca2+ recovery. With respect to cell shortening, the insulin-mediated protection was also abolished in presence of LY 290004, L-NMMA, or KT 5283. The results indicate that these signalling steps are
essential for the insulin-mediated protection against development of hypercontracture.

While L-NMMA and KT 5823 were used at concentrations known to be inhibitory, the effective concentration for LY 294002 had to be determined. The results show that a minimal concentration of 1 \( \mu \text{M} \) of the PI 3-kinase inhibitor, LY 294002, was required to reverse the insulin-mediated protection against development of hypercontracture (cell length as % of end-hypoxic length: control 61.6 \( \pm \) 3.2; insulin 76.3 \( \pm \) 2.9; insulin + LY 294002 (100 \( \mu \text{M} \)) 72.5 \( \pm \) 4.4; insulin + LY 294002 (1 \( \mu \text{M} \)) 63 \( \pm \) 3.7\(*)\); insulin + LY 294002 (10 \( \mu \text{M} \)) 58 \( \pm \) 3.4\(*\); \( n=26 \); \(*p<0.05 \) vs. insulin).

When applied alone to cells during reoxygenation, the inhibitors had no effect on cell length or cytosolic Ca\(^{2+}\) kinetics (cell length as % of end-hypoxic length reoxygenation: control 56.7 \( \pm \) 1.6; LY 294002 54.0 \( \pm \) 1.4\(*\); L-NMMA 55.8 \( \pm \) 2.0\(*\); KT 5823 55.2 \( \pm \) 3.6\(*\); \( n=10 \); a=not significant vs. control; fura-2 ratio after 2 min reoxygenation [a.u.]: control 1.05 \( \pm \) 0.03; LY 294002 1.13 \( \pm \) 0.03\(*\); L-NMMA 1.09 \( \pm \) 0.02\(*\); KT 5823 1.14 \( \pm \) 0.03\(*\); \( n=10 \); a=not significant vs. control). A multifactor analysis (two-way ANOVA) was also performed to test for interaction between insulin and inhibitor treatment. The result showed both for cytosolic [Ca\(^{2+}\)] and cell length that the insulin-mediated effects were only present in the absence of inhibitor (\( p<0.001 \), two-way ANOVA).

4.2. Signalling sequence

We analysed the phosphorylation of NO synthase in situ by immunofluorescence with or without the presence of the PI 3-kinase inhibitor LY 290004, since PI 3-kinase/Akt can activate NO synthase by increasing its phosphorylation. As shown in Fig. 3, insulin indeed increased the phosphorylation of NO synthase (eNOS) during the first 2 min of reoxygenation. Application of the PI 3-kinase inhibitor significantly abolished this insulin-mediated effect. With use of the fluorescence indicator DAF, we monitored generation of NO in reoxygenated cells as an activity assay for NO synthase in situ. Upon reoxygenation, an increase in DAF fluorescence was observed that was fully inhibited in the presence of L-NMMA. This rise during reoxygenation was significantly augmented in the presence of insulin. As shown in Fig. 4, insulin failed to augment NO production during reoxygenation when PI 3-kinase or NO synthase was inhibited. These data indicate that PI 3-kinase is an essential step in the pathway of insulin-mediated activation of NO synthase.
4.3. Effect of insulin signalling on SR function

In order to investigate whether the insulin-induced acceleration of Ca\(^{2+}\) recovery in reoxygenated cardiomyocytes is due to an effect on the SR, SERCA was inhibited with thapsigargin (Fig. 5). The presence of thapsigargin alone slowed the recovery of cytosolic [Ca\(^{2+}\)] in comparison to control conditions. This can be expected since, under these conditions, the SR can no longer serve as an intermediate store for intracellular Ca\(^{2+}\) sequestration. Of greater importance in the context of our experiments is the finding that in the presence of the SERCA inhibitor, insulin administration no longer has an effect on Ca\(^{2+}\) recovery, which indicates that this insulin effect is dependent on SERCA function.

As SERCA activity is dependent on the phosphorylation state of its regulatory protein phospholamban, the latter was analyzed during normoxia, hypoxia and reoxygenation in the presence or absence of insulin and inhibitors of the insulin-mediated signalling cascade. As shown in Fig. 6, phospholamban phosphorylation did not significantly change in hypoxia and reoxygenation under control conditions. Insulin treatment, however, significantly increased the phosphorylation of phospholamban in the first 5 min of reoxygenation. The increase in phospholamban phosphorylation under insulin treatment was attenuated in the presence of LY 290004, L-NMMA, or KT 5823.

Application of the inhibitors alone during reoxygenation did not significantly alter the phosphorylation state of phospholamban (pPLB after 5 min reoxygenation as %; control 100 ± 0; LY 290004 90 ± 19\(^{a}\); L-NMMA 85 ± 23\(^{a}\); KT 5823 83 ± 31\(^{a}\); n = 4, a = not significant vs. control). These results indicate that PI 3-kinase, NO synthase and PKG are involved.
Since increased phospholamban phosphorylation enhances the activity of SERCA and may thereby increase the amount of Ca\(^{2+}\) sequestered into the SR, we investigated the relative loading of the SR with Ca\(^{2+}\) at the end of reoxygenation by analysing the amount of Ca\(^{2+}\) released from the SR upon application of caffeine. We found that treatment of cells with insulin during 15 min of reoxygenation caused a threefold increase in the caffeine-releasable Ca\(^{2+}\) in the SR. As shown in Fig. 7, the caffeine-releasable Ca\(^{2+}\) was no longer elevated in insulin-treated cells when the PKG inhibitor was present. These data indicate that insulin-mediated acceleration of Ca\(^{2+}\) recovery is due to an augmentation of Ca\(^{2+}\) sequestration into the SR caused by activation of SERCA through PKG.

5. Discussion

The aim of the present study was to investigate on the cellular level whether administration of insulin at the time of reoxygenation interferes with reoxygenation-induced hypercontracture of cardiomyocytes and, if so, whether this is due to signalling through PI 3-kinase, NO synthase and PKG. Our data show, firstly, that protection provided by insulin against reoxygenation injury, observed previously in whole hearts, is indeed due to a direct effect on the cardiomyocytes. Secondly, the data also demonstrate for the first time that insulin exerts a rapid effect during this initial, vulnerable period of reoxygenation, and that this is an effect on the deleterious mechanism of reoxygenation-induced hypercontracture, i.e., a mechanism leading to rapid cell necrosis in the tissue. Thirdly, we identified Ca\(^{2+}\) sequestration by the SR as the target of insulin signalling via PI 3-kinase, NO synthase and PKG.

As shown previously, reoxygenation-induced hypercontracture is triggered by oscillatory elevations of cytosolic Ca\(^{2+}\) in the early phase of reoxygenation [10–12]. Rapid movement of Ca\(^{2+}\) between cytosol and SR causes these Ca\(^{2+}\) oscillations. This futile shifting of Ca\(^{2+}\) occurs because upon re-energisation of the cell, SERCA, with its large transport capacity for Ca\(^{2+}\), is activated much more rapidly than the energy-driven sarcolemmal extruders for cytosolic Ca\(^{2+}\). Interventions that reduce the frequency or amplitude of these SR-dependent Ca\(^{2+}\) oscillations also reduce the extent of reoxygenation-induced hypercontracture [12,14]. Based on these insights, we developed the hypothesis that the protective effect of insulin signalling may be due to a targeting of SR function as the end-effector of protection. The following findings support this hypothesis. (i) When insulin was present in the first minutes of reoxygenation, the initial drop of cytosolic Ca\(^{2+}\) concentration proceeds more rapidly than otherwise. It was shown before that this initial [Ca\(^{2+}\)] drop is mostly due to a rapid Ca\(^{2+}\) uptake by the SR [11–14]. In the present study, this was also seen in the slow-down of Ca\(^{2+}\) recovery in reoxygenated cells when thapsigargin was administered. (ii) When SERCA was inhibited by thapsigargin, the specific effect of insulin on
Ca\(^{2+}\) recovery was gone. (iii) The phosphorylation of the regulatory protein of SERCA, phospholamban, was increased in the presence of insulin. Since phospholamban phosphorylation causes an increase in SERCA activity, this finding again hints at SERCA activation in the presence of insulin [17]. (iv) The caffeine-releasable SR Ca\(^{2+}\) storage capacity was increased in reoxygenated cardiomyocytes exposed to insulin.

The signalling sequence leading to the insulin-induced rapid SR Ca\(^{2+}\) sequestration first involves activation of PI 3-kinase and NO synthase. When either was inhibited, the acceleration by insulin of the initial, SR-dependent phase of Ca\(^{2+}\) recovery was abolished. Inhibition of PI 3-kinase prevented the activation of NO synthase by insulin, which is characterised by an increase in eNOS phosphorylation and the increase in NO production. Intermediate between PI 3-kinase and NO synthase activation is likely to be an activation of Akt, as the latter is able to directly phosphorylate eNOS [18]. Downstream signalling of NO synthase involves PKG, the target of cGMP produced by an NO-activated guanylyl cyclase. When PKG was inhibited, the insulin-induced increase in phospholamban phosphorylation and in SR Ca\(^{2+}\) sequestration was no longer seen; however, the data do not show that phospholamban is the direct target of PKG. These latter findings are in agreement with those of a recent study in which we demonstrated the same mode of action for cGMP-mediated signalling in reoxygenated cardiomyocytes stimulated by ANF receptor agonists or cell-permeable PKG activators [12].

To summarise our data, we propose the following scheme as an explanation for the insulin-mediated protection against reoxygenation-induced hypercontracture. (i) Insulin activates NO synthase via the PI 3-kinase/Akt pathway. (ii) An activation of NO synthase leads to activation of PKG, which increases phosphorylation of phospholamban and consequently activates SERCA. (iii) An activated SERCA allows the SR to sequester larger amounts of cytosolic Ca\(^{2+}\). (iv) An augmented Ca\(^{2+}\) sequestration into the SR causes an accelerated recovery of cytosolic Ca\(^{2+}\) control and prevents excessive myofibrillar activation, e.g., hypercontracture. Thus, this study has identified the signalling mechanism by which insulin provides protection against acute reoxygenation-induced hypercontracture in cardiomyocytes.

Presently, the opening of mitochondrial permeability transition pores (MPTP) is also discussed as a key mechanism of myocardial reperfusion injury [19]. Our results do not rule out an interaction between the insulin-dependent survival pathway and MPTP, since the SR-dependent control of cytosolic [Ca\(^{2+}\)] in reoxygenated cells is likely to affect the probability of MPTP opening. Further studies are needed, therefore, to analyse whether MPTP opening in reoxygenated cardiomyocytes is secondary to SR-dependent cytosolic Ca\(^{2+}\) oscillations.

Some considerations on the limitation of the cellular experimental model as used here seem appropriate. Use of isolated cardiomyocytes permits identification of mechanisms specific to the muscular cell type, but one cannot gain insight into the role of other cells, e.g., vascular cells or leukocytes, in reperfused myocardium. In addition, simulated ischemia and reperfusion in isolated cardiomyocytes do not reflect the complete scenario of ischemia-reperfusion in the whole heart; however, previous studies have proven that several important triggers for reperfusion injury, such as changes in energy metabolism and cellular Ca\(^{2+}\) control, can be studied in great detail using this model. Finally, restriction of our investigations to the first minutes of reoxygenation is sufficient to study the causes of reoxygenation-induced hypercontracture but not of apoptosis. Therefore, our results do not exclude the possibility that insulin applied to reoxygenated cardiomyocytes exerts also a direct, anti-apoptotic effect.

The identification of PI 3-kinase/Akt-eNOS as a survival pathway is of clinical importance, since eNOS can be activated not only by insulin but also by other agents such as HMG-CoA reductase inhibitors (statins) and G-protein-coupled receptor ligands such as adenosine receptor agonists or bradykinin [20,21].

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