Modulation of Purinergic Receptors is Protective Against Hypoxia/Reoxygenation Injury in AC16 Cardiomyocytes

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It has been reported that extracellular ATP concentrations during postischemic reperfusion activate cardiomyocyte purinergic receptors thus modulating both cardiac function and survival. This study is purported to assess the effects of purinergic modulation on the viability of a adult human ventricular derived cell line (AC16 cardiomyocytes) viability after hypoxia/reoxygenation (H/R). Cultured AC16 cardiomyocytes were subjected to 5 hours of hypoxia in a hypoxic chamber or by mineral oil layering. During the 1 h reoxygenation cells received no additional intervention or were treated with increasing ATP doses (10mM, 100mM and 1 mM) in the presence vs. the absence of either 100mM or 300mM suramin (a purinergic inhibitor). Cell viability was evaluated by MTT colorimetric assay. Administration of 100mM ATP (but not with 10mM) induced a significant improvement of cellular relative viability (RV) in experiments performed in the hypoxic chamber, an effect that was completely inhibited by suramin. Treatment with 1mM ATP and 1mM ATP (but not with 10mM and 100mM), elicited a significant increase of cellular RV equally in cardiomyocytes belonging to the control group and the ones exposed to both H/R protocols, regardless the presence or the absence of the purinergic inhibitor (100mM or 300mM). In conclusion, purinergic stimulation elicits cardioprotection in the settings of H/R injury, the effect being dependent both on the ATP concentration and the type/severity of the hypoxic insult.

Keywords: hypoxia/reoxygenation, purinergic modulation, cardiomyocytes

According to the World Health Organization, coronary heart disease continues to be the leading cause of global mortality, ischemia/reperfusion (I/R) injury playing the key role in its pathogenesis. Although intracellular [1] and extracellular ATP concentrations are very low in physiological settings [2], during postischemic reperfusion, the necrotic and/or apoptotic cells in the ischemic area release in the extracellular space high concentrations of ATP and other nucleotides that act as signaling mollecules via the activation of two families of purinergic receptors: P_2X which are ligand-gated ion channels and P_2Y which are metabotropic G protein-coupled receptors [3]. Whereas P_2X receptors are mainly activated by ATP, the ones that belong to the P_2Y class are stimulated by ATP, UTP, ADP, UDP and UDP-glucose [4]. Almost all tissue types express P_2 receptors, including the heart. Purinergic signaling plays an important role in the regulation of cardiovascular functions, being implicated in vasoconstriction/vasodilation, development of smooth muscle cells and endothelial cells, angiogenesis, vascular remodelling, platelet aggregation, coagulation and inflammation [5]. Recent findings have proven the unequivocal role of purinergic modulation in cardioprotection. Indeed, ATP and UTP are able to induce a significant antiinflammatory effect via P_2Y receptors in the setting of myocardial ischemia/reperfusion [6] besides their capability to modulate the inotropic and chronotropic properties [7]. Moreover, P_2 receptors have also been reported to directly regulate the viability of cardiomyocytes [8]. Accordingly, the present study was aimed at assessing the effects of purinergic modulation on the viability of AC16 cardiomyocytes subjected to hypoxia/reoxygenation (H/R).

Experimental part

Material and methods

Reagents

ATP, suramin, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and the mineral oil were purchased from Sigma. Dulbecco's Phosphate Buffered Saline (DPBS) 10X free from Calcium and Magnesium was obtained from Dominique Dutscher. DPBS supplemented with calcium and magnesium, Dulbecco's Modified Eagle Medium (DMEM)/F-12 and DMEM without glucose were purchased from Gibco. Trypsin was bought from GE Healthcare Life Sciences.

Cell culture

AC16 cardiomyocytes, derived from adult human ventricular myocytes were maintained in a humidified incubator gassed with 5% CO_2 at 37°C. Cells were grown in DMEM/F-12 supplemented with 10% fetal bovine serum and 1% mixture of penicillin/streptomycin. Two

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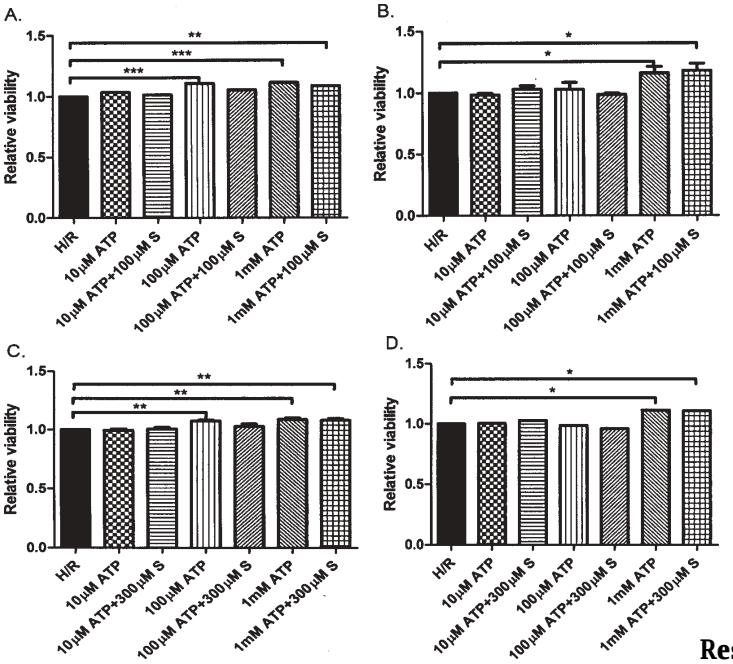

Fig. 1. Bar graphs showing the relative viability of hypoxia/reoxigenated cells treated with increasing concentrations of ATP and different concentrations of suramin: (A) Hypoxia induced via the hypoxic chamber + 100μM suramin; (B) Hypoxia induced via mineral oil layering + 100μM suramin; (C) Hypoxia induced via the hypoxic chamber + 300μM suramin; (D) Hypoxia induced via mineral oil layering + 300μM suramin (n = 4-5)

experimental groups were formed: control which received no hypoxic intervention and H/R which was subjected to hypoxia/reoxygenation. One day before the experiment, control and H/R cells were plated in 24-well plates using DMEM/F-12 and kept in the normoxic incubator.The induction of hypoxia was performed by replacing the DMEM/F-12 with DPBS supplemented with calcium and magnesium, followed by either: a) exposure to oxygen and nutrient deprivation in a modular incubator chamber (Invivo 200, Ruskinn Technology; 1% O_2, 5% CO_2, 94% N_2); or b) mineral oil layering, obtained by the addition of 600μL of mineral oil in each well, after which the cells were kept in the normoxic incubator. Hypoxia lasted for 5 h followed by 1 hof reoxygenation using DMEM, when, in accordance to the treatment the cells received, for each experimental group, 5 sub-groups were formed: 1) ∅ - untreated cells; 2) 10μM ATP - cells receiving 10μM ATP; 3) 10μM ATP+100μM S or 10μM ATP+300μM S, respectively - cells receiving 10μM ATP coupled with either 100μM or 300μM suramin; 4) 100μM ATP - cells receiving 100μM ATP; 5)100μM ATP+100μM S or 100μM ATP+300μM S, respectively - cells receiving 100μM ATP coupled with either 100μM or 300μM suramin; 6) 1mM ATP - cells receiving 1mM ATP; 7) 1mM ATP+100μM S or 1mM ATP+300μM S, respectively - cells receiving 1mM ATP coupled with either 100μM or 300μM suramin.

MTT assay

After 1 hour of reoxygenation for both the control and H/R groups DMEM was replaced by 500ìL MTT solution/well maintained at 37^{0}C and the cells were kept for 2 h in the normoxic incubator (as described by Mosmann, 1983). Then, the MTT was substituted with 400ìL DMSO/well, the plates wrapped in aluminium foil and left on a turning plate to ensure complete solubilization. After 45 min the absorbance was read at 540nm wavelength using the Biotek EL800 Absorbance Microplate Reader and the Microplate Data Collection & Analysis Software Gen 5.

Statistics

Cellular viability was expressed relative to the absorbance of the non-treated cells (relative viability – RV). Group comparisons were performed by one-way analysis of variance (ANOVA) and Dunnett's post-hoc multiple comparison test (GraphPad Prism version 5.0). Values for $p < 0.05$ were considered statistically significant.

Results and discussions

The major finding of this paper is that ATP elicited a dose-dependent increase in AC16 cardiomyocyte viability, an effect that is, at least partly, mediated via the P_2 receptors and dependent on the type/severity of the ischemic insult.

We firstly assessed the effects of 10μM, 100μM and 1mM ATP in cells subjected or not to hypoxia/reoxygenation, in the presence versus the absence of 100μM suramin (fig. 1A). The addition of 10μM ATP did not induce a significant change of RV in hypoxic cells and their corresponding controls, regardless the presence of suramin (100 μM). At variance, 100μM of ATP significantly increased ($P<0.001$) the RV of hypoxic cells, an effect that was completely inhibited by suramin. However, when applying an increased ATP concentration (1mM ATP) a significant enhancement of RV was observed in both hypoxic ($P<0.001$) and control cells ($P<0.01$); in the presence of suramin, this protective effect was diminished in the hypoxic cells ($P<0.01$) but not in the control ones (fig. 1A).

In order to evaluate whether these effects can be recapitulated using an alternative protocol, hypoxia was induced by mineral oil layering (fig. 1B). At variance from the above mentioned results, neither 10μM nor 100μM ATP elicited a significant change in the RV of controls or hypoxic cells as compared to the untreated groups, regardless the presence or the absence of suramin. However, the concentration of 1mM ATP increased cellular RV in both control ($P<0.01$) and hypoxic cells ($P<0.05$). The protective effect was partly blunted in controls ($P<0.05$) but not in the hypoxic group, in the presence of suramin. Since the administration of suramin (100μM) was not able to completely inhibit the cardioprotection elicited by 1mM ATP, a second set of experiments using a higher dose of the purinergic inhibitor (300μM) was performed in both hypoxic conditions. For the hypoxia/reoxygenation experiments using the hypoxic chamber, no different results were obtained in the presence of a higher suramin concentration vs. the lower one in the same hypoxic conditions, regardless the ATP concentration (fig. 1C). We further evaluated the effects of ATP alone versus co-administration of 300μM suramin in the case of hypoxia induced by mineral oil layering (fig. 1D). Similar results as in the case of 100μM suramin were recorded: no significant difference was noticed for the 10ìM and 100μM ATP concentrations, regardless the higher suramin concentration (300μM) while the 1mM ATP elicited a

significant improvement ($P<0.05$) of RV that was not affected by suramin.

Our data show that if hypoxia was induced using the hypoxic chamber, the concentration of 100μM ATP was sufficient to increase the viability of cells. This result is in agreement with the findings of Urban et al [10] who reported that administration of ATP is capable to prevent the ischemia-induced apoptosis of human cardiac endothelial cells via the activation of the P_2Y receptors. Similarly, short-term activation of P_2Y_2 and P_2Y_4 receptors by UTP has been reported to decrease cell death caused by hypoxia in neonatal rat cardiomyocytes [11]. These receptors, (especially P_2Y_2) are also activated by ATP, therefore presumably ATP would have the same beneficial effect.

The protective effect of ATP is clearly dependent on purinergic signaling, as 100μM or 300μM suramin is capable to completely suppress it. Interestingly, when ATP was applied in the highest concentration (1 mM) the effect was only partially influenced by suramin, an observation suggesting that high doses of ATP are beneficial when briefly administered during the postischemic reperfusion. Suramin is a broad-spectrum antagonist of the P_2 receptors, blocking P_2X_1, P_2X_2, P_2X_3, P_2X_5, P_2Y_2, P_2Y_{11} and to a lesser extent P_2Y_4 and P_2Y_6 [12]. We speculate that the lack of complete inhibition of these latter 2 receptors accounts, at least to some extent, for the persistence of the beneficial effect of high ATP in the presence of suramin.

Indeed, several studies have revealed that activation of P_2Y_4 [8] elicited cardioprotection and that purinergic signaling via this receptor, via P_2Y_6 receptor [13], and also via P_2X_4 receptor (not inhibited by suramin, [14]), increased myocardial contractility, a beneficial effect in heart failure after myocardial infarction. It has been postulated that the increased release of ATP and UTP upon post-ischemic reperfusion is aimed at improving myocardial contractility via purinergic signaling through the aforementioned receptors [15]. However, we have to mention as a limitation of our study that we have not measured the expression of these receptors in the human ventricular-derived AC16 cardiomyocyte line.

In the case of mineral oil layering-induced hypoxia, we observed cardioprotection only for the maximal ATP concentration (1mM) and this effect was less significant than for the experiments performed in the hypoxic chamber, suggesting that the amount of ATP required to maintain cellular viability is directly proportional to the severity of the ischemic insult. Finally, our results revealed that the effect of 1mM ATP on the relative viability of cardiomyocytes is not abolished by either 100μM or 300μM suramin. Thus, it is tempting to speculate that, when available in sufficient amounts, ATP is able to promote cell survival regardless the degree of inhibition of suramin-dependent purinergic receptors. As previously mentioned, this effect can be ascribed to a certain extent to the stimulation of P_2Y_4 (only partly inhibited by suramin) and P_2X_4 (not inhibited by suramin) receptors upon post-ischemic reoxygenation. We cannot exclude the fact that cardioprotection can be also attributed to the accumulation of adenosine as result of the rapid ATP degradation in the extracellular environment [16]. Indeed, it is a well established fact that adenosine, acting on the A_1 and A_{2A} [17] but also on the A_{2B} [18] receptors, elicits cardioprotection against myocardial ischemia/reperfusion, a hypothesis being worth further investigation.

Conclusions

In AC16 cardiomyocytes, purinergic stimulation elicits cardioprotection in the settings of H/R injury, the effect being dependent both on the ATP concentration and the type/severity of the hypoxic insult.

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