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Cardioprotective effects of adenosine A_1 and A_3 receptor activation during hypoxia in isolated rat cardiac myocytes

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Abstract

Adenosine (ADO) is a well-known regulator of a variety of physiological functions in the heart. In stress conditions, like hypoxia or ischemia, the concentration of adenosine in the extracellular fluid rises dramatically, mainly through the breakdown of ATP. The degradation of adenosine in the ischemic myocytes induced damage in these cells, but it may simultaneously exert protective effects in the heart by activation of the adenosine receptors. The contribution of ADO to stimulation of protective effects was reported in human and animal hearts, but not in rat hearts. The aim of this study was to evaluate the role of adenosine A_1 and A_3 receptors (A_1R and A_3R), in protection of isolated cardiac myocytes of newborn rats from ischemic injury. The hypoxic conditions were simulated by exposure of cultured rat cardiomyocytes (4–5 days *in vitro*), to an atmosphere of a N_2 (95%) and CO_2 (5%) mixture, in glucose-free medium for 90 min. The cardiotoxic and cardioprotective effects of ADO ligands were measured by the release of lactate dehydrogenase (LDH) into the medium. Morphological investigation includes immunohistochemistry, image analysis of living and fixed cells and electron microscopy were executed. Pretreatment with the adenosine deaminase considerably increased the hypoxic damage in the cardiomyocytes indicating the importance of extracellular adenosine. Blocking adenosine receptors with selective A_1 and A_3 receptor antagonists abolished the protective effects of adenosine. A_1R and A_3R activation during the hypoxic insult delays onset of irreversible cell injury and collapse of mitochondrial membrane potential as assessed using DASPMI fluorochrom. Cardioprotection induced by the A_1R agonist, CCPA, was abolished by an A_1R antagonist, DPCPX, and was not affected by an A_3R antagonist, MRS1523. Cardioprotection caused by the A_3R agonist, Cl-IB-MECA, was antagonized completely by MRS1523 and only partially by DPCPX. Activation of both A_1R and A_3R together was more efficient in protection against hypoxia than by each one alone. Our study indicates that activation of either A_1 or A_3 adenosine receptors in the rat can attenuate myocyte injury during hypoxia. Highly selective A_1R and A_3R agonists may have potential as cardioprotective agents against ischemia or heart surgery.

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Keywords

adenosine receptors; cardiomyocyte; cardioprotection; hypoxia; light and electron microscopy

Introduction

Adenosine (ADO) is a metabolite of adenine nucleotides, which has multiple physiological and pathological functions in numerous types of cells *in vitro* and *in vivo*. ADO exerts its effects via activation of four receptor subtypes that have been cloned and designated A_1, A_{2A}, A_{2B} and A_3 [1]. ADO is released during myocardial ischemia and hypoxia and is one of the plausible modulators in the protection of the ischemic myocardium [2]. The observation that a brief hypoxic period can increase the endurance against subsequent ischemic injury was termed ischemic preconditioning. Although the mechanism of preconditioning was elusive, there were various indications that activation of the ADO A_1 and/or A_3 receptors (A_1R, A_3R) was involved [3, 4]. The protection afforded by preconditioning was simulated by a brief prior exposure to nanomolar concentrations of the A_3R agonist in an isolated rabbit heart [5]. This phenomenon also occurs in chick ventricular myocytes [6, 7]. Stambaugh *et al.* [7] have shown that activation of A_1R and A_3R during hypoxia can protect against injury in cardiac ventricular myocytes cultured from chick embryos. In rats, the efficiency of A_3R agonists were studied in hypoxic conditions and controversy results were obtained. Lasley *et al.* [8], reported hemodynamic effects of A_3R agonists in isolated rat hearts, that were blocked by A_{2A}R antagonist and only partially by A_3R antagonist, and these effects were not found in the isolated rabbit heart. Moreover, Cave *et al.* [9] did not find cardioprotective effects mediated by adenosine receptors in rat hearts. On the contrary, Nojiri *et al.* [10] and Headrick [3] concluded that adenosine is of primary importance in mediating the cardioprotection in rat. We have shown recently that A_3R is expressed in rat's newborn cultured cardiac myocytes and its activation by high doses (≥10 μM) induce apoptosis [11, 12]. The well-documented species variability in A_3R activity, together with the several studies that proposed that A_3R are not expressed in the ventricular myocardium in adult rats [13, 14], directed us to this investigation. The aim of the present study was to establish an experimental model of acute hypoxic condition in isolated rat cardiac myocytes and to evaluate the role of A_1R and A_3R in mediating the adenosine-induced protection in cardiomyocytes at the cellular and ultrastructural levels.

Materials and methods

Cell culture

Hearts of 2–3 day-old rats were removed under sterile conditions and washed 3 times in phosphate buffered saline (PBS) to remove excess blood cells. The hearts were minced and then gently agitated in a solution of proteolytic enzymes, RDB (Biological Institute, Ness-Ziona, Israel), which was prepared from a fig tree extract. The RDB was diluted 1:100 in Ca^{2+} and Mg^{2+}-free PBS at 25°C for a few cycles of 10-min each, as described previously [12]. Dulbecco's modified Eagle's medium (DMEM) containing 10% horse serum (Biological Industries, Kibbutz Beit Haemek, Israel) was added to supernatant suspensions containing dissociated cells. The mixture was centrifuged at 300 g for 5 min. The

supernatant phase was discarded, and the cells were suspended again. The suspension of the cells was diluted to 1.0×10^6 cells/ml and 1.5 ml were placed in 35-mm plastic culture dishes on collagen/gelatin-coated coverglasses. The cultures were incubated in a humidified atmosphere of 5% CO_2, 95% air at 37°C. Confluent monolayer exhibiting spontaneous contractions were developed in culture within 2 days. Myocyte cultures were washed in serum-free medium BIO-MPM-1 (Kibbutz Beit Haemek, Israel) containing 5-mg/ml glucose and incubated in this medium for further 48 h. After 48 h in serum-free medium the experiments were performed.

Hypoxic conditions

Myocyte cultures were washed in serum and glucose-free medium (DMEM) before incubating with DMEM in the presence of ligands under hypoxic conditions. The cardiomyocyte cultures were exposed to hypoxia of varied duration. In the first set of experiments myocyte damage after 1 and 2 h hypoxia were compared in the presence or absence of adenosine deaminase (ADA). The second set of experiments was performed to test the influence of hypoxia for 75 min to cultured myocytes in the presence of adenosine A_1R and A_3R antagonists. Finally, in the third set of experiment the effects of adenosine A_1R and A_3R agonists were studied after 90-min hypoxia. Hypoxic conditions were carried out in an hypoxic incubator where O_2 was replaced by N_2 (95%) and CO_2 (5%) mixture, in glucose-free media. The hypoxic damage was characterized at the end of the hypoxic period by morphological and biochemical evaluations.

Experiments with A_1R and A_3R ligands

CCPA (A_1R agonist), Cl-IB-MECA (A_3R agonist), DPCPX (A_1R antagonist), 8-SPT (A_1R and A_3R antagonist) and MRS1523 (A_3R antagonist) at various concentrations were introduced to cell cultures 10-min prior to and during the hypoxic conditions. When both agonist and antagonist introduced to the medium, the cells were pre-treated first with the antagonist and after 10 min with the agonist. When the cells were treated with A_1 and A_3 agonists, both agonists were co-administered 10 min before hypoxia and as well during the hypoxia.

α-Sarcomeric actin staining

Cells on coverslips were stained for immunohistochemical demonstration of α-sarcomeric actin using mouse monoclonal anti-α-sarcomeric actin (C-5) and goat anti-mouse biotinylated immunoglobulin conjugated with extra avidin-peroxidase. The chromogen 3-amino-9-ethylcarbazole (AEC) was used as described previously [11, 12].

Transmission electron microscopy

The cells were fixed with 2.5% gluteraldehyde in 0.1 M sodium cacodylate for 1 h, postfixed in 1% osmium tetroxide in the same buffer for 1 h, en bloc [11]. The cultures were dehydrated in an ascending series of alcohols, infiltrated in Epon-Araldite epoxy resin, and heat polymerized. En face (with respect to the culture substratum) sections were cut on an LKB ultramicrotome, poststained with uranyl acetate (saturated solution) and lead citrate

[11], and examined in a JEOL-1200 transmission electron microscope at an operating voltage of 80 kV.

Enzyme release – lactate dehydrogense (LDH)

Protein content and lactate dehydrogenase activity were determined according to El-Ani *et al.* [15]. Briefly, 25 μl of the supernatant was transferred into 96-well dish and the LDH activities were determined using LDH-L kits (Sigma, St. Louis, MO, USA) as described by the manufacturer. The product of the enzyme was measured spectrometrically at a wavelength of 340 nm as described previously [16]. The results are expressed as a fold of the control in the same experiment. Experiments were done in 4–8 replicas each and were repeated at least 3 times.

Propidium iodide assay

The assay is based on vital binding of propidium iodide to nuclei of cells whose plasma membranes have become permeable due to cell damage. The assay was performed according to Nieminen *et al.* [17]. Cell number was evaluated by using a Scan-Array 2 Image Analyzer (Galai, Israel). The analyzer consisted of an Axiovert 135TV microscope (Zeiss, Germany) and a black and white Sony camera interfaced to an image analysis computer [12].

Examination of mitochondria in living cells

The method was according to Shneyvays *et al.* [12]. The accumulation of DASPMI dye in the mitochondrial matrix space is known to be dependent on the presence of high membrane potential of the mitochondrial inner membrane. Living cells grown on round coverslips were exposed to DASPMI, dissolved in PBS (at a final concentration 10 μg/ml) for 15 min. Then, the coverslips were washed and mounted on chambers containing dye-free medium. DASPMI fluorescence was excited at 460 nm, and the emission wavelength was 540 nm.

Chemicals

The highly selective ADO A_3 agonist, Cl-IB-MECA, was gift from the National Institute of Mental Health Chemical Synthesis and Drug Supply Program. The selective ADO A_3 antagonist MRS1523 was synthesized as described by Li *et al.* [18]. DASPMI (4-Di-1-ASP, D-288) was acquired from Molecular Probes Inc., and other reagents were purchased from Sigma Chemical.

Statistical analysis

Results are expressed as mean ± S.E.M. Data were analyzed by analysis of variance (ANOVA) with application of a *post-hoc* Tukey-Kramer test. $P < 0.05$ was accepted as indicating statistical significance.

Results

To simulate ischemic conditions, the cardiomyocyte cultures were exposed to hypoxic periods of varied duration. In our system, a 2-h hypoxic exposure, but not a 1-h exposure, was sufficient to cause injury to the myocytes, as measured by morphological and biochemical criteria (LDH release). Exposure of similar cultures to the same conditions in

the presence of adenosine deaminase (ADA) caused toxic effects even within 1-h of hypoxia (Fig. 1). These results may indicate the release of endogenous adenosine during hypoxic conditions, which protects the myocytes only during short-term hypoxic exposure (1 h). Preliminary results indicated that in our system, a 90-min hypoxic period is the appropriate duration for studying the effect of adenosine receptor ligands (data not shown). The effects of A_1R and A_3R activation on LDH release from cardiac myocytes under hypoxic conditions for 75 min in the presence of antagonist (Fig. 2a), and for 90-min in the presence of agonist (Fig. 2b) were determined. In order to characterize the cardiotoxic effects, antagonists to A_1R (DPCPX), and to A_3R (MRS1523), or the non-selective adenosine receptor antagonist (8-SPT) were used. The antagonists increased the LDH release, indicating that protective activity of endogenous adenosine, which released from the cells during ischemia, abolished by the antagonists (Fig. 2a). The effect of DPCPX appeared more pronounced, even at low concentrations (10 nM), than the cardiotoxicity in the presence of the MRS1523, which exhibited a lesser degree of LDH release at the same concentration (Fig. 2a). 8-SPT was only effective at higher antagonist concentrations (≥1 μM).

The ability to attenuate myocyte injury during prolonged hypoxia was measured by exogenously activating the A_1R and A_3R by specific agonists (CCPA and Cl-IB-MECA respectively, Fig. 2b). The cardioprotective ability of the A_3R agonist, Cl-IB-MECA, was observed at 10 nM, while at higher concentrations (≥1 μM) the protective ability decreased. The A_1R agonist, CCPA, exhibited protection in a concentration-dependent manner. Activation of both A_1R and A_3R together was more efficient in protection against hypoxia than by each other (Fig. 2b). The concentration-dependent effects and specificity of the selective antagonists DPCPX and MRS1523 on cardioprotection induced by the selective agonists, CCPA, (Fig. 3a) and Cl-IB-MECA were studied (Fig. 3b). Decrease in LDH release to the culture media suggest that both A_1R and A_3R agonists (CCPA and Cl-IB-MECA, respectively) prevented hypoxia-induced injury. The cardioprotection caused by A_1R agonist CCPA, was abolished by A_1R antagonist, DPCPX, in a dose dependent pattern, and was not affected by A_3R antagonist, MRS1523. Moreover, the protection achieved by the A_3R agonist Cl-IB-MECA was inhibited effectively by the A_3R antagonist, MRS1523, while only slightly by high concentrations (> 1 μM) of the A_1R antagonist DPCPX (Fig. 3b).

The morphological changes in the rat myocyte cultures treated under hypoxic conditions were followed by immunohistochemical staining of α-sarcomeric actin with hematoxylin counterstaining (Fig. 4). In rat primary cardiac cultures, the flattened contractile cells exhibited strands of well organized striated myofibrils run in various directions (Fig. 4A), and the myocytes possessed one or two nuclei. Hypoxic conditions caused damage of the cardiac cells, beginning with vacuolization of cytoplasm, concentration of cytoplasm around the nucleus and the loss of striation in myofibrils (Fig. 4B). Further alterations were characterized by a complete degeneration of the myofibrils and a significant decline in the α-sarcomeric actin staining, the appearance of enlarged vacuoles, and at the end stage, disruption of the cell membranes. Some cells containing pyknotic or fragmented nuclei and punctuated α-sarcomeric actin staining patterns, edematous areas in the cytoplasm and around the nucleus. Some cells exhibited sign of complete destruction (necrotic debris), their cytoplasm and nucleus material was greatly condensed and they exhibited disintegrated α-sarcomeric actin immunoreactivity. This degenerative process was almost completely

inhibited in the presence of either the A_1R agonist CCPA (Fig. 4C) or A_3R agonist Cl-IB-MECA (Fig. 4E). The A_1R antagonist DPCPX abolished the protection by CCPA (Fig. 4D), and the A_3R antagonist MRS1523, abolished the protection by A_3R agonist (Fig. 4F).

Fluorescent staining was used to differentiate between the cardiotoxicity and cardioprotective effects in living cells. Both propidium iodide (red) and DASPMI fluorescence (glossy yellow) were applied to the cells. Propidium iodide is used to identify disrupted cell membranes. DASPMI is specific for the membrane potential of mitochondria (vital staining) (Figs 4G–4L). Two types of mitochondrial patterns were observed in normoxic conditions. The first, longitudinal oriented and stretched mitochondria in subsarcolemmal areas in the cytoplasm, and the second, oval shape mitochondria in the perinuclear and intramyofibrillar regions (Fig. 4G). These cells maintained cellular integrity; propidium iodide, a marker for identification of necrotic cells, did not penetrate the cells (Fig. 4G). In hypoxic conditions the cellular membranes were damaged in many myocytes, which enabled the entrance of the propidium iodide red dyes and allows to quantify the percent of killed cells. The mitochondrial damage was characterized by the loss of the intensive glossy yellow fluorescence staining exhibited by DASPMI dye, which represent the dissipation of the mitochondrial membrane potential (Fig. 4H). Parallel changes in LDH release and percent of killed cells (Fig. 5), suggested that many cultured myocytes exhibited irreversible injury during the 90-min hypoxia. Figure 5 demonstrated that A_1R agonist CCPA and A_3R agonist Cl-IB-MECA, can protect cultured cardiomyocytes against hypoxia-induced injury. Activation of both A_1R and A_3R together was more efficient in protection against hypoxia than by each one alone. The degenerative alterations in surviving cells were almost completely inhibited in the presence of either the CCPA or Cl-IB-MECA (Figs 4I and 4K). Furthermore, the hypoxic-mediated injury was increased with the A_1R antagonist, DPCPX in the presence of CCPA (Fig. 4J) or the A_3R antagonist, MRS1523 in the presence of Cl-IB-MECA (Fig. 4L). The A_1R antagonist, DPCPX (not shown), did not affect the protection by Cl-IB-MECA.

The ultrastructural characteristics of control normoxic cells included intact nuclei with delicate heterochromatin against a pale background, dense and well-developed mitochondria (MT), and an intact sarcolemma. The myofibrils (MF) were relaxed, well developed and organized with a clear striations in longitudinal arrangements (Fig. 6A). In-contrast, the cultures exposed to hypoxic conditions (Fig. 6B) and/or exposed concomitantly to A_3R agonist and antagonist (MRS1523) (Fig. 6D), exhibited severe cardiotoxic features. The extent of the damage was rather heterogeneous among the myocytes, but the presence of cellular injury was characteristic for all myocyte population. The nuclear chromatin was clumped with condensed heterochromatin and convoluted perinuclear membrane that often was split and edematous areas were evident. Many nuclei were reduced in sizes. The diameters of these shrunken nuclei decreased to half or one-third of the normal ones. The hypoxic damage was characterized by clumping of nuclear chromatin, sarcolemmal rupture, intermyofibrillar edema and increased vacuolation (Figs 6B and 6D). No signs of apoptotic degeneration of cardiomyocytes were found. The mitochondria became pale and swollen, accompanied by rupture of the internal septa and membranes. The arrangements of sarcomeres, myofibrils and myofilaments were lost and the integrity of the actin and myosin filaments was not visualized. In high magnification, holes were noted in the cell membranes

(arrows). These typical oncotic and necrotic structural damage in the myocyte cultures were significantly attenuated by A_3R agonist Cl-IB-MECA (100 nM) under 90-min hypoxic conditions as indicated by preservation of mitochondria, conservation of myofilaments structure, and maintenance of cellular integrity (Fig. 6C).

Discussion

Over the past decade substantial experimental data have been obtained indicating that ADO has multiple physiological and pathological functions in various tissues and organs [19]. ADO reduces reversible and irreversible ischemia/reperfusion injury by preconditioning the heart and thereby reducing the size of myocardial infarctions [20, 21]. Administration of ADO to the coronary vessels after prolonged ischemia also limited the infarct size in isolated heart [22, 23]. The ADO-mediated cardioprotection phenomena raises questions as to whether the beneficial effects are due to direct activation of ADO receptors present in cardiac myocytes or mediated through the neutrophils, vascular or neuronal elements that are implicated in the coronary vasculature exposed to ischemic conditions. ADO levels were found to be elevated in heart failure and chronic heart failure [24]. Ischemia in the intact heart [25] or hypoxia in ventricular heart cells cultured from chick embryos mediates the release of endogenous ADO, which may precondition the heart and result in cardioprotection [6].

The first goal of our study was to evaluate a model of hypoxia in neonatal rat cardiomyocyte cultures and to assess the cardioprotection mediated by activation of ADO receptors. Exposure of myocytes to hypoxia for 2-h resulted in significant myocyte injury. This damage was not evident after 1 h. The addition of ADO deaminase (ADA) during the 1-h hypoxic period caused pronounced myocyte injury that was augmented after 2-h. These results suggest that in our hypoxic model, endogenous ADO was released and inhibited injury to the myocytes, while the ADA caused degradation of ADO and increased cytotoxicity. Therefore, during short hypoxia, no morphological and biochemical protective changes may be evident, due to the release of endogenous ADO, which is not sufficiently beneficial during longer periods (> 2 h). These data may point the importance of evaluating the exact period of hypoxic conditions (90-min, in our system) to enable the measurements of both cardiotoxic and cardioprotective effects mediated by ADO ligands. The same hypoxic duration has been used in other *in vitro* studies in isolated chick ventricular cultures [6, 7, 26].

A_1R and A_3R stimulation has been implicated in cardioprotection in several species, including rabbit [5], human [27], chicken [6, 7, 26] and others. However, in the rat there remains a controversy about whether ischemic preconditioning and cardioprotection are mediated by these receptors [9, 10, 13, 28]. Therefore, the second goal of the present study was to assess the involvement of A_1R and A_3R in cardiotoxicity and cardioprotection under 90-min hypoxic conditions. In the rat cardiomyocyte cultures, the protective effect of ADO was mainly mediated through activation of the A_1R and A_3R, since DPCPX (A_1R antagonist), MRS1523 (A_3R antagonist) and 8-SPT (both A_1R and A_3R antagonist), blocked the cardioprotective effect of ADO.

The ability of the MRS1523 to reverse the protective effect of ADO was more pronounced than that of DPCPX at the low concentrations. It can be suggested that the cardioprotective potential of the 90-min A_3R activation exceeds that of A_1R activation at the same concentrations, or the difference may be due to another type of second messengers. Another explanation can be related to the high selective ability of the MRS1523 to both human and rat A_3R [18] (in rat it corresponds to selectivities of 140- and 18-fold vs. A_1 and A_{2A} receptors, respectively [20]). This specificity may belong to the A_3R only (within limits of selectivity of the MRS1523), while DPCPX mainly inhibits the activation of the A_1R with an additional potential to inhibit the A_3R at high concentrations. The ability of the 8-SPT to inhibit ADO receptor activation was observed only at high concentration (≥10 μM) in preconditioning experiments in the intact heart [2, 19] and in myocytes cultures [4, 6]. This phenomenon was also documented in prolonged hypoxic conditions [7]. The high concentration can be related to the low affinity of the ligand. Activation of the A_1R and A_3R by a low level of endogenous agonist has a protective role in hypoxic conditions. Preconditioning and chronic exposure to low concentrations of agonist may cause upregulation and sensitization of the A_3R and their protection ability. In this study, the activation of both A_1R and A_3R by their own specific agonist (CCPA and Cl-IB-MECA, respectively) induced cardioprotective effects in prolonged hypoxic conditions and attenuated rat cardiac myocyte injury *in vitro*. Although low concentrations of the A_3R agonist, Cl-IB-MECA, protected the cardiomyocytes, the protection declined significantly at 1 μM and higher concentrations (≥10 μM), which may cause toxicity and apoptosis [11, 12, 29]. Using the model of neonatal rat isolated cardiomyocytes, we have demonstrated that activation of the A_1R and A_3R may result in cardioprotection during 90 min hypoxic conditions. The study describes an in vitro model of hypoxic conditions where the neuronal elements, circulating blood, hemodynamic parameters and their involvement in mediating the cardioprotection are essentially precluded. The results of this study provide conclusive evidence that A_1R and A_3R activation in cardiomyocytes delays onset of irreversible cell injury following hypoxia. The observations of the present study confirm that in myocytes pretreated with adenosine receptor agonists, morphological injury developed more slowly than in hypoxic (non-treated) cells. Most of the alterations observed in prolonged hypoxia may be attributed to the loss of critical metabolites such as ATP. Mitochondrial membrane potential provides the driving force for ATP synthesis. The potential is generated by the supply of NADH through the matrix dehydrogenases and electron flux through electron transport chain [30]. As was shown in this study, hypoxia did not produce acute decrease in mitochondrial potential according to maintaining of DASPMI fluorescence up to terminal stage, when abrupt decrease or cessation of emission was observed, which indicate the complete collapse of the potential in dying cells (see Figs 4H and 4L). Budinger *et al*. [30], suggests that inhibition within the electron transport chain during hypoxia would therefore result in a decrease in electron flux and a depolarization of the membrane, while a sudden inhibition of ATP utilization or an inhibition of the ATP synthase produces hyperpolarization. Maintaining of membrane potential during acute hypoxia suggests that ATP utilization and ATP synthesis remained closely matched in surviving cells. Both sarcomeres length and mitochondrial swelling depend on the actual ATP content [31, 32]. The loss of mitochondrial function inevitably leads to cell necrosis. As was shown in this study collapse of membrane potential leads to permeability of sarcolemma to propidium

iodide, sign of irreversible cell injury (see Figs 4H and 4L). Thus, for understanding cardioprotective activity of adenosine compounds we should understand the mechanism of irreversible mitochondria damage.

It is known that reactive oxygen species can be generated in ischemic/reperfused hearts in the cytosol and/or one-electron reduction process of the respiratory chain in mitochondria [33]. The oxygen radicals may induce a lipid peroxidation of mitochondrial membranes and their formation may result in mitochondrial injury and cell necrosis. Characteristic for cardiomyocytes that they contain less catalase, glutathione peroxidase and superoxide dismutase than other cells [34]. Recent evidence implicates the ADO A_1Rs and A_3Rs in the activation of antioxidant enzymes. It has been demonstrated that stimulation of adenosine receptors activates the antioxidant enzyme system in rat cardiac myocytes [35, 36], that might explain the cardioprotection.

In order to clarify the controversy whether adenosine can protect rat cardiomyocytes in hypoxia [9, 10, 13, 27, 28], we have characterized the specificity of the A_1R and A_3R. The presence of CCPA (A_1R selective agonist) during 90-min hypoxic conditions resulted in cardioprotection. This effect was abolished by the A_1R antagonist DPCPX and not affected by the A_3R antagonist MRS1523. Furthermore, the protection achieved by A_3R agonist Cl-IB-MECA was inhibited by pretreatment with A_3R antagonist, but not by the A_1R antagonist. Activation of each of ADO receptors A_1R and A_3R resulted in delaying of irreversible cardiomyocyte damage. Cave *et al.* [9], showed the lack of protection against contractile dysfunction in isolated rat hearts, but found a significant decrease in creatine kinase leakage by adenosine pretreatment, that confirms our results in culture. Protection against contractile dysfunction seems to involve a combination of several receptors and downstream signaling cooperation in heart tissue and not only ADO receptors.

In conclusion, low doses of highly selective ADO agonist may have therapeutic potential in treatment of cardiac ischemia and other cardiac disorders. Highly selective A_1R and A_3R ligands may represent novel potent cardioprotective agents and co-activation of both A_1R and A_3R was more potent than that produced by activation of either receptor individually. These agonists may have therapeutic potential during infarct-producing ischemia and may reduce the size of myocardial infarction.

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Abbreviations

ADO	adenosine
A_1R, A_3R	adenosine receptor subtype 1 or 3
CCPA	2-chloro-N^6-cyclopentyladenosine

C1-IB-MECA	2-chloro-N^6-(3-iodobenzyl) adenosine-5′-N-methyluronamide
CK	creatine kinase
DASPMI	2-(p-dimethylaminostyryl) pyridyl methyl iodide
DPCPX	8-cyclopentyl-1-3-dipropylxanthine
LDH	lactate dehydrogenase
MRS1623	5-propyl-2-ethyl-4-propyl-3-(ethylsulfanylcarbonyl)-6-phenylpyridine-5-carboxylate
8-SPT	8-sulphophenyl-theophylline

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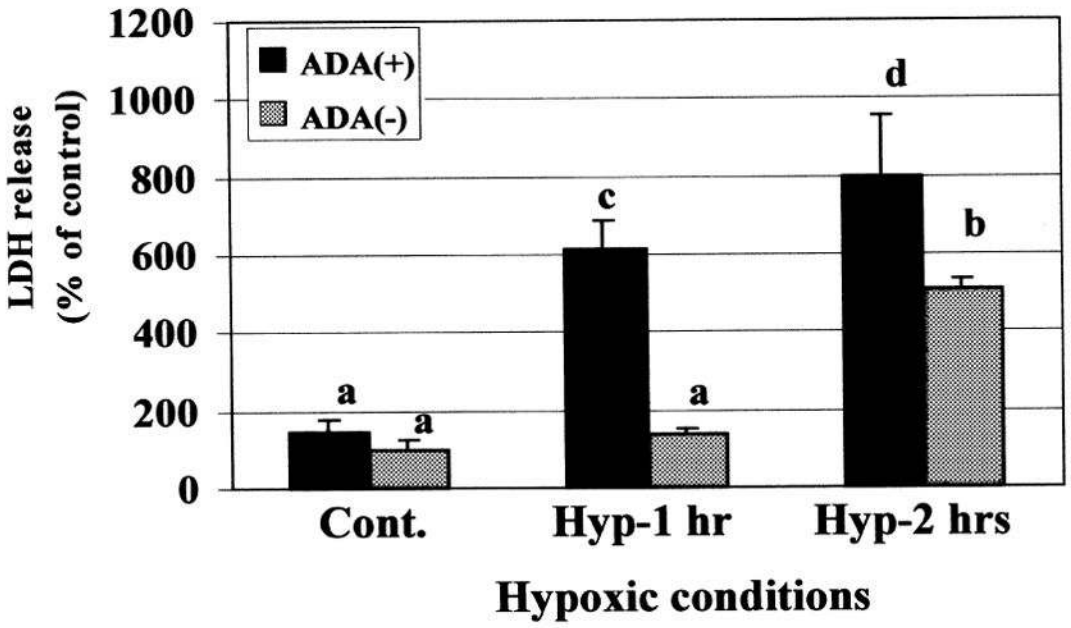

Fig. 1.

Duration of hypoxic conditions and the effect of adenosine deaminase (ADA) (5 units/ml) on endogenic adenosine release from cultured cardiomyocytes after prolonged exposure to hypoxic conditions. The LDH release was determined immediately after hypoxia. One hundred percent were considered as the release in the control cultures. Data are expressed as mean ± S.E.M. of at least 3 replicates in 3 separate experiments. Means with the same letter are not significantly different ($p < 0.05$) according to a *post-hoc* Tukey-Kramer test.

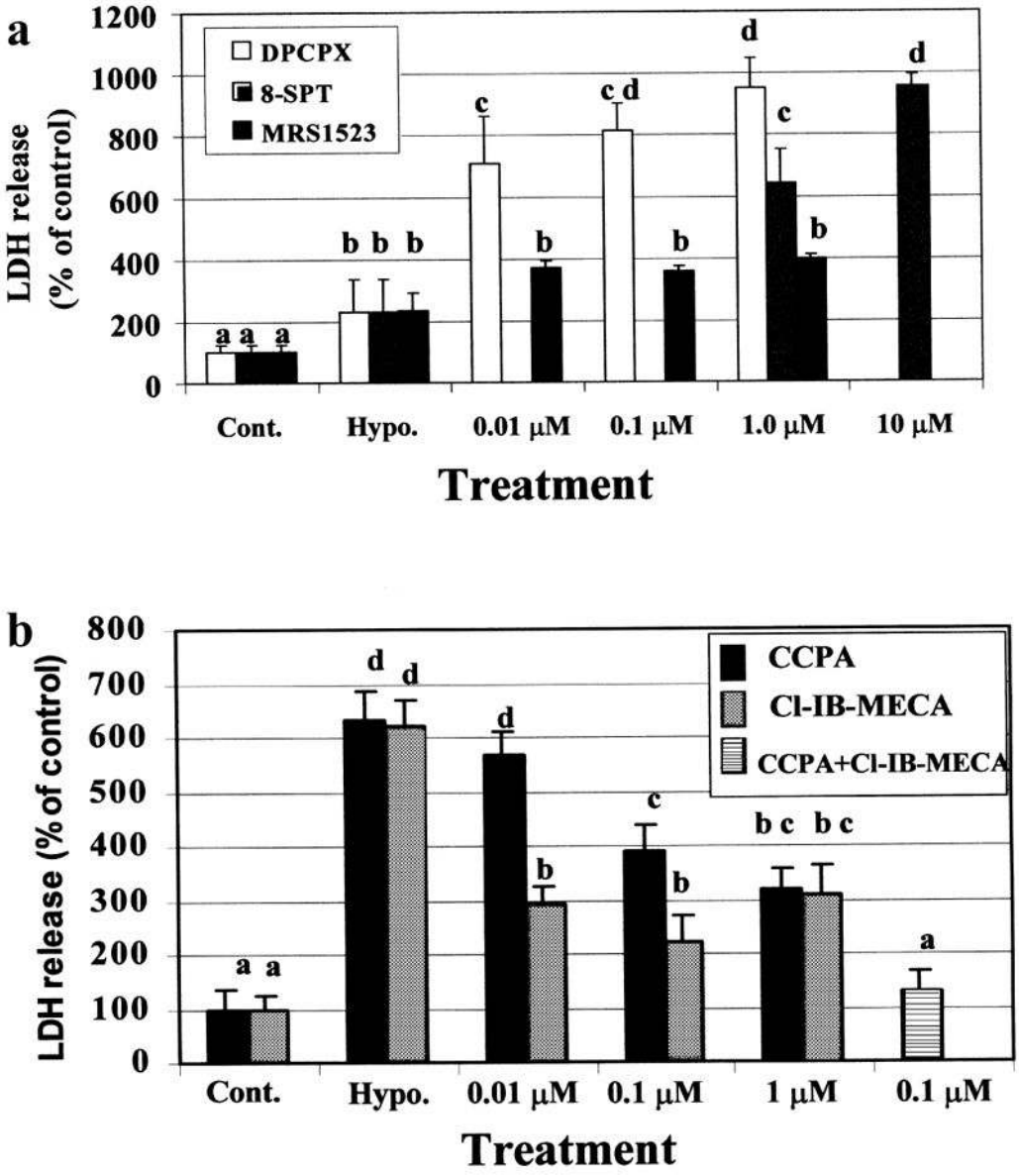

Fig. 2.

Effect of Adenosine A_1R and A_3R ligand in cardiac myocytes, under hypoxic conditions for 75-min in the presence of antagonist (a), and for 90-min in the presence of agonist (b). The LDH release was determined immediately after hypoxia. One hundred percent were considered as the release in the control cultures. Data are means of at least 3 replicates in 5 separate experiments. Means with the same letter are not significantly different ($p < 0.05$) according to a *post-hoc* Tukey-Kramer test.

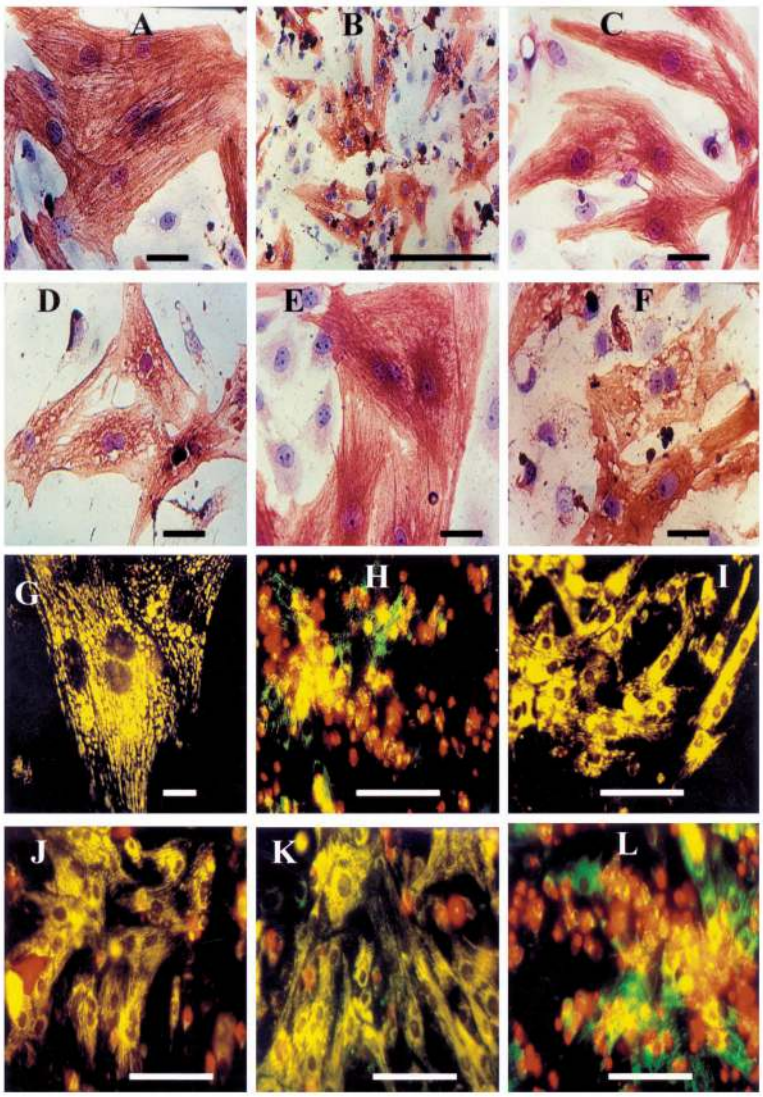

Fig. 3.

Dose dependent effect of A_1R (DPCPX) and A_3R (MRS1523) antagonists on the cardioprotection induced by A_1R (CCPA – 0.1 μM) (a) and A_3R (Cl-IB-MECA – 0.1 μM) (b) agonists. The LDH release was determined after 90 min hypoxia. The 100% were determined as the release in the control cultures. Data are means of at least 3 replicates in 3 separate experiments. Means with the same letter are not significantly different ($p < 0.05$) according to a *post-hoc* Tukey-Kramer test.

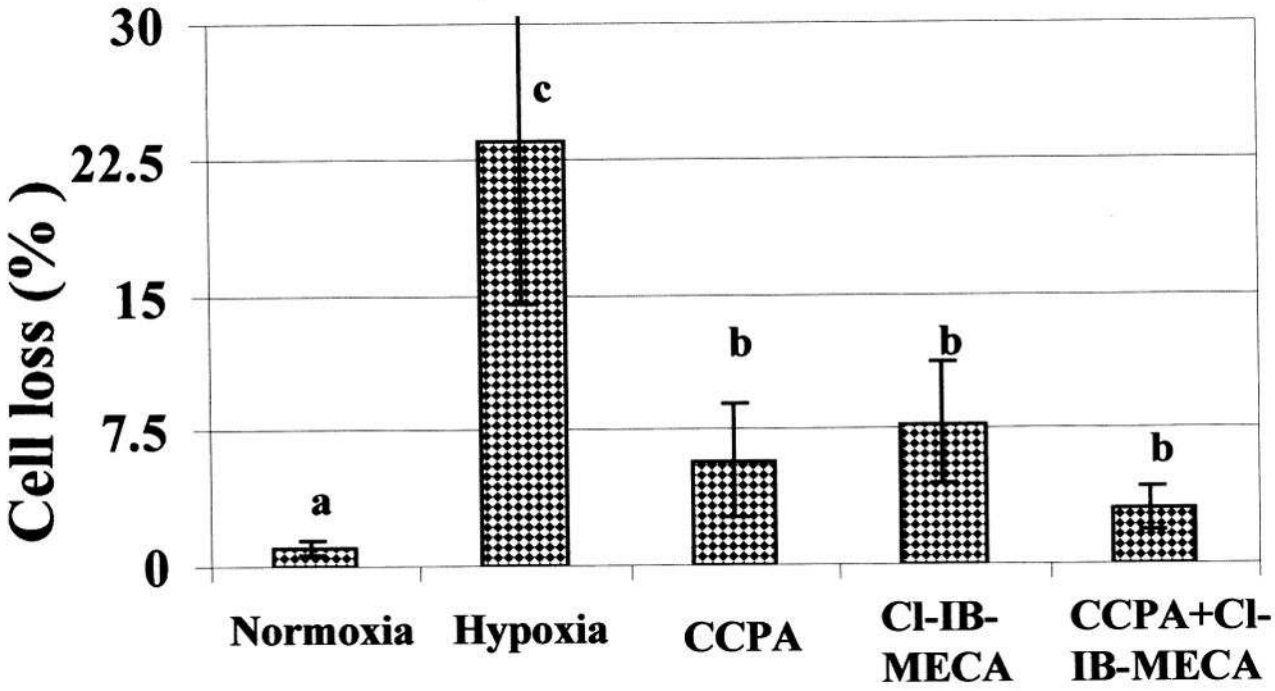

Fig. 4.

Cardiotoxicity and cardioprotection demonstrated by morphological investigations. (A–F) Immunohistochemical staining of α-sarcomeric actin in rat cardiomyocyte cultures followed by hematoxylin counterstaining. (A) Control – normoxic conditions; (B) 90-min hypoxic conditions; (C) Presence of A_1R agonist (CCPA – 0.1 μM) during hypoxic period; (D) CCPA was introduced together with A_1R antagonist (DPCPX – 1 μM) during hypoxic period; (E) Presence of A_3R agonist (Cl-IB-MECA – 0.1 μM) during hypoxic period; (F) Presence of A_3R agonist (Cl-IB-MECA – 0.1 μM) together with A_3R antagonist (MRS1523 – 1 μM) during the hypoxic period; (G–L) The accumulation of DASPMI dye in the mitochondrial matrix space (vital staining) in conjunction with propidium iodide binding to nuclei of cells whose plasma membranes have became permeable in rat cardiomyocyte cultures. (G) Control – normoxic conditions; (H) 90-min hypoxic conditions; (I) Presence of A_1R agonist (CCPA – 0.1 μM) during hypoxic period; (J) CCPA was introduced in presence of A_1R antagonist (DPCPX – 1 μM) during hypoxic period; (K) Presence of A_3R agonist (Cl-IB-MECA – 0.1 μM) during the hypoxic period; (L) Presence of A_3R agonist (Cl-IB-MECA – 0.1 μM) together with A_3R antagonist (MRS1523 – 1 μM) during hypoxic period. The results were obtained in at least 3 replicates in 3 separate experiments.

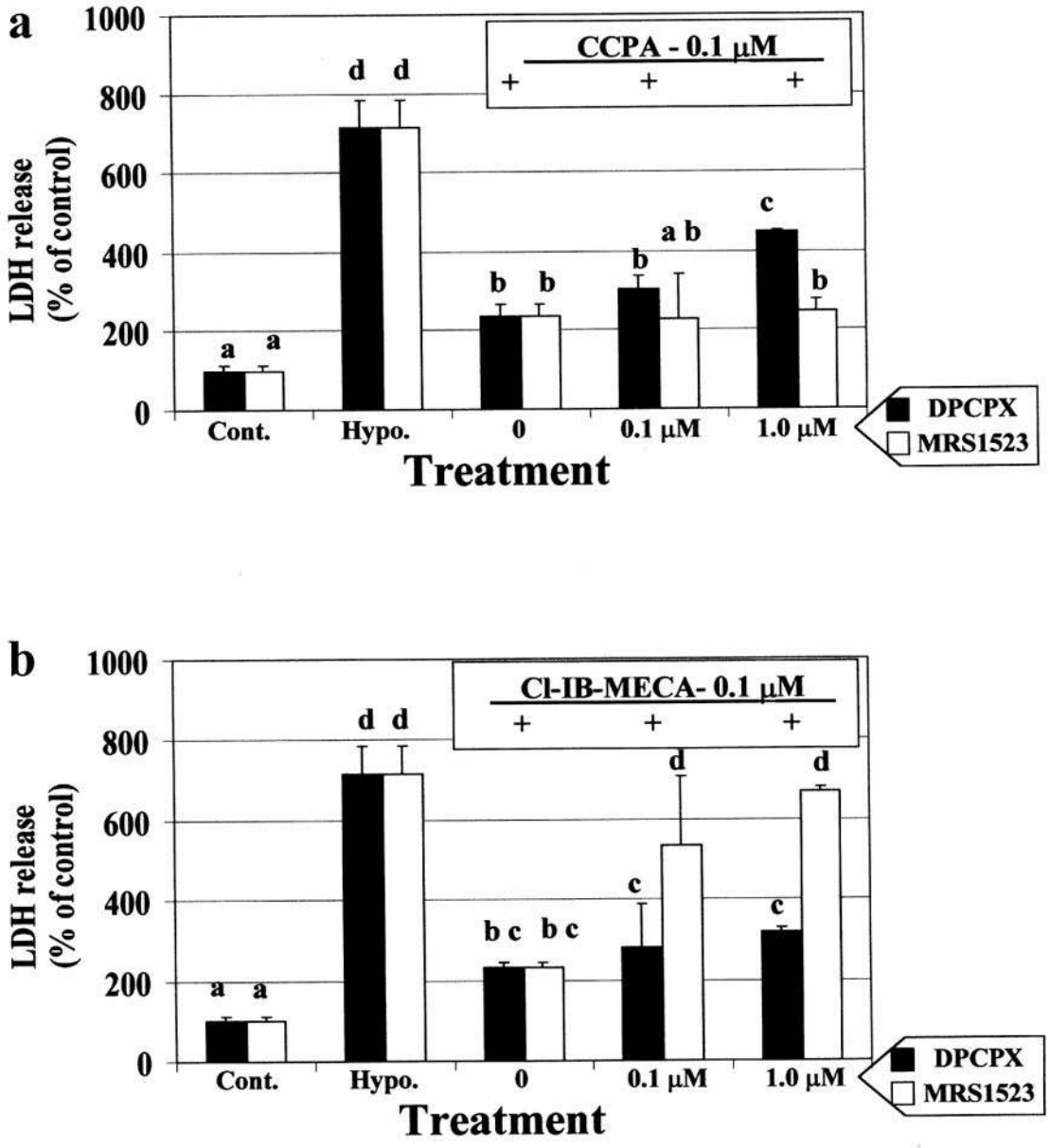

Fig. 5.

Effect of Adenosine A_1R and A_3R agonists on cardiac myocytes loss after hypoxia for 90-min. A_1R agonist (CCPA – 0.1 μM), or A_3R agonist (Cl-IB-MECA – 0.1 μM) were introduced 10 min before and during the 90 min hypoxia. Data are means of at least 3 replicates in 3 separate experiments. Means with the same letter are not significantly different ($p < 0.05$) according to a *post-hoc* Tukey-Kramer test.

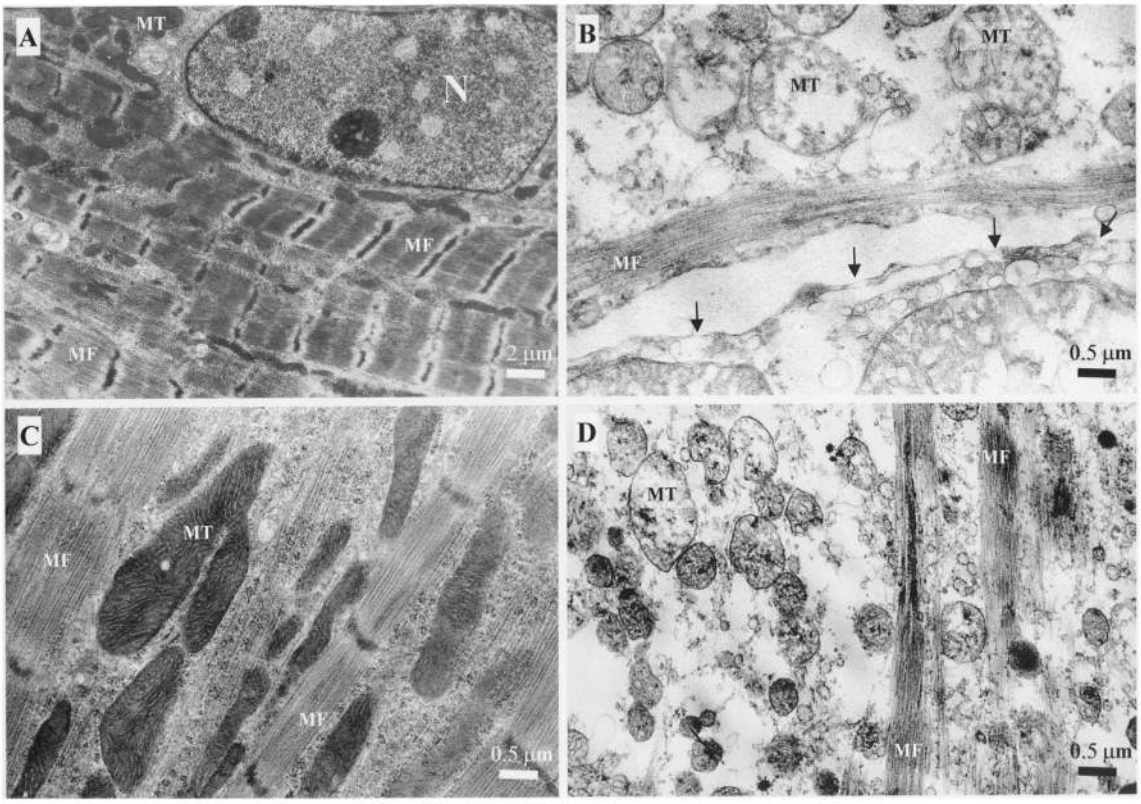

Fig. 6.

Electron micrographs of cardiac cells after hypoxic conditions. (A) Control cardiomyocyte in normoxic conditions; (B) Hypoxic conditions for 90-min. Gaps in the cell membrane (arrows); (C) A_3R agonist (Cl-IB-MECA – 100 nM) exposure for 10-min before and during the 90-min of hypoxic conditions; (D) A_3R agonist (Cl-IB-MECA – 100 nM) and A_3R antagonist (MRS1523 – 1 μM) exposure for 10-min before and during the 90-min of hypoxic conditions. Nucleus (N), myofibrils (MF) and mitochondria (MT).