EFFECT OF ADENOSINE ON ISOPROTERENOL-INDUCED HYPERTROPHY IN VITRO. A PRELIMINARY STUDY

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Abstract

Persistent β1-adrenergic stimulation is thought to induce cardiac hypertrophy, alteration in calcium regulation and Cx43 over-expression. Adenosine is a negative feedback inhibitor of adrenergic stimulation in the heart, protecting it from toxic effects of overstimulation.

The aim of our study evaluate the effects of adenosine administration on Cx43 and pCx43 expression and $[Ca2+]i$ in Isoproterenol-treated cardiomiocytes by using H9c2 cells.

Adenosine co-treatment reduced cellular hypertrophy, Cx43, pCx43 and CaMKII over-expression, and reduced intracellular Ca2+ overload.

These results suggest that the beneficial activity of adenosine on cardiomyocytes underlie the reduction of Cx43-dependent calcium overload and CaMKII over-expression, implying a potential therapeutic in cardiac hypertrophy.

Key words: Adenosine, connexin 43, hypertrophy, Ca2+ homeostasis, CaMKII, Isoproterenol, H9c2 cells

Introduction

Increased sympathetic tone plays a pivotal role in heart failure by contributing to the progression of myocardial dysfunction due to arrhythmia and hypertrophy. Cardiac hypertrophy is a primary disease of the myocardium characterized by a structural and functional remodelling of the heart with increase in heart weight and force of contraction, both related to the activation of Gap junctions[1], strictly relied upon the expression levels of Cx43[2]. In support, a previous study has shown that under pathological conditions gap junction disordering is involved in the abnormal Ca2+ activities in injured heart [3].

So far, it has been well established that adenosine and adrenergic receptors are involved in the regulation of contractile and metabolic function of the heart[4][5]. Adenosine attenuates the β1-adrenergic signal cascade by reducing the β1-adrenergic receptor-induced elevation of cAMP levels and PKA activity [6]. More recently, our group demonstrates that adenosine reduces Cx43 over-expression induced by β1-adrenergic receptor stimulation by accelerating Cx43 ubiquitination and proteasome degradation[7].

This study aimed to investigate the activity of adenosine in preventing and/or reducing the hypertrophy-like effects in cardiomyocytes undergoing long term administration of Isoproterenol. Here, we observed that the administration of adenosine proved of beneficial activity on H9c2 cells which counted lower phosphorylation of Cx43 and intracellular Ca2+ overload.

Methods

2.1 Cell culture

H9c2 cells were purchased from the American Tissue Culture Collection (Manasas, VA, USA.) Cells were subcultured weekly in 100-mm Corning dishes containing 10 ml Dulbecco's modified Eagle's Medium (DMEM; Gibco) with 10% fetal bovine serum (FBS; Gibco) and antibiotics (25 U/ml penicillin and 25 U/ml streptomycin). Cells were seeded at a density of 3.5x104 cells per 35-mm well of twelve-well plates, starved for 24h in DMEM containing 1% FBS and treated as described below.

To evaluate the effects of adenosine in preventing and/or reducing the hypertrophy induced by

isoproterenol, in our experiments we performed three different experimental protocols represented schematically in Figure 1. To avoid the degradation of adenosine by the enzyme adenosine deaminase, adenosine administration was always preceded by erythro-9-Amino-β-hexyl--α-methyl-9H-purine-9-ethanol hydrochloride erythro-9-(2-Hydroxy-3-nonyl)-adenine hydrochloride (EHNA, 10 μM), a specific inhibitor of the enzyme.

2.2 Protein content per cell measurement

Cells treated as previously described were collected by scraping into 100 μl RIPA buffer. Protein concentrations were measured using Bradford protein assay kit (Bio Rad, Italy). Cell protein content was determined by dividing the total amount of protein by the cell number. The Trypan blue exclusion test of cell viability is used to determine the number of viable cells present in a cell suspension.

2.3 Western blot analysis

Total intracellular proteins were extracted from the cells by freeze/thawing in lysis buffer (Tris–HCl 50 mM pH 7.4) containing 10 mM NaF, 150 mM NaCl, 1% Nonidet P40, 1 mM phenylmethylsulfonylfluoride, 1 mM sodium orthovanadate, leupeptin (10 μg/ml) and trypsin inhibitor (10 μg/ml). Protein content was estimated according to Bio-Rad protein assay (BIO-RAD, Milan Italy) and 50 μg protein/lane were loaded onto an acrylamide gel and separated by SDS-PAGE in denaturing conditions. Blots were incubated with primary antibody anti-Cx43, or anti-pCx43 Ser368 (Cell Signaling, DBA Italy), or anti-CaMkII or anti-actin, used as reference protein (Santa Cruz Biotechnology, DBA Italy). After incubation with the primary antibodies and washing in TBS/0.1% Tween, the appropriate secondary antibody, either anti-rabbit or anti-mouse was added for 1h at room temperature. Immunoreactive protein bands were detected by using enhanced chemilumunescence reagents (ECL) and exposed to Kodak X-Omat film (both from GE Healthcare, Italy). Films were then subjected to densitometric analysis using a Gel-Doc 2000 system (BIO-RAD).

2.4 Measurement of intracellular Ca2+ signalling

Intracellular Ca2+ concentrations were measured by using the ratiometric fluorescent indicator dye Fura 2-AM, the membrane-permeant acetoxymethyl ester form of Fura 2 (Sigma-Aldrich), as previously described [8] with minor revisions. Briefly, confluent H9c2 cell monolayers grown on culture dishes were incubated at 37°C in Hank's balanced salt solution (HBSS) containing 5 µM Fura 2-AM for 45 min. After the incubation period, cells were washed with the same buffer to remove excess Fura 2-AM and incubated in buffer for 15 min to allow hydrolysis of Fura 2-AM into its active-dye form, Fura 2. Cells were then transferred into the cuvette of a spectrofluorimeter (Perkin-Elmer LS-50). The excitation wavelength was alternated between 340 and 380 nm, and emission fluorescence was recorded at 515 nm. The fluorescence ratio was calculated as F340/F380 nm.

2.5 Statistical analysis

Statistical analysis was performed with the aid of commercially available software GraphPad Prism4 (GraphPad Software Inc., San Diego CA). Results are expressed as mean ± SEM. Student's t test was used for statistical analysis. $P < 0.05$ was considered statistically significant.

Results

Protein content measurement in single cells, a marker of cellular hypertrophy[9], indicated that ISO induced a significant ($P < 0.05$) increase in protein content in the cells. In contrast, adenosine co-administration significantly ($P < 0.05$) reduced this increase. No significant differences in protein content between ISO- and DMEM/1%FBS-treated cells were observed when adenosine was administered 96 h after ISO (Table 1).

Western blot analysis revealed that Cx43 expression was significantly ($P < 0.05$) higher than control cells in ISO-treated cells. Adenosine co-administration significantly ($P < 0.05$) reduced Cx43 over-expression. No significant effects were observed when adenosine was administered 96 h after ISO (Fig. 2a). ISO significantly ($P < 0.05$) increased the expression of Cx43 phosphorylated on Ser368 (pCx43), whereas adenosine significantly ($P < 0.05$) reduced pCx43 over-expression both when administered simultaneously and 96 h after ISO (Fig. 2b).

In addition, spectrofluorimetric analysis, using the fluorescent marker FURA-2 AM, showed that, in the Ca2+-free medium, ISO treatment significantly ($P < 0.05$) increased basal levels of [Ca2+]i. In fact, ionomycin administration evoked an increase in [Ca2+]i significantly reduced compared with control cells. Adenosine administration restores Ca2+ impairment. As reported in Fig. 3a, no significant differences were observed between ISO-treated cells and control cells either when adenosine was administered simultaneously or 96 h after ISO. CaMkII expression was significantly ($P < 0.05$) higher in ISO-treated cells. Adenosine significantly ($P < 0.05$) reduced CaMkII over-expression both when administered simultaneously and 96 h after ISO (Fig. 3b).

Discussion

The present study demonstrates that adenosine is able to reduce isoproterenol-induced hypetrophy-like effects in the cardiomyoblasts H9c2. In fact, our data show that the co-administration of adenosine: i) reduced protein content in single cell; ii) reduced Cx43, pCx43 and CaMkII expression; iii) restores Ca2+ homeostasis.

β-adrenergic signalling can induce myocyte death and cardiac decompensation, culminating in heart failure. Some studies suggest that protein kinase A (PKA) is the mediator of β-adrenergic-induced myocyte apoptosis by altering Ca2+-regulation, whereas others have suggested that Ca2+/calmodulin-dependent kinase II (CaMkII) can mediate β-adrenergic-induced myocyte death through a PKA-independent process. It is thought that the antiadrenergic action of adenosine serves as a mechanism of cardioprotection against cardiotoxicity which accompanies excessive adrenergic stimulation[10]. Fenton and co-workers[6] reported that the activation of adenosine A1 receptor attenuates β1-adrenergic-dependent cascade by an antiadrenergic action, reducing the β1-adrenergic receptor-induced elevation of cAMP levels and PKA activity. More recently a direct action of adenosine A1 receptor on Cx43 turn-over in Isoproterenol-treated cells has been demonstrated by our group [7].

The key question addressed in this study is whether adenosine was able to prevent and/or reduce the hypertrophy induced by long term administration of isoproterenol.

Treatment with isoproterenol for 96 hours induced a significant increase of the amount of proteins synthesized from single cells, a cellular marker of hypertrophy. Our data demonstrate that adenosine is able to revert this effect, confirming what was reported in the literature, namely that adenosine has an antiproliferative activity[11][12] .Though, the administration of adenosine did not alter cell viability at 96 hours (data not shown). However, in our case, this data serves to corroborate our experimental system and the data obtained subsequently.

The effect of adenosine on Cx43 and pCx43 expression was evaluated by Western blot analysis. Our data indicates that isoproterenol induced an increase in the expression level of these proteins and that adenosine, administered concurrently to isoproterenol, reduced the observed over-expression.

Previous studies have shown that under pathological condition gap coupling is disordered and involved in the abnormal Ca2+ activities that potentially generate lethal arrhythmias and hyperconstriction in ventricles, suggesting a functional role of the gap junction/intercellular communication in the regulation of Ca2+ signalling in diseased heart[3][13]. In our experimental model we observed an increase in basal [Ca2+]i in isoproterenol-treated cells. Adenosine co-administration restores basal [Ca2+]i. β-adrenergic-induced increase in Ca2+ overload, associated to CaMKII activation[14]. Our data demonstrate that adenosine co-administration is also able to reduce CaMKII over-expression induced by isoproterenol.

In conclusion, adenosine beneficial effects as anti-hypertrophic agent underlie, not only the reduction of cAMP levels via A1 receptor, but also on the alteration of the phophorylated isoform of Cx43, reduced amounts of the intracellular Ca2+ and CAMKII activation, responsible of tissue remodelling observed in cardiac hypertrophy[15][16].

Acknowledgments

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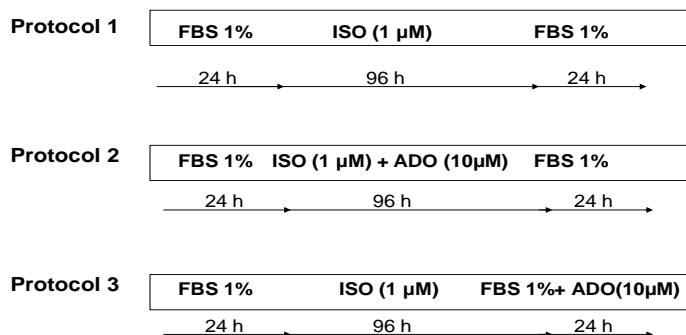
Figure 1

Figure 1. Schematic representation of experimental protocols H9c2 cells were treated i) with ISO for 96h and the kept in culture for 24h in DMEM/FBS 1% (protocol 1); ii) or with ISO and ADO for 96h and then kept in culture for 24h in DMEM/FBS 1% (protocol 2); iii) or with ISO for 96h and then with ADO for 24h (protocol 3).
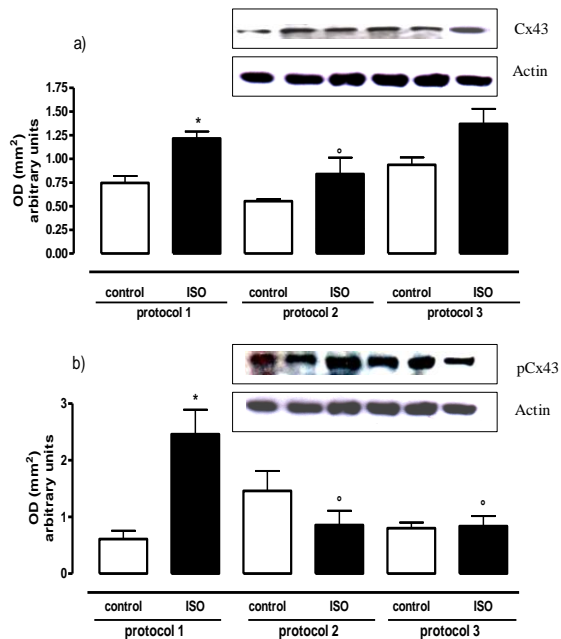
Figure 2. Effect of adenosine and isoproterenol treatments on Cx43 (a) and pCx43 (b) expression. H9c2 cells were treated i)with ISO for 96h and the kept in culture for 24h in DMEM/FBS 1% (protocol 1); ii) or with ISO and ADO for 96h and then kept in culture for 24h in DMEM/FBS 1% (protocol 2); iii) or with ISO for 96h and then with ADO for 24h (protocol 3). Cx43 and pCx43 expression was measured by Western blot analysis. Results are espressed as mean ± S.E.M. from at least three independent experiments. *denotes P<0,05 vs control.
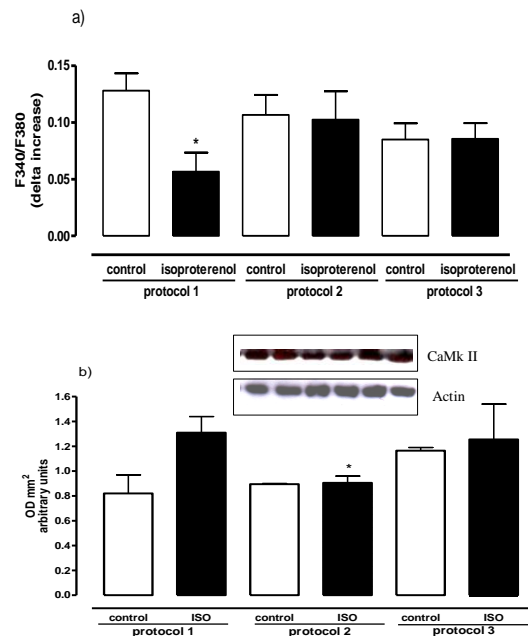
Figure 3. Effect of adenosine and isoproterenol treatments on [Ca^{2+}]i concentrations (a) and on CaMK II expression (b). H9c2 cells were treated i)with ISO for 96h and the kept in culture for 24h in DMEM/FBS 1% (protocol 1); ii) or with ISO and ADO for 96h and then kept in culture for 24h in DMEM/FBS 1% (protocol 2); iii) or with ISO for 96h and then with ADO for 24h (protocol 3). Intracellular calcium concentration was evaluated in Ca^{2+}-free medium. Results were expressed as mean ± s.e.m. of delta (δ) increase of FURA 2 ratio fluorescence (340/380 nm) from at least three independent experiments. Data were analyzed by analysis of variance test, and multiple comparison were made by Bonferroni's test. # denotes P<0.05 versus control. CaMKII expression was measured by Western blot analysis. Results are espressed as mean ± S.E.M. from at least three independent experiments. *denotes P<0,05 vs control.

	Protocol 1		Protocol 2		Protocol 3	
	control	**ISO**	**control**	**ISO**	**control**	**ISO**
Proteins µg/well	2.32±1.169	5.180±0.403 *	3.757±0.487	3.278±0.484#	3.18±0.338	4.433±0.998
Cells/well (x 10⁴)	7.11±6.66	5.88±1.222	3.26±0.70	3.55±1.11	6.51±0.32	6.22±1.22
Proteins ng/cells	0.107±0.091	0.080±0.025	0.122±0.027	0.084±0.039	0.080±0.009	0.079±0.026

Table 1. Effect of treatment with adenosine on Isoproterenol-induced hypertrophy.
Values are expressed as mean ± S.E.M. from at least three independent experiments.
* denotes P<0.05 versus control; # denotes P<0.05 versus Isoproterenol protocol 1.