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ORIGINAL ARTICLE

Adenosine receptor expression in an experimental animal model of myocardial infarction with preserved left ventricular ejection fraction

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Abstract Adenosine, a purine nucleoside and a "retaliatory metabolite" in ischemia, is ubiquitous in the body and increases 100-fold during ischemia. Its biological actions are mediated by four adenosine receptors (ARs): A_1, A_{2A}, A_{2B} and A_3. The aim of this study was to determine possible myocardial alterations in AR expression in an experimental animal model of myocardial infarction (MI) with a preserved left ventricular (LV) ejection fraction. LV tissue was collected from sexually mature male farm pigs with MI ($n = 6$) induced by permanent surgical ligation of the left anterior descending coronary artery and from five healthy pigs (C). mRNA expression of A_1R, $A_{2A}R$, $A_{2B}R$, A_3R and TNF-α was determined by real-time PCR in tissue collected from border (BZ) and remote zones (RZ) of the infarcted area and from LV of C. BZ, RZ and samples of C were stained immunohistochemically to investigate A_3R immunoreaction. After 4 weeks a different regulation of ARs was observed. A_1R mRNA expression was significantly lower in the infarct regions than in controls (C = 0.75 ± 0.2, BZ = 0.05 ± 0.2, RZ = 0.07 ± 0.02 $p = 0.0025$, $p = 0.0016$, C vs. BZ and RZ, respectively). Conversely A_3R was higher in infarct areas (C = 0.94 ± 0.2, BZ = 2.85 ± 0.5, RZ = 3.48 ± 1.0, $p = 0.048$ C vs. RZ). No significant differences were observed for $A_{2A}R$ (C = 1.58 ± 0.6, BZ = 0.42 ± 0.1, RZ = 1.37 ± 0.6) and $A_{2B}R$ (C = 1.66 ± 0.2, BZ = 1.54 ± 0.5, RZ = 1.25 ± 0.4). A_3R expression was confirmed by immunohistochemical analysis and was principally localized in cardiomyocytes. TNF-α mRNA results were: C 0.41 ± 0.25; BZ 1.60 ± 0.19; RZ 0.17 ± 0.04. The balance between A_1R and A_3R as well as between $A_{2A}R$ and $A_{2B}R$ was consistent with adaptative retaliatory anti-ischemic adenosinergic changes in the infarcted heart with preserved LV function.

Keywords Myocardial infarction · Adenosine receptor · mRNA expression · Real-time PCR

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Introduction

Infarcted left ventricle with preserved ejection fraction (EF) is a clinically relevant disease [1]. After an acute myocardial infarction (MI) the magnitude of left ventricular (LV) remodeling and the extent of scarring are key predictors of cardiac performance and long-term prognosis [2]. However, in infarcted hearts with preserved LV function, the processes that underlie myocardial remodeling are likely multifactorial and still poorly defined [3–5] although coronary revascularization plays the major role [6].

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Biological and mechanical stressors such as ischemia, hypoxia, cellular ATP depletion, free radicals, pressure and volume overload, catecholamines and cytokines burst, and the renin-angiotensin axis activation may independently and simultaneously lead to different degrees of LV dysfunction after acute and severe coronary flow limitation. Several endogenous self-protective mechanisms naturally occurring to counteract LV remodeling fail in part to prevent or limit cellular injury.

Adenosine, the catabolite of ATP, acts as an endogenous cardioprotectant in different cardiovascular diseases, such as MI and congestive heart failure [7, 8].

To the best of our knowledge, it is mainly released from the cardiac cells when the myocardial oxygen supply is too low, i.e., during ischemia, hypoxia or enhanced oxygen consumption [9]. In fact, the adenosine extracellular concentration in body fluid may increase 100-fold under long-standing ischemia [10].

Even though adenosine can directly enter into the cardiomyocytes and promote ATP resynthesis in support of cell functions, its physiological actions are mainly mediated through the binding of selective receptors, which are classified into three subtypes [11].

Adenosine A_1 receptors (A_1R) are responsible for the inhibition of adenylate cyclase via activation of G_i proteins; A_2 receptors ($A_{2A}R$, $A_{2B}R$) are responsible for stimulation of adenylate cyclase via activation of G_s proteins, and A_3 receptor (A_3R) activation is thought to increase phospholipase C activity via G_o or G_q proteins [11–13]. Since A_1R and A_3R are mainly distributed on myocardial cells, and A_2Rs are located on coronary vascular smooth muscle cells, adenosine may substantially modulate cardiac function as a whole [8, 14]. Although the single role of each adenosine receptor in the heart is not fully understood, the signaling that underlies the myocardial adaptation to ischemic insult seems to be related to the cross talk among several types of adenosine receptors located on different cardiac cells.

The effects of A_1R and probably of A_3R appear to be associated with actions on the myocyte, whereas the effects of A_2R seem to be related to vasodilation and actions on cells in the vascular compartment [15].

In sum, A_1R activation results in reduced O_2 requirements, increased energy supply and decreased Ca2+ overload [15]. A_3R activation may be involved in cardioprotection seen as early preconditioning and is probably mediated during ischemia although the mechanisms involved are uncertain; A_2Rs are mainly involved in the vasodilating and antiplugging effects of adenosine, which are particularly important during reperfusion.

A recent study has shown that AR subtypes may affect the magnitude of fibrosis [16]. For example, adenosine binding to $A_{2B}R$ inhibits collagenase production without affecting either stromelysin or tissue inhibitor of metalloprotease expression [17]. Thus, it is conceivable that adenosine may play a critical role in collagen synthesis and/or accumulation into the myocardium in the absence of hemodynamic factors.

The aim of this study was to map the local changes of myocardial AR expression in infarcted swine hearts with preserved LVEF. Our experimental model is consistent with the clinical condition of small LV infarct scar size (<15 % transmurality), which does not affect the global cardiac function (further parameters of in vivo hemodynamic alterations of this model were reported in our previous studies [18, 19]) (Table 1).

Methods

Study design and tissue collection

All animals received human cure, and the study protocols comply with the institution's guidelines.

This protocol was approved by the Italian Ministry of Health and was in accordance with Italian law (DL-116, 27 January 1992), which 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, revised 1996).

Fourteen healthy male sexually mature farm pigs (35–40 kg) were surgically instrumented in the Laboratory of Experimental Cardiology of Scuola Superiore Sant'Anna, Pisa, Italy, as previously described [18, 19]. They were randomized in two experimental groups: (1) MI ($n = 9$), (2) sham-operated animals (control, C: $n = 5$). Three MI animals died because of ventricular fibrillation after ligation of the left anterior descending (LAD) coronary artery. Four weeks after surgery, the cardiac performance of sedated

Table 1 Cardiac MRI measurements

	Normal	Myocardial infarction
Heart rate (beats/min)	90 ± 5.77	89.60 ± 8.2
Mean aortic pressure (mmHg)	118.33 ± 10.7	124.43 ± 11.5
ES volume (ml)	21.71 ± 5.5	24 ± 1.7
ED volume (ml)	60.86 ± 4.2	65.5 ± 4.5
Cardiac output (l/min)	4.62 ± 0.3	4.72 ± 0.4
Stroke volume (ml)	39.15 ± 1.34	41.5 ± 3.2
Ejection fraction (%)	64.32 ± 6.85	63 ± 1.5
End-systolic wall-thickening border zone (%)	64.16 ± 9.69	8.4 ± 16.6*
End-systolic wall-thickening remote zone (%)	68.45 ± 12.5	62 ± 9.3

* $p < 0.05$

animals was investigated by magnetic resonance imaging (1.5 T, Signa Excite HD; GE Medical Systems, Waukesha, WI) as reported earlier [18, 19].

Anesthetized pigs were killed by infusion of a cold cardioplegia solution to collect cardiac tissue for molecular and histological analysis [18, 19]. LV tissue samples were quickly harvested from the infarct border (BZ) and non-infarcted remote zones (RZ) of the MI group, and from healthy LV wall of the C group, and were immediately placed in ice-cold RNA*later* and stored at −80 °C.

Molecular analysis

RNA extraction

Total RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform method from LV tissue samples obtained from pig hearts with the Rneasy Midi kit (Qiagen S.p.A, Milano, Italy) as previously described [19–25]. The RNA samples were stored at −80 °C for subsequent gene expression studies.

Real-time PCR

Following DNAse treatment, first strand cDNA was synthesized with iScript cDNA Synthesis kit (Bio-rad, Hercules, CA, USA) using about 1 μg of total RNA as template. Real-time PCR was conducted on a 96-well iQ Cycler Real-time PCR detection system (Bio-rad, Hercules, CA, USA) in a 25-μl final volume, using 12.5 μl of iQ SYBR Green Supermix 2X (Bio-rad, Hercules, CA, USA) and 2 μl of cDNA as template. Annealing temperatures vary depending on the primer pair used (Table 2).

A series of PCRs were performed using primer pairs for A_1R, $A_{2A}R$, $A_{2B}R$, A_3R and TNF-α. Primer pairs were designed with Primer Express version 2.0 (Applied Biosystems), and details are given in Table 2. Specificity of each primer pair, i.e., absence of artifacts, multiple PCR products or primer dimers, and PCR yields were checked by agarose-gel electrophoresis and by melting analysis. All reactions were performed in duplicate.

Immunohistochemistry analysis

Five-μm-thick sections of LV tissue samples from infarct BZ, RZ and healthy myocardium were used for immunohistochemical analysis as previously described [19].

A_3R expression was detected using a rabbit anti-human adenosine A_3R antibody (ADORA3, MBL International Corp., MA, USA) diluted 1:100 in BSA-PBS. This antibody reacts with pig A_3R on paraffined sections as specified by the supplier. For each selected specimen, at least three serial sections 1/10 were examined using a semi-quantitative scale of immunoreactivity: no (−), low (+) and high (++) staining. Negative controls, obtained by incubating the specimens with BSA-PBS, omitting the primary antibody, were performed to test the specificity of the secondary biotinilated antibody. Representative photomicrographs were taken by a DFC480 digital camera (Leica Microsystem, Cambridge, UK).

Table 2 Primers pairs

Gene	Primers	Annealing T°	GeneBank
A_1R	5′-ATCAGGTTACTTGGTTCT-3′	57°	AY772411
	5′-ATCAGGTTACTTGGTTCT-3′		
$A_{2A}R$	5′-GATCAGCCTCCGCCTCAACGGCCA-3′	60.5°	AY772412
	5′-TCAGGACACTCCTGCTCTGTCCTG-3′		
$A_{2B}R$	5′-TGGTGTACTTCAACTTCCTG-3′	60°	AY772413
	5′-GATCTTGGCGTAGATGGC-3′		
A_3R	5′-GGTGAAGTGCCAGAAGTTGTG-3′	60°	AY772414
	5′-AGCATAGACGATAGGGTTCATCAT-3′		
TNF-α	5′-TGACCACCACCAAGAATT-3′	60°	NM_214022
	5′-TGTTCTGAAGTATTCCGATTG-3′		
GAPDH	5′-TCGGAGTGAACGGATTTG-3′	59°	AF017079
	5′-CCTGGAAGATGGTGATGG-3′		
CypA	5′-GGGTGGTGACTTCACACGCCA-3′	60°	AY266299
	5′-TTGGGAACCGTTTGTGTTGGGGC-3′		
TBP	5′-GATGGACGTTCGGTTTAGG-3′	59°	DQ178129
	5′-AGCAGCACAGTACGAGCAA-3′		
ACTB	5′-TCTGGCACCACACCTTCT-3′	60°	DQ178122
	5′-TGATCTGGGTCATCTTCTCAC-3′		

Primer pair used for housekeeping and target genes in real-time qPCR experiments

A_1R adenosine: A_1 receptor, $A_{2A}R$ adenosine: A_{2A} receptor, $A_{2B}R$ adenosine: A_{2B} receptor, A_3R adenosine: A_3 receptor, TNF-α: tumor necrosis factor-α, GAPDH: glyceraldehyde 3-phosphate dehydrogenase, CypA: cyclophilin A, TBP: TATA-Binding protein, ACTB: beta-actin

Data analysis and statistics

Several reference genes were tested, and GeNorm software was used to define the most stably expressed gene set, as previously described [26, 27]. The geometric mean of the four most stably expressed genes (CYC, TBP, ACTB, GAPDH) was used for normalization of each gene mRNA expression in the samples [19]. The relative quantification was performed by the ΔΔCt method using the iQ5 Software (BioRad). Differences between more than two independent groups were analyzed by Fisher's test after ANOVA. The results are expressed as mean ± SEM, and p values were considered significant when <0.05.

Results

We analyzed LV myocardial tissue samples from the left ventricle with infarct scar size corresponding to 13 ± 1 % of the LV wall mass, as shown in our previous studies [18, 19].

Four weeks after acute LAD ligation, we observed a different LV regulation of AR expression (Fig. 1). A_1R mRNA expression was significantly lower in the BZ than in controls. Conversely, A_3R was higher in both BZ and RZ. No significant changes were observed at the level of $A_{2A}R$ and $A_{2B}R$ receptors in each LV sample. A significant correlation ($r = 0.55$, $p = 0.04$) was observed between $A_{2A}R$ and $A_{2B}R$ mRNA level. A_3R expression in cardiomyocytes was detected by immunohistochemical analysis that showed low immunostaining (+) for all C samples and strong immunoreaction (+++) for all BZ and RZ samples (Fig. 2). A_3R expression was almost undetectable in endothelial cells (Fig. 2 squares).

The TNF-α mRNA myocardial level tended to be higher in the LV infarct BZ compared to RZ and normal myocardium (Fig. 3), yet no statistically significant differences were detected.

Discussion

The main result of this study was that the myocardial expression of all AR subtypes is differently modulated in distinct regions of infarcted swine left ventricle in the absence of hemodynamic factors, but not in healthy myocardium.

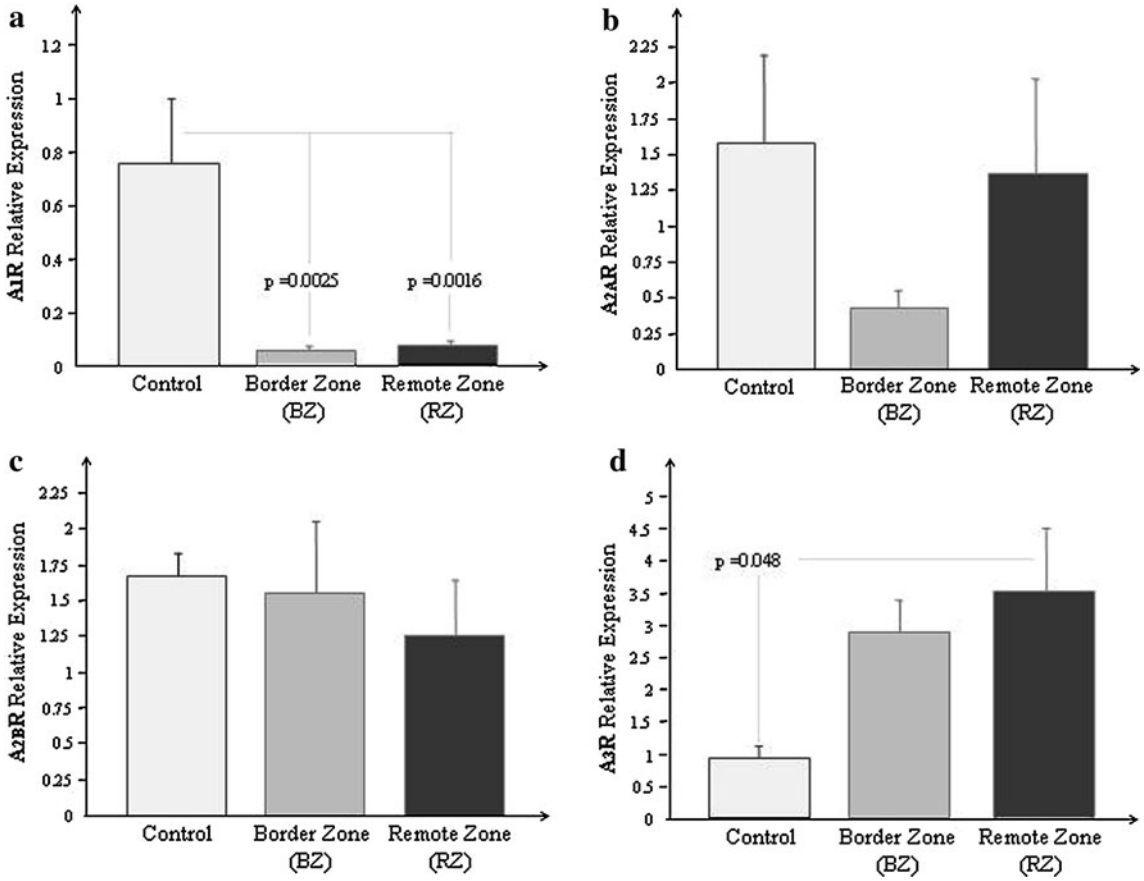

Fig. 1 **a** A_1R, **b** $A_{2A}R$, **c** $A_{2B}R$ and **d** A_3R mRNA expression measured by real-time PCR in the control (*white bars*), border zone (BZ) (*grey bars*) and remote zone (*black bars*)

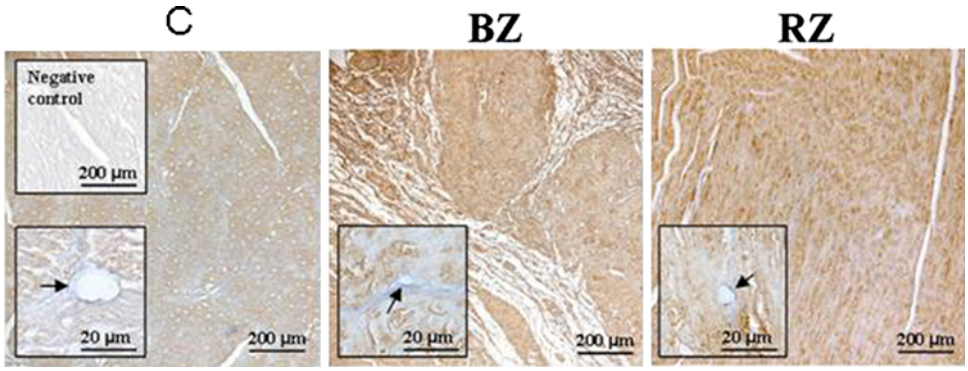

Fig. 2 A_3R immunohistochemical expression in the control (C), border zone (BZ) and remote zone (*brown color*). Negative control was obtained omitting the primary antibody. *Arrows* indicate endothelial cells

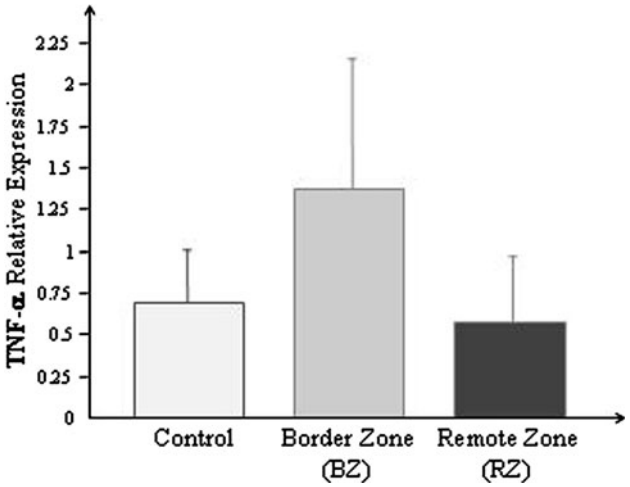

Fig. 3 TNF-α mRNA expression measured by real-time PCR in cardiac tissue of the control (*white bars*), border zone (BZ) (*grey bars*) and remote zone (*black bars*)

A transcriptional balance between A_1R and A_3R as well as $A_{2A}R$ and $A_{2B}R$ was observed, consistent with an adaptative retaliatory anti-ischemic adenosinergic mechanism after myocardial infarction even in the presence of a globally preserved LV function.

Our findings suggest that adenosine is a paracrine mediator of post-ischemic myocardial remodeling independent of mechanical stress.

The present study was partly in accordance with the fascinating hypothesis of Von Lubitz [28] that supports the existence of a concentration gradient of adenosine surrounding the ischemic infarct area. The ischemic "core" is characterized by diffuse necrosis and consequent massive formation of adenosine from catabolism of nucleic acids. It is conceivable that the extracellular concentrations of adenosine following myocardial necrosis are sufficient to induce a sustained regulation of the specific receptor apparatus of cardiomyocytes, in particular the A_3R receptors. Once the A_3Rs have been upregulated, they attenuate the inhibitory feedback on glutamate release by A_1R, resulting in further release of the neurotransmitter, hence in an enhancement of cell damage [29, 30].

The high adenosine concentrations could induce a desensitization of A_1R, which may explain the very low levels of mRNA expression observed for this receptor subtype in BZ and RZ as also suggested by a previous study in which a desensitization and internalization of A_1R were observed during periods of ischemia [31–33]. A_1R belong to the G protein-coupled receptor family but, unlike most in their family, A_1Rs have a long half-life [33] and seem to be resilient to desensitization [34]. In light of the evidence, A_3R seems to play a key role in the paracrine myocardial response during the ischemic remodeling process, yet its mechanism is still not well defined [35]. In this study the upregulated transcription of A_3R was also confirmed by immunohistochemical analysis where increased expression of A_3R in the infarct BZ and RZ compared to normal myocardial tissue from sham-operated animals was detected.

Since a similar and stable A_3R mRNA and protein expression was found in both LV regions, we further confirmed that the adenosinergic response of ischemic cardiomyocytes is modulated at the transcriptional level. However, these data are more controversial in the presence of significant deposits of collagen in the BZ region, which are absent in the RZ [18, 19]. We suggest that the local release of adenosine following MI causes an epigenetic domino effect on A_3R expression in series of cardiomyocytes, but the role is still to be proven.

The role of two A_2 subtypes under ischemic conditions is poorly understood, but is emerging as modulators of cardiovascular stress responses and inflammatory processes. Since both A_2 subtypes possess immunomodulatory and anti-inflammatory functions, they thus may well regulate the impact of inflammatory processes on ischemic and postischemic damage [36]. It has been speculated that under pathologic conditions, such as ischemia, $A_{2B}R$ may be upregulated to compensate for the downregulation of $A_{2A}R$-mediated responses [37]. In particular, $A_{2B}R$

upregulation has been detected in ischemic mouse hearts and in A_1R knockout animals [38, 39].

In this study, adenosine concentrations were not measured since this molecule is rapidly degraded into inosine, making its assessment technically very difficult, hence the physiological relevance of AR expression as an indirect marker of adenosine release.

The main limitation of the study is the lack of pharmacological approaches by adenosine agonists/antagonists to better comprehend the role of adenosine receptors in regulating myocardial infarction. However, data provided by this experimental model are valuable as a starting point for new studies focused on understanding and defining possible new pharmacological approaches in the setting of acute myocardial infarction in clinical practice.

Adenosine is one of the most intriguing cardioprotective molecules; data based on extensive literature on adenosinergic protection led Kitakaze et al. [8] to conclude in a recent paper: "we have asked what does stress to the heart do for adenosine, so now it is time to ask what adenosine can do for patients with heart disease."

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