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Original article

Involvement of CD73, equilibrative nucleoside transporters and inosine in rhythm and conduction disturbances mediated by adenosine A1 and A2A receptors in the developing heart☆

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ABSTRACT

We previously established that exogenous adenosine (ADO) induces transient arrhythmias in the developing heart via the adenosine A1 receptor (A_1AR) and downstream activation of NADPH oxidase/ERK and PLC/PKC pathways. Here, we investigated the mechanisms by which accumulation of endogenous ADO and its derived compound inosine (INO) in the interstitial compartment induce rhythm and conduction troubles. The validated model of the spontaneously beating heart obtained from 4-day-old chick embryos was used. Quantitative RT-PCR showed that enzymes involved in ADO and INO metabolism (CD39, CD73 and eADA) as well as equilibrative (ENT1, -3, -4) and concentrative (CNT3) nucleoside transporters were differentially expressed in atria, ventricle and outflow tract. Inactivation of ENTs by dipyridamole, 1) increased myocardial ADO level, 2) provoked atrial arrhythmias and atrio-ventricular blocks (AVB) in 70% of the hearts, 3) prolonged P wave and QT interval without altering contractility, and 4) increased ERK2 phosphorylation. Blockade of CD73-mediated phosphohydrolysis of AMP to ADO, MEK/ERK pathway inhibition or A_1AR inhibition prevented these arrhythmias. Exposure to exogenous INO also caused atrial ectopy associated with AVB and ERK2 phosphorylation which were prevented by A_1AR or A_{2A}AR antagonists exclusively or by MEK/ERK inhibitor. Inhibition of ADA-mediated conversion of ADO to INO increased myocardial ADO and decreased INO as expected, but slightly augmented heart rate variability without provoking AVB. Thus, during cardiogenesis, disturbances of nucleosides metabolism and transport, can lead to interstitial accumulation of ADO and INO and provoke arrhythmias in an autocrine/paracrine manner through A_1AR and A_{2A}AR stimulation and ERK2 activation.

1. Introduction

Adenosine, which derives from ATP degradation, is an important regulator of the function of the developing cardiovascular system [1–4] and accumulates under hypoxia [5]. Under physiological conditions, although the embryo develops normally in a low oxygen environment ($PO_2 \approx 0$–8 kPa), the heart shows both aerobic (mitochondrial oxidation) and anaerobic (glycolysis) energy-producing capacities [6,7]. Under hypoxia or anoxia, the imbalance between oxygen availability and utilization in the embryonic myocardium exacerbates both lactate and adenosine production. Adenosine which derives from both intra- and extracellular ATP catabolism can interact with the four subtypes of receptors, A_1AR, A_{2A}AR, A_{2B}AR and A_3AR [8]. Although ADO is protective against hypoxia in the developing embryo via A_1AR [9,10], exposure of the embryonic heart to adenosine can also disturb pacemaking and conduction via A_1AR. These arrhythmias occur through concomitant stimulation of NADPH oxidase and PLC, followed by downstream activation of ERK2 and PKC with L-type calcium channel and transient receptor potential canonical 3 (TRPC3) channel as possible targets [11–13]. In human as well as in mouse, activation of adenosine receptors can stimulate compensatory signaling pathways that lead to cellular resistance to subsequent hypoxia [14–16] and extracellular accumulation of adenosine is involved in either protective or deleterious processes [10,17]. Adenosine is mostly used as an anti-arrhythmic agent in patients, but can also induce bradyarrhythmias, tachyarrhythmias and atrio-ventricular blocks [18–20].

The extracellular (interstitial) concentration of adenosine is controlled by the balance between its enzymatic production and degradation into inosine and by trans-membrane transport processes [21,22]. The sequential action of ectonucleoside triphosphate diphosphohydrolase (eNTPDase, CD39) and 5′-nucleotidase (CD73) convert ATP to adenosine, whereas adenosine kinase (AK) and adenosine deaminase (ADA) are the

Abbreviations: ADO, adenosine; INO, inosine; AR, adenosine receptor; AVB, atrio-ventricular block; eADA, ecto adenosine deaminase.

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main players in adenosine metabolism [23]. ADA catalyzes the hydrolytic deamination of adenosine to inosine and ADA deficiency increase venous efflux of adenosine in mouse heart [24]. ADA is located both in the cytosol and on extracellular side of the membrane, and recent evidence suggests that ecto-ADA (eADA) can form a functional complex with A_1AR [25].

Transport of adenosine and inosine across cell membranes is mediated by nucleoside transporters, which are classified as either equilibrative (bidirectional) or concentrative and regulate intra- and extracellular adenosine levels [26,27]. The equilibrative nucleoside transporter (ENT) family, also known as SLC29, is a group of 4 plasmalemmal transport proteins, designated ENT1, -2, -3 and -4, which allow a diffusion process of nucleoside substrates like adenosine or inosine [28]. The predominant isoform ENT1 in human and mouse heart, has been shown to mediate protection against hypoxia by decreasing extracellular adenosine levels and thus adenosine receptors signaling [15]. Although the presence of ENT1 was demonstrated in the retina of the chick embryo [29,30], the expression and function of other ENTs isoforms are unknown in the embryonic chick heart model. Similarly, the expression of the subtypes of sodium-dependent concentrative nucleoside transporters CNT (CNT1, -2 and -3) belonging to the family SLC28, remains to be examined in the developing heart.

Adenosine is the most studied nucleoside; however, inosine is a contributing factor in protective responses in cells and tissues [31,32] and might be a biomarker of initial cardiac ischaemia [33]. Although inosine can bind to A_1AR, A_{2A}AR and A_3AR, the targeted subtypes are different depending on various models and conditions [34–41]. Inosine accumulates during hypoxia, is transported by ENT1 and -2 and involved in hypoxic preconditioning of cardiomyocytes, neuroprotection, anti-inflammatory processes and hepatic function [32,42–46].

The cellular mechanisms by which a rise of interstitial adenosine and inosine under pathological conditions such as intrauterine hypoxia can induce rhythm and conduction disturbances remain unclear. In particular, expression and roles of enzymes and transporters involved in nucleosides metabolism deserve to be investigated in the developing heart.

2. Materials and methods

2.1. Drugs, antibodies and reagents

Adenosine (ADO), inosine (INO), DPCPX (A_1AR antagonist), SCH58261 (A_{2A}AR antagonist), MRS1754 (A_{2B}AR antagonist), MRS1523 (A_3AR antagonist), EHNA (inhibitor of eADA), AOPCP (inhibitor of CD73), dipyridamole (DIP, inhibitor of ENTs) and U0126 (MEK inhibitor) were purchased from Sigma Aldrich. DIP was prepared in dimethyl sulfoxide (DMSO) and the other drugs in buffered medium. Final working solutions contained no more than 0.1% DMSO which had no effect on protein phosphorylation and cardiac function. For HPLC, all solvents were HPLC grade (Carlo Erba Reagenti, Italy). Goat anti-rabbit horseradish peroxidase-conjugated secondary antibody used for Western Blotting determinations was from GE Healthcare, anti-p44/42 MAPK (ERK 1/2) and anti-phospho-p44/42 MAPK (ERK 1/2 Thr202/Tyr204) antibodies were from Cell Signaling. In contrast to other species, ERK 1 is not detectable in the chicken [47].

2.2. Experimental procedures

Fertilized eggs from Lohman Brown hens were incubated for 96 hours at 38 °C and 95% relative humidity to obtain stage 24HH embryo according to Hamburger and Hamilton [48].

In order to assess arrhythmogenic effect of the used agents alone or in combination, isolated hearts, dissected atria or ventricles were placed in 24-plastic-wells containing 1 ml of buffered medium at 37.5 °C on the thermostabilized stage of an inverted microscope (Leica DMI3000 B, Wetzlar, Germany). Isolated hearts, atria or ventricles were subjected to 45 min stabilization, followed by 30 min pre-treatment with ADO receptors antagonists, AOPCP, EHNA or vehicle (control) (see online supplement Fig. S1). Then, the preparations were treated with INO or DIP in combination with the previous compounds. Atrial and ventricular beating rates were measured at 0, 7, 15, 30, 45 and 60 min in control and in treated hearts. The ratio of the number of arrhythmic hearts (all types of arrhythmias) to rhythmic hearts at a given time point was determined and reported in figures as "% of arrhythmic hearts." Samples were collected for western blotting at each time point.

In order to characterize the electrical and contractile activity, spontaneously beating hearts or isolated atria or ventricles were placed in the culture compartment of a stainless airtight chamber maintained at 37.5 °C as described elsewhere in detail [49]. Electrocardiogram (ECG) of whole hearts or electrogram (EG) of dissected atria and ventricles were performed in the chamber.

Arrhythmias were characterized mostly according to the classical diagnosis used in clinical human practice and were considered as present if documented at least once in a given recording [49]. The main arrhythmias observed were bradycardia, atrial ectopy and Wenckebach phenomenon associated with second-degree AVB. Atrial flutter/fibrillation was not observed in our embryonic model.

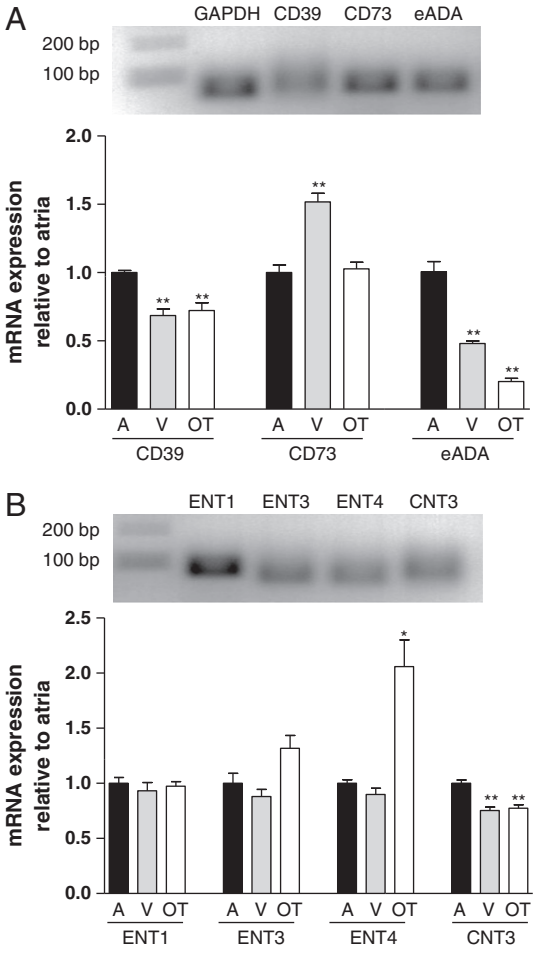

Fig. 1. Expression of enzymes CD39, CD73 and eADA (A) and transporters ENT1, -3, -4 and CNT3 (B) involved in ADO metabolism. PCR products in atria (upper panel) and quantification (lower panel) showed that CD39, CD73, eADA, ENT1, ENT3, ENT4 and CNT3 mRNA were differentially expressed in atria (A, black bars), ventricle (V, gray bars) and outflow tract (OT, white bars). GAPDH: housekeeping gene. Quantification was normalized by atrial value. *, $p < 0.05$ and **, $p < 0.01$ compared with expression in atria, $n = 3$ determinations; 3 biological duplicates for each determination. Size of different unique products: see online supplement Table S1.