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Altered expression of connexin 43 and related molecular partners in a pig model of left ventricular dysfunction with and without dipyrydamole therapy

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ABSTRACT

Gap junctions (GJ) mediate electrical coupling between cardiac myocytes, allowing the spreading of the electrical wave responsible for synchronized contraction. GJ function can be regulated by modulation of connexon densities on membranes, connexin (Cx) phosphorylation, trafficking and degradation. Recent studies have shown that adenosine (A) involves Cx43 turnover in A_1 receptor-dependent manner, and dipyridamole increases GJ coupling and amount of Cx43 in endothelial cells. As the abnormalities in GJ organization and regulation have been described in diseased myocardium, the aim of the present study was to assess the regional expression of molecules involved in GJ regulation in a model of left ventricular dysfunction (LVD). For this purpose the distribution and quantitative expression of Cx43, its phosphorylated form pS368-Cx43, PKC phosphorylated substrates, RhoA and A receptors, were investigated in experimental models of right ventricular-pacing induced LVD, undergoing concomitant dipyridamole therapy or placebo, and compared with those obtained in the myocardium from sham-operated minipigs. Results demonstrate that an altered pattern of factors involved in Cx43-made GJ regulation is present in myocardium of a dysfunctioning left ventricle. Furthermore, dipyridamole treatment, which shows a mild protective role on left ventricular function, seems to act through modulating the expression and activation of these factors as confirmed by in vitro experiments on cardiomyoblastic cell line H9c2 cells.

1. Introduction

Gap junctions (GJs) are clusters of transmembrane channels that mediate electrical coupling between cardiac myocytes, allowing the spreading of the electrical wave responsible for synchronized contraction in the heart [1]. These channels consist of two apposing connexon complexes, which allow the direct diffusion of ions and small molecules between adjacent cells [2]. Each connexon comprises six connexin (Cx) proteins, among which the isotype Cx43 is the most widely expressed as well as the most represented in cardiac myocytes gap junctions [3,4].

GJ functions can be regulated at different levels by a variety of mechanisms such as modulation of connexon densities on cell membranes and Cx phosphorylation, which leads to modification of channel conductance as well as Cx trafficking and degradation [5]. Protein kinase C (PKC) has been demonstrated to phosphorylate Cx43 on Ser368 and this state has been shown to induce a reduction in intercellular communication [6]. RhoA, a member of small GTPases, known as key regulator of many cell functions [7], regulates the permeability of Cx43-made GJs in cardiac myocytes [8]. A recent in vitro study has shown that adenosine, an important endogenous physiological modulator of heart function, involves Cx43 turnover in an A_1 receptor-dependent manner [9]. Furthermore, it has recently been demonstrated that the antiplatelet drug dipyridamole increases GJ coupling [10,11] and the amount of Cx43 [12] in smooth muscle and endothelial cells. Increased endogenous adenosine accumulation, achieved with chronic oral

Abbreviations: GJ, gap junction; Cx, connexin; PKC, proteine kinase C; PKCps, phosphorylated PKC substrate; A, adenosine; ARs, adenosine receptor subtypes; LVD, left ventricular dysfunction; RV, right ventricle; LV, left ventricle; LVEDD, left ventricle end diastolic diameter; LVESD, left ventricle end systolic diameter; LVFS, left ventricular fractional shortening; H&E, haematoxylin–eosin; pS368-Cx43, connexin 43 phosphrylated at serina 368; SEM, standard error of the mean.

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dipyridamole administration, is known to exert anti-inflammatory, pro-angiogenetic, anti-fibrotic and anti-apoptotic effects, targeting different adenosine receptors subtypes (ARs) (A_1, A_{2A}, A_{2B} and A_3) and cell populations (cardiomyocytes, endothelial cells, leukocytes, cardiac fibroblasts). Thus, a cardioprotective role of this drug in conditions as chronic congestive heart failure has been postulated [13–15].

For the reasons stated above and since the abnormalities in GJ organization and regulation have been implied in different myocardial diseases [1,16,17], the aim of the present study was to assess the regional expression of molecules involved in GJ regulation in a model of left ventricular dysfunction (LVD). For this purpose, the distribution and quantitative expression of Cx43, its phosphorylated form pS368-Cx43, PKC phosphorylated substrates, RhoA and ARs, were investigated in animals with LVD induced by high frequency right ventricular (RV)-pacing. The animals were under concomitant dipyridamole therapy (DP+) or placebo (DP−); sham operated minipigs (C-SHAM) were considered as controls. In addition, the possible dipyridamole signalling transduction pathway was explored by employing the cardiomyoblastic cell line H9c2.

2. Methods

2.1. Experimental animal protocol

Animal instrumentation and experimental protocols were approved by the Animal care Committee according to Italian legislation, following the National Institute of Health publication *Guide for Care and Use of Laboratory Animals*. The experiments were performed according to the guidelines of the European Communities Council Directive 2010/63/EU on the use of animals for scientific purposes.

Twelve minipigs (weight 35–40 kg) were randomly assigned to one of three experimental groups: (1) C-SHAM ($n = 4$), sham operated used as controls, (2) DP+ ($n = 4$), left ventricular dysfunction (LVD) under concomitant dipyridamole therapy (5 mg/kg/daily, Persantin®, Boehringer Ingelheim, Milan, Italy) or (3) DP− ($n = 4$), LVD under placebo therapy. LVD was induced by pacing at 200 bpm in the right ventricular (RV) apex according to previously published protocol [18].

All animals underwent a 2D and M-mode EchoDoppler examination immediately after single chamber pacemaker implantation (t_0) and after 4-weeks (t_4) of RV tachycardic pacing. Then all minipigs were sacrificed with an intravenous injection of 10 ml of KCl and left ventricular (LV) tissue samples were collected according to the guidelines of the American Heart Association [19] after the following sectioning (Fig. 1): from base to the apex, the LV was divided into 16 segments of which six segments in the basal, six segment in the mid portions (anteroseptal, inferoseptal, anterior, anterolateral, inferolateral and inferior walls) and four at the apex (septal, anterior, lateral and inferior walls). In particular, three segments closest to pacing site (8, 9 and 14) and three segments from the opposite site (5, 11 and 12) were collected for this study as reported in a previous published paper [20].

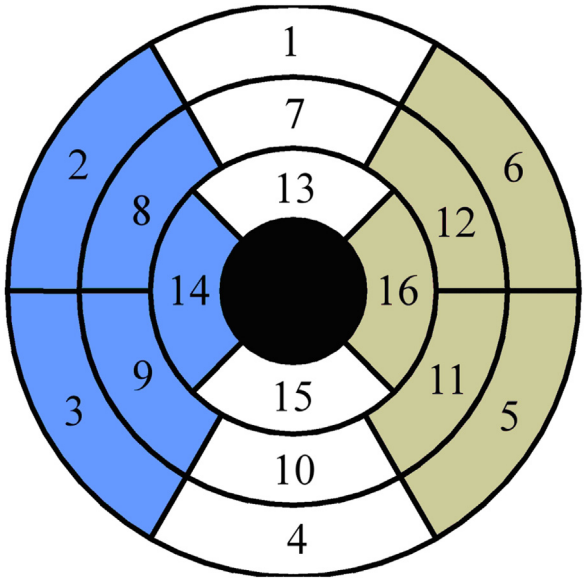

Fig. 1. A circumferential polar plot of the 16 segments (the 17 segment-apex is not considered and is coloured in black) representing the protocol used to group the LV segments in three regions. The septal, adjacent and lateral regions are respectively represented by 2, 3, 8, 9, 14 segments (pacing site), 1, 4, 7, 10, 13, 15 segments and 5, 6, 11, 12, 16 segments (opposite site).

Tissue samples were immediately placed in ice-cold RNAlater (Qiagen, Germany) or 10% formalin solution (Sigma–Aldrich, St. Louis, MO, USA) for following molecular and histological analysis, respectively.

2.2. Echocardiographic examination

Transthoracic echocardiography was performed by one experienced investigator who was blind to underlying treatment using commercially available General Electric Vivid-e equipment. Two dimensional and M-mode data were acquired from the parasternal long and short axis view at the level of papillary muscles and digitally stored for off-line analysis. LV end diastolic diameter (LVEDD), LV end systolic diameter (LVESD) and derived LV fractional shortening (LVFS) were measured in basal conditions (t_0) and after 4 weeks (t_4) pacing.

2.3. Histological analysis

The routinely formalin-fixed and paraffin-embedded LV samples were cut into 8 μ-thick sections, which were serially mounted on glasses. Immediately before use, slides were dewaxed, rehydrated and processed for both routine haematoxylin–eosin (H&E)

Table 1
Antibodies used in this study.

Antibody	Species	Source	Dilution	
			Tissue sections	Cultured cells
Anti-Cx43	Mouse monoclonal	Santa Cruz Biotechnology, Santa Cruz, CA, USA	1:5000	1:200
Anti-phospho-Cx43 (pS368-Cx43)	Rabbit polyclonal	Santa Cruz Biotechnology	1:100	1:50
Anti-RhoA	Mouse monoclonal	ThermoFisher Scientific, Waltham, MA, USA	1:300	
Anti-phospho-PKC substrate (PKCps)	Rabbit polyclonal	Cell Signalling Technology, Beverly, MA, USA	1:600	1:300
Anti-A_{2A}R	Mouse polyclonal	Novus Biological, Cambridge, UK		1:50
Biotinylated anti-mouse immunoglobulins	Goat polyclonal	Vector, Burlingame, CA, USA	1:200	1:200
Biotinylated anti-rabbit immunoglobulins	Goat polyclonal	Vector	1:200	1:200
Alexa Fluor®488 anti-mouse	Goat IgG	Life Technologies Italia, Monza, Italia		1:250
Alexa Fluor®568 anti-rabbit	Donkey IgG	Life Technologies Italia		1:250