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Original article

P2 receptors in human heart: upregulation of $P2X_6$ in patients undergoing heart transplantation, interaction with TNFα and potential role in myocardial cell death

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Abstract

ATP acts as a neurotransmitter via seven P2X receptor-channels for Na^+ and Ca^{2+}, and eight G-protein-coupled P2Y receptors. Despite evidence suggesting roles in human heart, the map of myocardial P2 receptors is incomplete, and their involvement in chronic heart failure (CHF) has never received adequate attention. In left myocardia from five to nine control and 5–12 CHF subjects undergoing heart transplantation, we analyzed the full repertoire of P2 receptors and of 10 "orphan" P2Y-like receptors. All known P2Y receptors (i.e. $P2Y_{1,2,4,6,11,12,13,14}$) and two P2Y-like receptors (GPR91 and GPR17) were detected in all subjects. All known $P2X_{1\text{-}7}$ receptors were also detected; of these, only $P2X_6$ was upregulated in CHF, as confirmed by quantitative real time-PCR. The potential significance of this change was studied in primary cardiac fibroblasts freshly isolated from young pigs. Exposure of cardiac fibroblasts to ATP or its hydrolysis-resistant-analog benzoylATP induced apoptosis. TNFα (a cytokine implicated in CHF progression) exacerbated cell death. Similar effects were induced by ATP and TNFα in a murine cardiomyocytic cell line. In cardiac fibroblasts, TNFα inhibited the downregulation of $P2X_6$ mRNA associated to prolonged agonist exposure, suggesting that, by preventing ATP-induced $P2X_6$ desensitization, TNFα may abolish a defense mechanism meant at avoiding Ca^{2+} overload and, ultimately, Ca^{2+}-dependent cell death. This may provide a basis for $P2X_6$ upregulation in CHF. In conclusion, we provide the first characterization of P2 receptors in the human heart and suggest that the interaction between TNFα and the upregulated $P2X_6$ receptor may represent a novel pathogenic mechanism in CHF.

Keywords: Heart failure, Receptors; Gene expression; Apoptosis; Cytokines

1. Introduction

Despite available therapies, chronic heart failure (CHF) remains the major cause of ill health in industrial societies [1]. This suggests that, besides neurohumoral factors and pro-inflammatory cytokines (ibidem [2]), other transmitter systems are involved and further therapeutic targets remain to be discovered.

Despite initial results demonstrating effects of extracellular nucleotides in the cardiovascular system [3–5], for several decades myocardial ATP has been merely regarded as a major fuel for cardiac contraction. Recent data confirming highly specific roles of adenine (ATP, ADP), uracil (UDP, UTP) and sugar nucleotides (UDP-glucose and UDP-

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galactose) in various organs and systems via cell-surface receptors [3,6] and demonstrating ATP release from myocardial sympathetic terminals and hypoxic cardiomyocytes [5] suggest new important functions of nucleotides in cardiac tissue. In particular, nucleotides act through seven ligand-gated channels for Na^+ and Ca^{2+} (the $P2X_{1-7}$ receptors) and eight G protein-coupled P2Y receptors (the $P2Y_{1,2,4,6,11,12,13,14}$ subtypes) related to intracellular cAMP or calcium release from intracellular stores [4,6,7]. The human genome may contain additional yet-unidentified P2Y receptors [6,8], since several "orphan" G protein-coupled receptors (oGPCRs) are structurally and phylogenetically related to P2Y receptors [9,10]. These receptors contain some structural aminoacid motifs that, in known P2Y receptors, seem to be important for ligand binding [6,8]. Thus, the "deorphanization" of these "P2Y-like" receptors and their identification in human tissues may help identifying new targets for novel therapeutic strategies to human diseases, including CHF. Conversely, $P2X_{1-7}$ receptors [7] can form homomeric and heteromeric channels allowing Na^+ and Ca^{2+} influxes, and have been implicated in fast excitatory transmission [7,11] and in apoptotic cell death [12].

Nucleotides exert multiple effects in the cardiovascular system [5,13]. ATP regulates coronary vascular tone synergistically with alpha-adrenoceptors [4], likely via P2X receptors inducing vasoconstriction, while in some coronary vessels it produces vasodilation through P2Y receptors (ibidem). Nucleotides induce rat cardiac myocyte contraction possibly through P2X receptors, and/or by increasing L-type Ca^{2+} currents (summarized in [13]). Positive inotropism by ATP has been also attributed to P2Y receptors [14]. In ferret ventricular myocytes, ATP can inhibit L-type Ca^{2+} currents, the action potential plateau and myoplasmic Ca^{2+} transient [13]. Similarly, both increases and decreases of Ca^{2+} transients by ATP have been described (ibidem). In patch-clamp experiments, the effect of ATP on the Ca^{2+} current was additive, with maximal stimulation induced by alpha-adrenergic agonist (ibidem). However, with the sole exception of $P2X_4$, which, upon myocardial-specific overexpression in the mouse, has been suggested to play a role in cardiac contractility [15], it is at present difficult to ascribe a given effect of ATP to a given receptor, and a complete map of P2X and P2Y receptors in human heart is lacking. On this basis, the present study was undertaken to analyze the full repertoire of P2 receptors in non-failing and failing human heart and to address the potential importance of detected changes in CHF. Results show a selective upregulation of the $P2X_6$ receptor channel in failing human heart and suggest that an interaction of this receptor with the proinflammatory cytokine TNFα may contribute to CHF pathogenesis.

2. Materials and methods

2.1. Subjects and heart tissue samples

Non-failing left ventricular tissue was harvested from five to nine donor hearts excluded from transplantation for technical reasons (mean ejection fraction: 65 ± 4%) at the Italian Homograph Bank at Monzino Cardiologic Center, Milan, Italy and stored at –80 °C (mean age of donors 46 ± 2.5 years; six males and three females). Non-necrotic tissue from 5 to 12 failing hearts was obtained from patients undergoing cardiac transplantation at Niguarda Hospital, Milan, Italy (mean age of patients 54 ± 3.7 years; seven males and five females). Causes of heart failure included idiopathic ($N = 7$) and ischemic cardiomyopathy ($N = 5$). Ejection fraction was < 20%. None was treated with left ventricular assist devices or received chronic intravenous inotropic support for at least 7 days immediately before transplant. Heart failure therapy consisted of angiotensin-converting enzyme inhibitors and diuretics in all patients. The study protocol was approved by the Ethics Committee of Monzino Cardiologic Center and Niguarda Hospital. The investigation conforms with the principles outlined in the Declaration of Helsinki.

A cardiomyocyte-enriched cell preparation was obtained from two CHF patients by heart tissue digestion with trypsin and collagenase, followed by centrifugation at 38 × *g* for 4 min. The pellet contained rounded myocytes while the supernatant contained non-myocyte heart cells (fibroblasts).

2.2. Bioinformatic analysis

P2 receptor sequences were obtained from GeneBank™ (www.ncbi.nlm.nih.gov/Entrez/). Sequences of oGPCRs were found at www.gpcr.org. Pig $P2X_6$ sequence was obtained from TIGR Pig Gene Index (accession number BF075096). BLAST searches were performed through www.ncbi.nlm.nih.gov/blast. Amino-acid sequences were aligned with ClustalX 1.8. Phylogenetic trees were generated with TreeView 1.5.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from endomyocardial biopsies (a total of 4–8 mg) by the guanidinium thiocyanate phenol–chloroform method [16]. A double extraction was used to eliminate small amounts of contaminating DNA. One microgram of RNA was reverse-transcribed and RT-PCR performed as previously described in [17]. Each sample was run in triplicate. Control samples lacking reverse transcriptase were processed in parallel with the same experimental protocol. All reagents were from Applied Biosystems (Milan, Italy). Amplifications were performed in a GeneAmp 9700 thermal cycler (Applied Biosystems) for 30–40 cycles (94 °C for 45 s, 45 s at an annealing temperature of 51–60 °C, 72 °C for 45 s), after denaturation at 94 °C for 2 min, using primers designed with Oligo4 (for $P2Y_{1,4,6}$ see [17], for $P2Y_2$ see [18], for all other primers and amplification conditions, see Table 1). Amplified products were size-separated by electrophoresis on a 1.5% agarose gel.

2.4. Real time-PCR

Real time-PCR was performed using SYBR Green technology on an ICycler thermal cycler (Biorad). The nucle-