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Journal of Molecular and Cellular Cardiology 39 (2005) 929–939
Journal of Molecular and Cellular Cardiology
www.elsevier.com/locate/yjmcc

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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Received 12 May 2005; received in revised form 31 August 2005; accepted 7 September 2005

Available online 19 October 2005

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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doi:10.1016/j.yjmcc.2005.09.002

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-

Table 1
Sequences of oligonucleotide primers, annealing temperatures and expected product length (bp) of amplification products for RT-PCR analysis of P2 receptors and P2Y-like orphan GPCRs

Primer	Sequence	T_m (°C)	Product length (bp)
$P2Y_{11}$	FW: 5′-ACTTCCTGTGGCCCATACTG RW: 5′-GCTGTCCCCAGACACTTGAT	56	499
$P2Y_{12}$	FW: 5′-CCCTCCAGAATCAACAGTTAT RW: 5′-CGCTTTGCTTTAACGAGTTC	52.4	1127
$P2Y_{13}$	FW: 5′-TGTGTCGTTTTTCTTCGGTG RW: 5′-TGCTGCCAAAAAGAGAGTTG	52.2	568
$P2Y_{14}$	FW: 5′-CGCAACATATTCAGCATCGTGT RW: 5′-GCTGTAATGAGCTTCGGTCTGA	50.9	102
GPR17	FW: 5′-GACTCCAGCCAAAGCATGAA RW: 5′-GGGTCTGCTGAGTCCTAAACA	59.6	1087
GPR26	FW: 5′-ATGAACTCGTGGAACGCGGGC RW: 5′-TCACTCAGACACCGGCAGAAT	61.6	1014
GPR34	FW: 5′-CCACAGAAGACAATGAGAA RW: 5′-TCAAAGAATTAAGAGGCTG	49.8	1207
GPR78	FW: 5′-ATGGGCCCCGGCGAGGCGCTG RW: 5′-TCAGTGTGTCTGCTGCAGGCA	63.2	1092
GPR80	FW: 5′-ATGAATGAGCCACTAGACTAT RW: 5′-TCAAGGGTTGTTTGAGTAACT	52	1014
GPR84	FW: 5′-TCAGCCTCTATCATGTGGAAC RW: 5′-CCTAATCTCCTATTACCTGGC	56.6	1277
GPR87	FW: 5′-CCACCACAATGAAAGAAAT RW: 5′-CCAACAAACAATAAAAGG	51.9	1209
GPR91	FW: 5′-ATGGCATGGAATGCAACTTG RW: 5′-CTGTTTCACAAGCCCCTCAC	54.5	1009
GPR102	FW: 5′-ATGACCAGCAATTTTTCCCAA RW: 5′-TTATTCTAAAAATAAACTAAT	48.5	1029
H963	FW: 5′-GGAATCCTGGCTTTATATCTTA RW: 5′-GGCAGAATTGGTAGCACA	51.6	1085
$P2X_1$	FW: 5′-GTTTGGGATTCGCTTTGA RW: 5′-TGGCTGAGAGGGTAGGAGAC	57.4	384
$P2X_4$	FW: 5′-CACCCACAGCAACGGAGTCT RW: 5′-TTTGATGGGGCTGTGGAGAG	57.8	793
$P2X_5$	FW: 5′-GCTGGAAACGGAGTGAAG RW: 5′-GCCTCCTGGGAACTGTCT	55.7	665
$P2X_6$	FW: CGCCAGCCCAAGTTCAGG RW: GCCTACAGAGCCACCCAG	57.9	440
$P2X_7$	FW: 5′-TGAAGGGAACCAGAAGACC RW: 5′-AACCAGGATGTCAAAACG	53.7	499
Pig P2X6	FW: 5′-GCACGCACAGCCACGGTATC RW: 5′-CCAGGTCGCAATCCCATCG	60.5	356
GAPDH	FW:5′-CCACCCATGGCAAATTCCATGGCA RW:5′-TCTAGACGGCAGGTCAGGTCCACC	58	593

otide sequences for forward and reverse primers were each designed in a different exon of the target gene sequence, eliminating the possibility of amplifying genomic DNA: $P2X_6$ (T_a = 60 °C) FW5′: ATGTGGCCGACTTCGTGAA, RW5′: TGGTCACCAAGAAGAACACGTT, 18S (T_a = 60 °C) FW5′: CGGCTACCACATCCAAGGAA, RW5′: CCTGTATTGTTATTTTTCGTCACTACCT. For each set of primers, a basic local alignment search tool (BLAST) revealed that sequence homology was obtained only for the target gene. Prior optimization was conducted for each set of primers, which consisted of determining optimal primer and $MgCl_2$ concentrations, the template concentration and verifying the efficiency of the amplification. To confirm the specificity of the amplification, the PCR product was subjected to a melting curve analysis and agarose gel electrophoresis. PCR amplification (performed in triplicate in a total reaction volume of 25 μl) was allowed to proceed 50 cycles, each consisting of denaturation at 95 °C for 15 s, annealing and extension at 60 °C. Results were normalized to 18S transcription to compensate for variation in input RNA amounts and efficiency of RT.

2.5. Western blotting (WB) analysis

Heart specimens were ground to powder, resuspended in 20 mM HEPES, 20 mM NaCl, 5 mM EDTA, 1% CHAPS with protease inhibitors, homogenized (PT 1200, Polytron), sonicated (20 s, three times) and centrifuged (13,000 rpm at 4 °C). Proteins in supernatants were electroforetically-separated and transferred to nitrocellulose as previously described in [17]. After incubation at 4 °C in blocking buffer, membranes were incubated with primary antibodies followed by horseradish peroxidase-conjugated goat anti-rabbit or rabbit anti-goat (for $P2X_6$ and $P2Y_6$) IgG. Bands were visualized by ECL (Amersham Pharmacia Biotech). The following antisera were used: $P2Y_{1,2,4,12}$ and $P2X_{1,2,4,7}$ (1:200, Alomone Labs, Israel); $P2Y_6$ and $P2X_6$ (1:100, Santa Cruz Biotechnology, CA, USA); $P2X_3$ (1:1000, Neuromics, Minneapolis, MN, USA); ERK1/2 (1:500, Calbiochem, Germany). Specificity of bands was tested in the presence of neutralizing peptides (μg ratio 1:1 between peptide and antiserum).

2.6. Isolation and culture of pig cardiac fibroblasts

Left ventricular tissue from healthy young pig heart was minced and digested at 37 °C in 0.1% trypsin and 200 U/ml collagenase (type IV, Sigma-Aldrich, St. Louis, MO, USA) for 10 min. Cells from the third to ninth digestions were plated on 100-mm culture dishes in Dulbecco's modified Eagle medium (DMEM, Invitrogen, Milan, Italy) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich) and incubated for 2 h at 37 °C in a humidified incubator with 10% CO_2. Unattached cells (cardiomyocytes) were discarded, and attached cells grown in DMEM + 10% FBS. For viability studies, 1×10^4 cells at passages 3–5 were seeded on polylysine-coated glass coverslips, exposed 3 days later to the pharmacological agents described in Figure legends and stained with Hoechst 33258 dye. Cell number in an identical area/coverslip was then measured as described in [19].

2.7. HL-1 cardiomyocytes

HL-1 cardiomyocytes were a kind gift from Professor W.C. Claycomb, LSU Health Sciences Center, New Orleans, LA, USA [20]. Cells were cultured in Claycomb medium supplemented with 10% FCS (both from JRH Biosciences, UK) following Professor Claycomb's instructions. 48×10^3 cells were seeded in 35 mm plates. The next day, medium was replaced

and, after 8 hours, HL-1 cells were pre-treated 16 hours with TNFα (10 ng/ml) followed by addition of ATP (500 μM final concentration).

2.8. Micrographs and flow cytometry

For flow cytometric analysis, cells were plated in 35-mm dishes (1×10^5 cells in the case of pig fibroblasts; for HL-1 cardiomyocytes, see above) and, after either 24 or 72 hours (see Figure legends) micrographs taken with a CCD Video Camera Module (Sony). Afterwards, the same cultures were collected and percentage of apoptosis and necrosis in the total cell population (i.e. adhering + detached cells) was determined by flow cytometric analysis of propidium iodide-stained nuclei [19].

2.9. Statistical analysis

Data are expressed as mean ± S.E.M. Differences were evaluated by the StatView program, using analysis of variance (ANOVA) followed by Fisher's or Scheffe's test. $P < 0.05$ was considered significant. A semi-quantitative densitometric analysis of RT-PCR data were obtained by normalizing values for each subject to the corresponding value of housekeeping GAPDH (data expressed in arbitrary units (AU), area × optical density (OD)). In a similar way, the software KDS IS440CF was used to normalize densitometric WB data to the corresponding values of ERK1/2, which undergoes no quantitative changes in CHF [21].

3. Results

3.1. Selection of "P2Y-like" receptors

"P2Y-like" oGPCRs were selected based on: (a) sequence identity with known P2Y receptors, and (b) presence in TM6 and TM7 of aminoacids critical for nucleotide binding (see [6] and references therein [22,23]). Ten oGPCRs (GPR17, GPR26, GPR34, GPR78, GPR80, GPR84, GPR87, GPR91, GPR102 and H963, see Fig. 1) were selected. While this study was in progress, GPR80 and GPR91 were reported to be activated by two citric acid cycle intermediates, α-ketoglutarate and succinate, respectively [24].

3.2. Map of P2Y and "P2Y-like" receptors in non-failing and failing human hearts

For all receptors, mRNAs and (based on antibodies availability) protein levels were determined in individual samples of left ventricular wall from control and CHF subjects (indicated with numbers 1–8 in Fig. 2). Transcripts for all P2Y subtypes (see also below) were detected (Fig. 2). The $P2Y_1$ receptor was also recognized as two immunoreactive bands

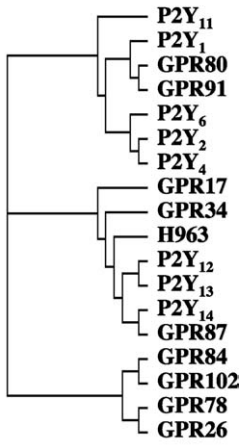

Fig. 1. Phylogenetic tree showing the relationship among known human P2Y receptors (human $P2Y_{1,2,4,6,11,12,13,14}$) and the 10 "P2Y-like" oGPCRs identified here. Sequences were aligned using ClustalX.

(43 and 50 kDa) which both disappeared upon incubation with the corresponding neutralizing peptide, likely representing two isoforms or differently-glycosylated receptors. The $P2Y_2$ transcript was detected as an amplification product of 432 bp (Fig. 2). For this receptor, WB analysis could not be utilized to confirm the presence of the receptor protein, since the bands recognized by a commercially available anti-$P2Y_2$ antibody did not disappear upon incubation with the corresponding neutralizing peptide. However, the $P2Y_2$ transcript was consistently found in eight control and eight CHF subjects (Fig. 2), suggesting that this receptor is indeed expressed in human myocardium, in line with previous literature data in [18]. $P2Y_4$ was recognized as a single specific 55 kDa band, in agreement with previous data in [25] (Fig. 2). $P2Y_6$ yielded two specific bands of 110 and 50 kDa (Fig. 2). Thus, $P2Y_{1,2,4,6,11,12,13,14}$ are all expressed in human heart (see Section 2). Semi-quantitative analysis after normalization of individual values to an internal housekeeping gene or protein showed no statistically significant differences in any P2Y receptor between control and CHF subjects. Of the 10 identified "P2Y-like" receptors (Fig. 1), only GPR17 and GPR91 were found; however, here too, no statistically significant differences were detected between control and CHF subjects (data not shown).

3.3. Map of P2X receptors in non-failing and failing human hearts

All P2X receptors were found in the human heart. Regarding RT-PCR, mRNAs for all P2X receptors were detected, except for $P2X_2$ and $P2X_3$, for which no specific amplification products were obtained with either published in [26] or with other primers specifically designed for this study (data not shown). However, both $P2X_2$ and $P2X_3$ proteins are present in human heart (Fig. 3), suggesting that negative RT-PCR results may be due to very low number of transcript copies. Regarding WB (Fig. 3), $P2X_1$ receptor was recognized as a doublet of 60 kDa bands, probably representing

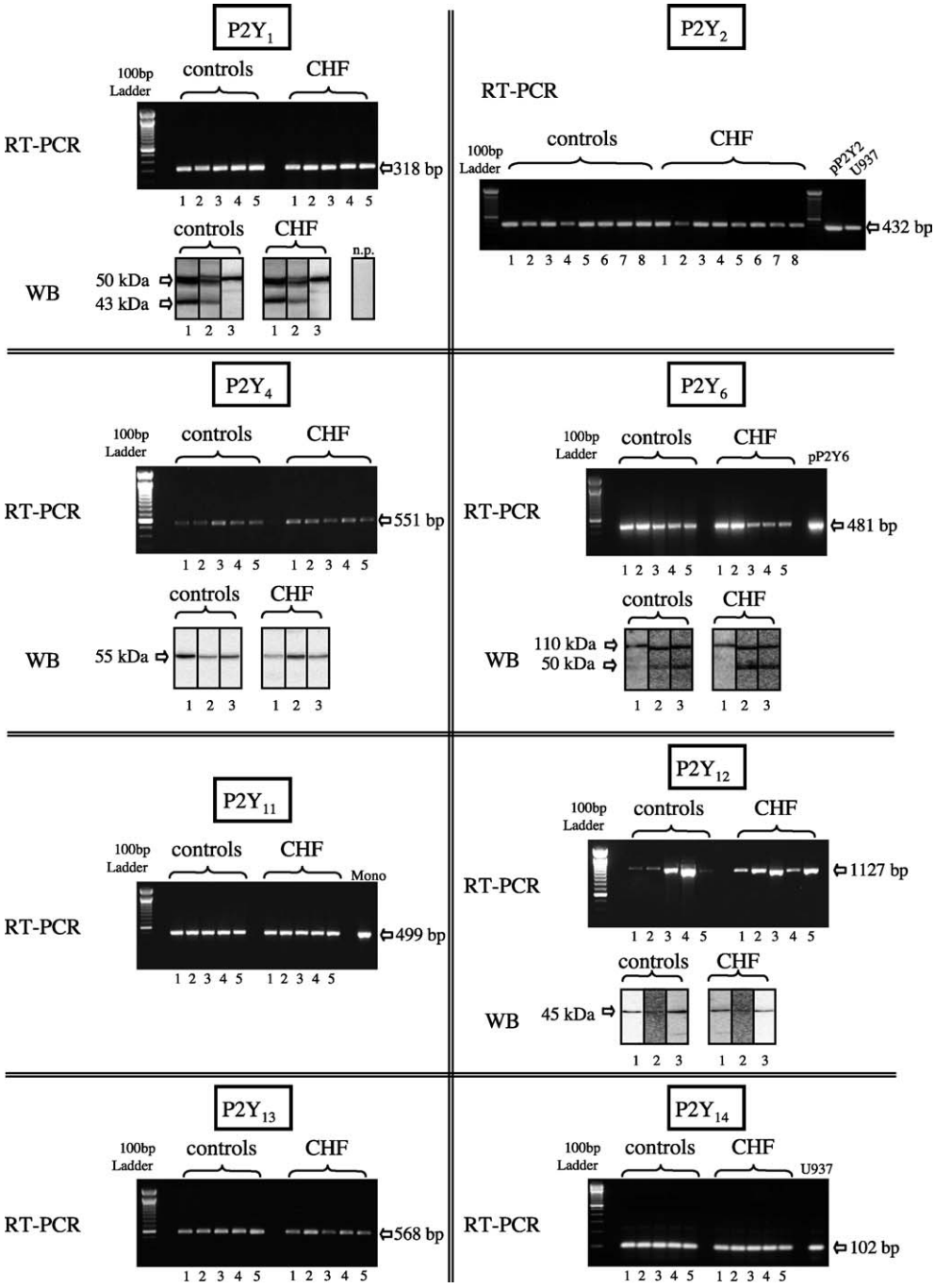

Fig. 2. Analysis of known P2Y receptors in human non-failing and CHF hearts. For each receptor, a representative RT-PCR ($N = 5$ at least) and/or WB ($N = 3$) analysis is shown. For $P2Y_{11}$, $P2Y_2$ and $P2Y_{14}$, positive control cells known to express these receptors were analyzed in parallel (monocytes for $P2Y_{11}$ and U937 cells for $P2Y_2$ and $P2Y_{14}$; for $P2Y_2$, a plasmid containing the coding sequence of the human receptor was also utilized). Similar data were obtained in three to five independent determinations/subject. No statistically significant differences were found after semi-quantitative densitometric analysis (see text). n.p. = neutralizing peptide; Mono = human monocytes; $pP2Y_2$ = $P2Y_2$ plasmid.

two different glycosylation states. $P2X_2$ was recognized as a 40 kDa specific band, in agreement with previous studies [27,28]. As expected [29], $P2X_3$ was recognized as a specific 55 kDa band. $P2X_4$ was detected as two 50 and 60 kDa bands, which may again represent two differently-glycosylated forms. $P2X_5$ receptor protein was not detected by the antisera we used. $P2X_6$ was recognized as a single specific 50 kDa band. $P2X_7$ was detected as a specific 45 kDa band, which differs from the previously reported 80 kDa form [17]; this may represent a deleted receptor which does not match to any known variants. Semi-quantitative densitometric analysis of data of control and CHF subjects after normalization against an internal housekeeping gene (see Section 2) did not reveal any difference in P2X receptors, with the only exception $P2X_6$, which was significantly ($P < 0.05$) upregulated in failing hearts with respect to healthy donor hearts (Fig. 3; see

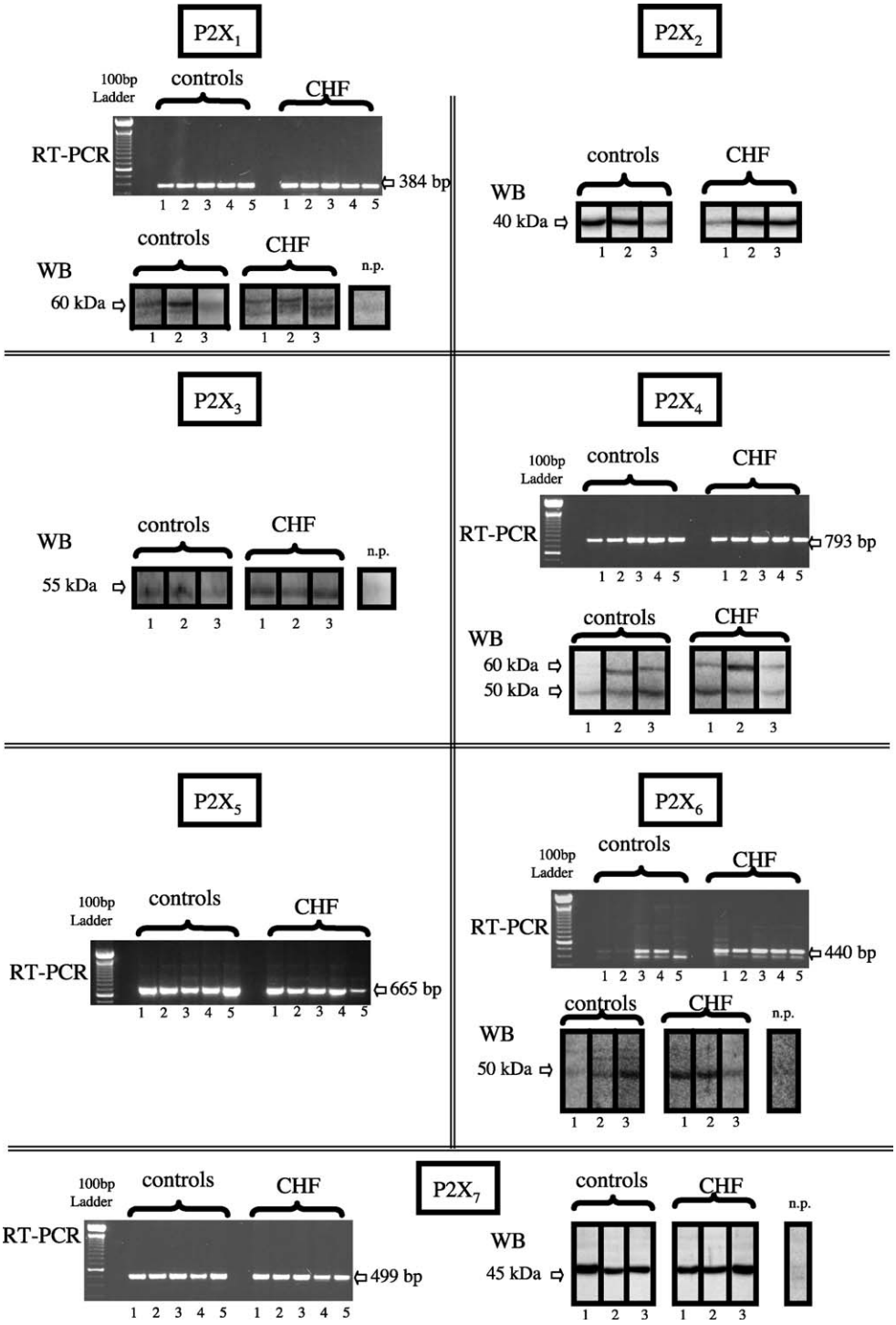

Fig. 3. Analysis of known P2X receptors in non-failing and CHF hearts. For each receptor, a representative RT-PCR ($N = 5$) and/or WB ($N = 3$) analysis is shown. Similar data were obtained in three to five independent determinations/subject. n.p. = neutralizing peptide.

also representative RT-PCR and WB analysis in one control and one CHF subject in Fig. 4A, B). Semi-quantitative densitometric analysis of data from five RT-PCR and three WB analysis showed a significant increase of $P2X_6$ in CHF patients (Fig. 4A,B). To further confirm this finding, we performed quantitative real time-PCR for the $P2X_6$ receptor in the heart from nine control subjects and 12 CHF patients. Marked and statistically-significant increase ($P = 0.019$) of $P2X_6$ receptor in myocardial tissue from CHF patients was found (Fig. 4C). Regarding the cellular population expressing this receptor subtype in human myocardium, $P2X_6$ was detected by RT-PCR in both cardiac fibroblasts and in a cardiomyocyte-enrinched cell population freshly isolated from two CHF patients (data not shown).

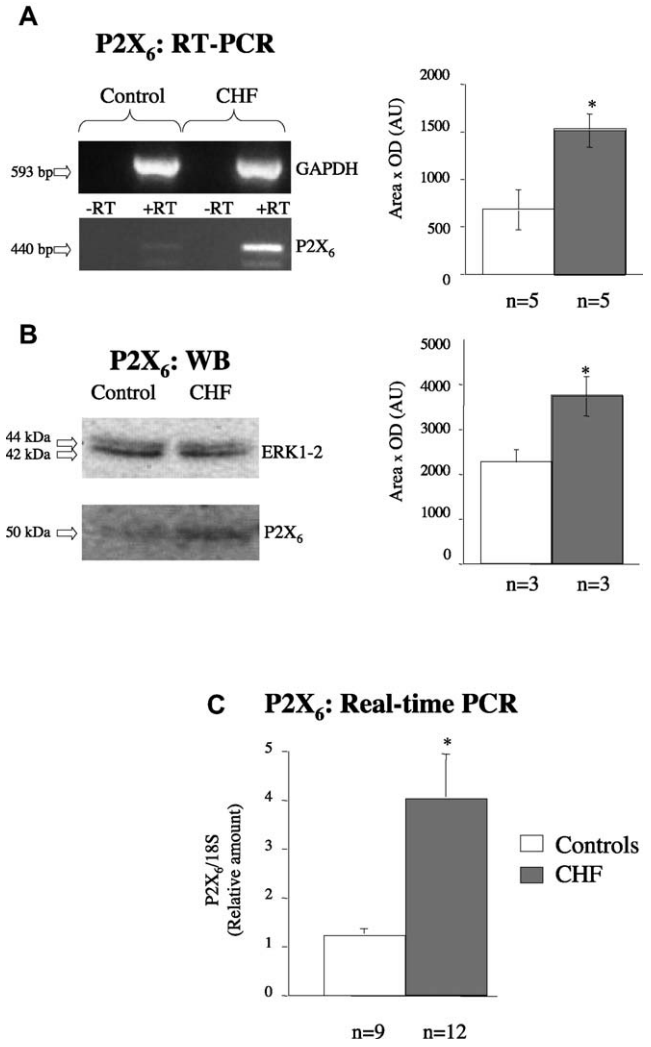

Fig. 4. Upregulation of $P2X_6$ in CHF patients. Representative RT-PCR (panel A) and WB analysis (panel B) of $P2X_6$ in one control and one CHF subject with corresponding GAPDH and ERK1/2 expression (see Section 2). Right panels: semi-quantitative densitometric analysis (mean ± S.E.M.) of data pooled from five (RT-PCR) and three (WB) non-failing and CHF hearts. $P < 0.05$ versus control. Panel C: quantitative real time-PCR of $P2X_6$ performed in nine control and 12 CHF hearts. $P < 0.05$ versus control.

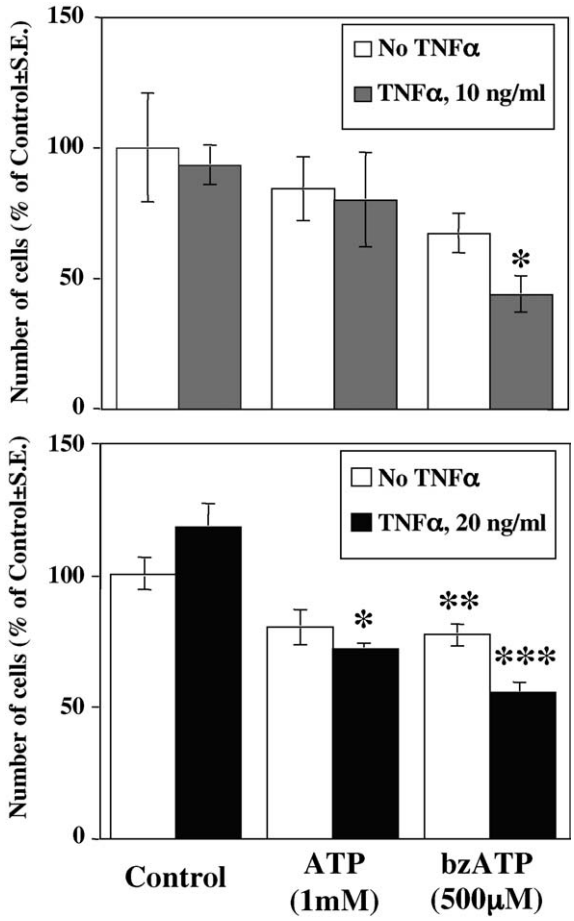

Fig. 5. Effects of a 72 hour exposure of primary pig cardiac fibroblasts to TNFα (10 ng/ml in the upper panel and 20 ng/ml in the lower panel) alone and/or in combination with ATP (1 mM) or the relatively selective P2X-agonist benzoyl-ATP (bzATP) (500 μM). Data are reported as cell number in culture and represent the mean ± S.E.M. of four replicates for the upper panel and five replicates for the lower panel from two independent experiments. *, **, ***$P < 0.05$ with respect to TNFα, control, TNFα and BzATP, respectively.

3.4. Effect of ATP and TNFα on the survival of cultured pig cardiac fibroblasts and murine cardiomyocytes

Activation of P2X receptors can sometimes result in calcium overload, and, eventually, cell death [30]. We thus postulated that the $P2X_6$ upregulation detected in CHF patients might contribute to cell death via a chronic amplification of receptor function. To test this hypothesis, we could not utilize human cells, since it is very difficult to freshly isolate (and maintain in culture) fibroblasts (and, to a greater extent, cardiomyocytes) from the adult human heart, especially for the long time periods required (days) for viability studies. Thus, for this second part of the study, we decided to take advantage on one side of cardiac fibroblasts freshly isolated from healthy young pigs (an experimental model where the long-term consequences of P2X receptor activation on cardiac cell survival could be investigated), and, on the other side, of the only available cardiomyocyte cell line (i.e. murine HL-1, see below). Pig cardiac primary fibroblasts proved to be adequate to our purposes, since they indeed express the $P2X_6$ receptor showing a 78% identity with human $P2X_6$, the receptor selectively undergoing upregulation in CHF patients. These cells at least express another P2X receptor subtype which is unchanged in the failing heart (i.e. $P2X_7$; see also below and Fig. 8). A 3-day exposure of these cells to the endogenous agonist ATP or the relatively hydrolysis-resistant agonist benzoylATP (BzATP, 500 μM) [7] decreased cell number compared to untreated control cultures, an effect that reached statistical significance for BzATP (cell number was 82.3 ± 7.4% of control in ATP-exposed cells, $P = 0.0957$ and 77.5 ± 4.8% of control in BzATP-exposed cells, $P < 0.05$, $N = 8$) (Fig. 5). Exposure to TNFα, a cytokine previously implicated in myocardial dysfunction [2], applied here at 10 or 20 ng/ml, had no effect "per se" on cell number (93.1 ± 7.2% and 117.9 ± 8.6% of control, $N = 4$ and $N = 5$, respectively), but potentiated cell number reduction by ATP or BzATP. For example, in the presence of both TNFα (10 ng/ml) and BzATP,

cell number was 43.8 ± 7.1% of control vs. 67 ± 7.4% for BzATP alone ($P < 0.05$ versus TNFα alone, $n = 4$). In the presence of 20 ng/ml TNFα, cell number reduction by ATP was enhanced from 79.5 ± 6.7 to 71.2 ± 2.3 (% of control, $P < 0.05$ versus TNFα alone) and, for BzATP, from 76.7 ± 4.3 to 54.6 ± 4.2 (% of control, $P < 0.05$ versus both TNFα and BzATP alone). The observed reduction in cell number was due to cell death, as evidenced by morphological signs of cytotoxicity (vacuolization, cell disorganization) in cultures exposed to BzATP alone, and to ATP and BzATP in the presence of TNFα (see left-hand micrographs in Fig. 6). The higher cytotoxicity of BzATP is likely due to higher resistance to hydrolysis in culture with respect to rapidly-degraded ATP. To determine the nature of cell death (i.e. apoptosis and/or necrosis), we analyzed the total cell population (adhering + detached cells) by flow cytometry. In control cultures, most cells were found in the Go/G1 phase, with a small proportion in the S and G2 phases (see right-hand cytogram in Fig. 6); as usually found for primary cultures, a small percentage (6.6%) of cells showed fragmented apoptotic nuclei (corresponding to the hypodiploid DNA peak), and 4.5% of cells were necrotic (see % in Fig. 6). Exposure to TNFα (10 ng/ml) or ATP alone induced no changes in cell distribution or in percentage of apoptotic and necrotic cells (Fig. 6). In contrast, exposure to BzATP alone markedly increased the percentage of apoptotic (35.1%) and necrotic (22.3%) cells relative to controls. Induction of apoptosis and necrosis was further increased when BzATP was combined with TNFα (43.7% and 24.1% of cells, respectively). Despite lack of significant effect when used alone, ATP also increased the percentage of apoptotic cells (16.7%) when added together with TNFα (Fig. 6).

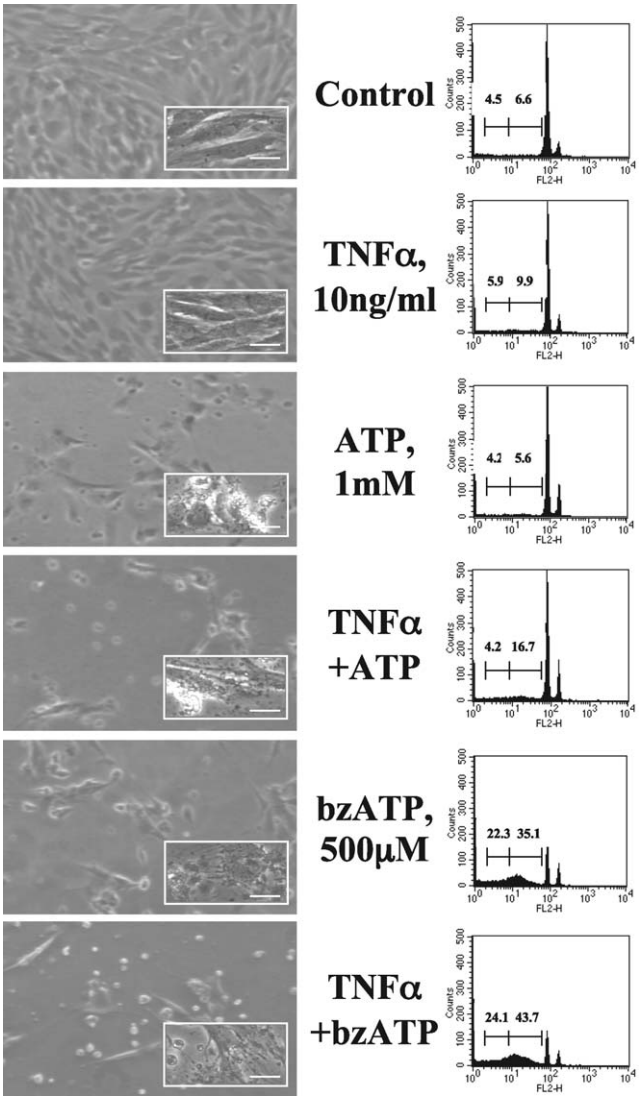

Fig. 6. ATP-, BzATP- and TNFα-induced death of pig cardiac fibroblasts in culture. Cells were exposed to ATP (1 mM), BzATP (500 μM), TNFα (10 ng/ml) alone or in combination, as indicated, and 72 h later, micrographs were taken on adhering cells (left) and flow cytometric analysis was performed on the total cell population (right). Bar = 25 μm. See text for details.

Comparable results were obtained in murine HL-1 cardiomyocytes, a cell line that has been shown to closely reproduce the characteristics of human cardiomyocytes, in terms of receptor expression and function [20]. Also these cells express $P2X_6$ (data not shown), the receptor undergoing selective upregulation in the myocardium of CHF patients. Exposure of HL-1 cells to ATP induced cell death, as demonstrated by cell number reduction, vacuolization, cell disorganization (Fig. 7A) and increased necrosis and apoptosis, as determined by flow cytometry analysis (Fig. 7B, C). Cardiomyocytes seemed to be more susceptible to ATP-induced death with respect to cardiac fibroblasts, since these effects were obtained with ATP alone, when utilized at a lower concentration for a shorter period of time (500 μM for 24 h, with respect to 1 mM for 72 h for cardiac fibroblasts). Also in this case, TNFα had no effect "per se" but markedly potentiated ATP-induced toxicity (Fig. 7A–C). The percentage of necrotic and apoptotic cells was increased from 1.7% ± 0.5 apoptosis and 3.4% ± 0.8 necrosis in control cardiomyocytes to 24% ± 8.4 apoptosis and 26.8% ± 8.5 necrosis in cultures exposed to both agents (Fig. 7C).

3.5. Selective effect of TNFα on $P2X_6$ receptor mRNA in cardiac fibroblasts

Prolonged exposure of receptors to agonists leads to receptor desensitization and loss of function, an event that often represents a defense mechanism to avoid excessive receptor activation [7,31]. Desensitization also occurs for some P2X receptors upon prolonged exposure to ATP [7]. We thus decided to test whether, in the cardiac fibroblast experimental model, potentiation of ATP-induced cytotoxicity by TNFα could be due to interference of this cytokine with agonist-induced $P2X_6$ receptor regulation. Indeed, prolonged exposure of cardiac fibroblasts to ATP or BzATP profoundly down-regulated the transcript encoding for $P2X_6$ (which is upregulated in human failing hearts, see above), without any effect on $P2X_7$ (which is unchanged in CHF) (Fig. 8). TNFα (which itself had no effect on either transcript, Fig. 8) markedly prevented nucleotide-induced down-regulation of $P2X_6$. TNFα had no effect either alone or in combination with ATP or BzATP on $P2X_7$ (Fig. 8).

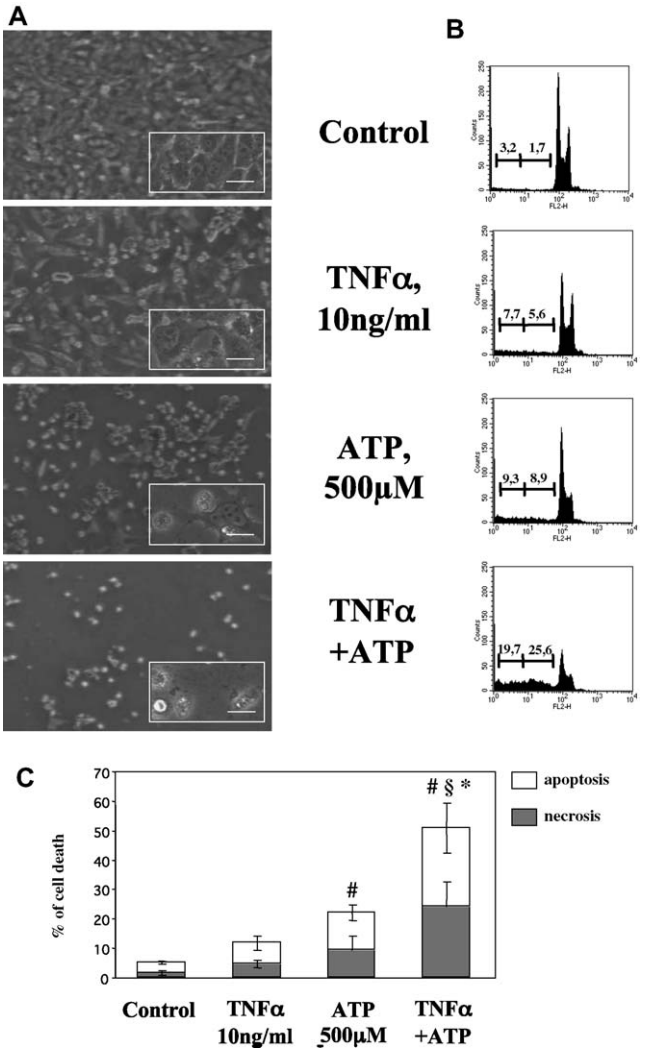

Fig. 7. TNFα potentiation of ATP cytotoxicity in murine HL-1 cardiomyocytes. Cells were exposed to ATP (500 μM) or TNFα (10 ng/ml) alone or in combination, as indicated, for 24 h. At the end of treatment, micrographs of adhering cells were taken (panel A) and flow cytometric analysis was performed on the total cell population (panel B). Bar = 25 μm. Panel C shows the percentage of necrotic and apoptotic cells. #$P < 0.05$ vs. control, *$P < 0.05$ vs. ATP, §$P < 0.05$ vs. TNFα, mean ± S.E.M. of five independent experiments.

4. Discussion

The present study provides, for the first time, a complete map of P2Y and P2X receptors expressed in human heart. All cloned P2 receptors were detected. Of the 10 identified P2Y-like receptors, only GPR17 and GPR91 were found to be expressed. Of these two receptors, GPR91 has been recently demonstrated not to be a P2Y receptor [24]. No functional information is yet available for GPR17.

The large repertoire of myocardial P2 receptors is consistent with the P2 receptor heterogeneity reported in other tissues [17], suggesting differential recruitment of receptors depending upon different functional states. The presence of both P2X and P2Y receptors is consistent with the hypothesis that ATP positive inotropic effects may depend not only on P2X receptors (allowing calcium influx from the extracellular environment), but also on P2Y receptors, which release calcium from intracellular stores [14].

Our results also extend previous studies showing expression of $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors in human fetal heart [32] and of $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ in rat whole heart, neonatal cardiac fibroblasts, neonatal and adult cardiac myocytes [33]. The slight discrepancies of our data with respect to above results may be due to either species differences (rat versus human) or utilization of different heart regions (ventricular tissue versus whole heart), and/or development-associated changes. In particular, differences in P2Y receptor expression with respect to human fetal heart [32] suggest that some receptors are expressed throughout life (e.g. $P2Y_4$ and $P2Y_6$) while some others may be only expressed during adulthood (e.g. $P2Y_1$, $P2Y_{11}$, $P2Y_{12}$ $P2Y_{13}$ and $P2Y_{14}$, although these more recently cloned receptors were not investigated before). Interestingly, we also detected the $P2Y_{14}$ receptor for nucleotide glycosides. The demonstration that this receptor is important in immune responses [34,35] may unveil interesting roles in the immunological mechanisms involved in myocardial dysfunction development.

Consistent with our results, $P2X_1$, $P2X_3$, $P2X_4$ and $P2X_5$ were previously described in mammalian heart [32,36]. Changes of both P2X and P2Y receptors in the failing heart were previously detected [18]. However, in that study receptor levels in the non-failing heart could not be compared, as no data from control subjects were provided. The present results show no differences of P2Y receptor levels in control and CHF patients. However, this does not rule out the possibility that these receptors undergo functional changes in CHF. Our data providing a complete map of myocardial P2Y receptors represent the molecular background and the starting point for studies aimed at assessing in more detail the roles of these receptors in the heart and their modulation in CHF.

Instead, a statistically significant greater expression of $P2X_6$ was found by both RT-PCR and WB analysis in failing hearts, irrespective of the type of cardiomyopathy (idiopathic or ischemic), indicating that this change may represent a final alteration common to both types of dysfunction. This change was also confirmed by quantitative real time-PCR performed in nine control subjects and 12 CHF patients. $P2X_6$ functions almost exclusively as an heteromeric channel [11] together with $P2X_2$ and $P2X_4$ (both present in human heart) inducing a remarkably robust Ca^{2+} influx. It might be speculated that P2X receptors are upregulated in CHF in an attempt to ameliorate myocardial function by promoting calcium entry. However, our results suggest that $P2X_6$ upregulation may also contribute to cardiac dysfunction progression, since, in experimental models of both cardiac fibroblasts and cardiomyocytes maintained in culture, ATP or bzATP (alone and to a major extent, in combination with TNFα, which is also increased in CHF), induced apoptosis and necrosis. Interestingly, other P2 receptor subtypes might play completely different roles in cardiomyocyte viability. For example, in a recent paper, it was demonstrated that UTP could indeed protect rat cardiomyocytes against hypoxic stress induced by

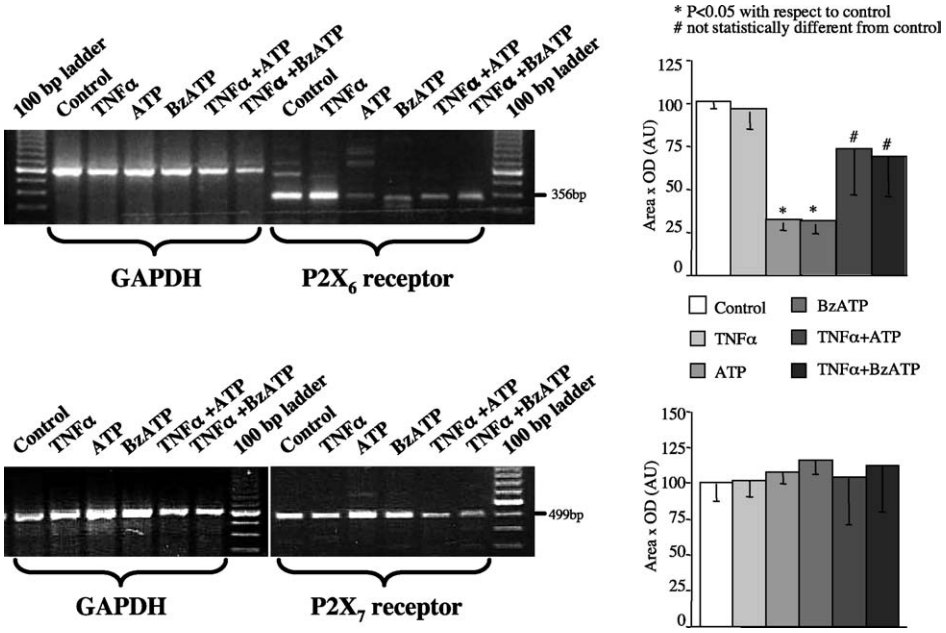

Fig. 8. TNFα prevents agonist-induced downregulation of $P2X_6$ mRNA in pig cardiac fibroblasts. Cells were exposed to TNFα (10 ng/ml), ATP (1 mM), BzATP (500 μM) alone or in combination as indicated, and 5 h later, $P2X_6$ and $P2X_7$ mRNAs were evaluated by RT-PCR in parallel with GAPDH. Left: data from one representative experiment; right: semi-quantitative densitometric analysis (mean ± S.E.M.) from three pooled experiments. *$P < 0.05$ vs. control; #not different from control.

cell injury in vitro, likely via activation of the $P2Y_2$ receptor subtype [37], whose expression is not changed in the hearts of CHF patients (the present study). It could be speculated that cardiomyocyte survival may be regulated by proapoptotic $P2X_6$ receptors and protective $P2Y_2$ receptors, and that an unbalance between these two receptor subtypes may also participate to increased sensitivity to hypoxic/toxic stimuli.

As demonstrated for other responses in CHF, P2X receptor upregulation may initially act as a compensatory mechanism but eventually become deleterious. In line with previous data on agonist-induced desensitization [7,31], in the cardiac fibroblasts experimental model, prolonged in vitro exposure to a nucleotide, a condition reproducing the higher ATP release occurring in hypoxic heart [38], decreased $P2X_6$ transcript selectively without effect on $P2X_7$. This probably represents a means to avoid excessive $P2X_6$ activation which may result in calcium overload and cell death [39]. When ATP or BzATP were added to cells together with TNFα, no downregulation of $P2X_6$ was observed, likely resulting in potentiation of ATP effects. We speculate that, in the failing heart, where cells are concomitantly exposed to elevated concentrations of TNFα and ATP [40,38], this cytokine can prevent ATP-induced $P2X_6$ downregulation (thus abolishing a defense mechanism aimed at avoiding excessive receptor activation) and lead to ATP-dependent cytotoxicity. A similar mechanism may lead to $P2X_6$ upregulation in CHF patients, who display elevated TNFα serum concentrations [40]. These mechanistic aspects might be elucidated in detail in future studies, by likely employing simpler experimental models such as a human fibroblast cell line in culture.

Our data extend the role of ATP in myocardium from that of a mere energy provider to that of a physiologically important transmitter that may also participate to heart failure. Our data also suggest that TNFα may be toxic only when combined with nucleotides, highlighting this interaction as a novel pathogenic mechanism in heart disease. Despite evidence correlating TNFα with myocardial disease, and despite benefits in small-scale studies, recent large-scale studies in CHF patients showed no clinical benefit from TNFα blockade [41]. Uncertainties about the mechanism of action of this cytokine in myocardial disease may have hampered the proper design of these trials. The hypothesis that TNFα may act, at least in part, by interfering with myocardial P2 receptors regulation could add innovative twists to clinical studies and unveil novel therapeutic strategies to a continuing medical need.

Acknowledgments

The authors warmly thank Professor William Claycomb, LSU Health Sciences Center, New Orleans, LA, USA, for the kind gift of HL-1 cells. The authors are grateful to Professor G.E. Rovati (University of Milan) for kindly providing U937 cells. Grant numbers and sources of support: Progetto Strategico “Ipertrofia ventricolare sinistra e scompenso cardiaco: alterazioni funzionali e meccanismi molecolari” granted to M.P.A. by the Italian Ministry of Health for 2002–2004 and by research project of national interest (PRIN-COFIN2004) No. 2004062711 to Professor Luciana Mussoni, Department of Pharmacological Sciences, University of Milan, by Italian Ministry of Education.

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