In Focus: Heart

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Opposite effects of uracil and adenine nucleotides on the survival of murine cardiomyocytes

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Abstract

We previously showed that the human heart expresses all known P2X and P2Y receptors activated by extra-cellular adenine or uracil nucleotides. Despite evidence that, both in humans and rodents, plasma levels of ATP and UTP markedly increase during myocardial infarction, the differential effects mediated by the various adenine- and uracil-preferring myocardial P2 receptors are still largely unknown. Here, we studied the effects of adenine and uracil nucleotides on murine HL-1 cardiomyocytes. RT-PCR analysis showed that HL-1 cardiomyocytes express all known P2X receptors (except for $P2X_2$), as well as the $P2Y_{2,4,6,14}$ subtypes. Exposure of cardiomyocytes to adenine nucleotides (ATP, ADP or BzATP) induced apoptosis and necrosis, as determined by flow-cytometry. Cell death was exacerbated by tumour necrosis factor (TNF)-α, a cytokine implicated in chronic heart failure progression. Conversely, uracil nucleotides (UTP, UDP and UDPglucose) had no effect *'per se'*, but fully counteracted the deleterious effects induced by adenine nucleotides and TNF-α, even if added to cardiomyocytes after beginning exposure to these cell death-inducing agents. Thus, exposure of cardiomyocytes to elevated concentrations of ATP or ADP in the presence of TNF-α contributes to cell death, an effect which is counteracted by uracil-preferring P2 receptors. Cardiomyocytes do not need to be 'primed' by uracil nucleotides to become insensitive to adenine nucleotides-induced death, suggesting the existence of a possible 'therapeutic' window for uracil nucleotides-mediated protection. Thus, release of UTP during cardiac ischaemia and in chronic heart failure may protect against myocardial damage, setting the basis for developing novel cardioprotective agents that specifically target uracil-preferring P2Y receptors.

Keywords: apoptosis ● heart failure ● P2Y receptors ● cytokines ● signal transduction

Introduction

Extracellular purine and pyrimidine nucleotides are primordial molecules that serve multiple biological processes [1]. They are responsible for transmission of genetic information, provide currency units for biological energy transfer, and are also released from cells to provide integral elements of extracellular signalling. Within such 'purinergic' system, specificity and regulation is dictated by the source of extracellular nucleotides and by the expression of specific receptors on target cells: the P2 receptors.

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Two distinct families of P2 receptors are currently recognized: the ligand-gated P2X receptors, and the G protein-coupled P2Y receptors [2–5]. So far, seven distinct P2X receptor subtypes (P2X$_{1-7}$) and eight P2Y receptors (the P2Y$_{1,2,4,6,11,12,13,14}$ subtypes) have been cloned and characterized (ibidem). Recently, a new putative member of the P2Y receptor family, previously known as the 'orphan' receptor GPR17, has been identified [6]. P2X receptors can form homomeric and heteromeric channels allowing Na$^+$ and Ca^{2+} influxes, and have been implicated in fast excitatory transmission [4] and, under some circumstances, in apoptotic cell death [7]. Conversely, activation of metabotropic P2Y receptors leads to modulation of adenylyl cyclase and/or phospholipase C, depending upon the specific P2Y subtype [5, 8]. While P2X receptors primarily respond to adenine nucleotides (*i.e.* ATP and ADP), P2Y receptors can be further subdivided into (*i*) the adenine-nucleotide-preferring receptors (human and rodent P2Y$_1$, P2Y$_{12}$ and P2Y$_{13}$, and human P2Y$_{11}$), (*ii*) the uracil-nucleotide-preferring receptors, responding to either UTP or UDP (human P2Y$_4$ and P2Y$_6$), (*iii*) receptors of mixed selectivity, which respond to both adenine and uracil nucleotides (human and rodent P2Y$_2$, rodent P2Y$_4$) and, (*iv*) receptors responding solely to sugar nucleotides, such as UDPglucose and UDPgalactose (P2Y$_{14}$) [5]. Interestingly, the newly identified P2Y-like receptor (GPR17) is solely activated by uracil nucleotides, with a pharmacological profile which is intermediate between P2Y$_6$ and P2Y$_{14}$ [6].

Both P2X and P2Y receptors are widely distributed in human tissues and are known to mediate fundamental roles in the cardiovascular system [9]. At myocardial level, despite very early reports on the cardiac effects of adenine nucleotides and nucleosides [10], and demonstration that ATP is released from myocardial sympathetic terminals and hypoxic cardiomyocytes [9], the effects mediated by specific myocardial P2 receptors are just now beginning to be elucidated. Adenine nucleotides induce rat cardiac myocyte contraction possibly through P2X receptors, and/or by increasing L-type Ca^{2+} currents (summarized in [1]). Positive inotropism by ATP has been also attributed to P2Y receptors [11]. Besides adenine nucleotides, uracil nucleotides have been also recently reported to act as positive inotropic agents in rat and mouse cardiomyocytes [12,13]. The utilization of stable selective agonists has allowed the pharmacological characterization of the specific P2 receptors involved in the positive inotropic effects induced by uracil nucleotides. Most important, nucleotide plasma levels were measured to evaluate whether UTP is released in patients with coronary heart disease. Besides ATP, venous plasma levels of UTP were increased (57%) in patients with myocardial infarction, giving the first evidence for UTP release in man. Based on these results, authors concluded that extracellular pyrimidines (UTP and UDP) could be important inotropic factors involved in the development of chronic heart failure, a disease that still remains a major cause of ill health in industrial societies countries [14,15].

However, UTP may also play a significant role in protecting the ischaemic heart against injury [16,17]. Short-term activation of UTP receptors indeed protects newborn rat cardiomyocytes against hypoxic damage [16] and significantly reduces the cell death caused by hypoxia *via* activation of P2Y$_2$ and P2Y$_4$ receptors [17].

Thus, besides influencing cardiac contractility, P2 receptors may also directly regulate the viability of myocardial cells. In a recent study, we have shown that the full repertoire of known P2X and P2Y receptors is expressed in the human heart from both healthy donors and patients with chronic heart failure (CHF) undergoing cardiac transplantation [18]. We also showed that the ionotropic P2X$_6$ receptor was up-regulated in CHF subjects, as confirmed by quantitative real time-PCR [18]. 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-analogue benzoylATP (BzATP) induced apoptosis. Tumour necrosis factor (TNF)-α (a pro-inflammatory cytokine implicated in CHF progression) [19] exacerbated cell death. In cardiac fibroblasts, exposure to TNF-α inhibited the down-regulation of P2X$_6$ mRNA induced by prolonged agonist exposure, suggesting that, by preventing ATP-induced P2X$_6$ desensitization, TNF-α may abolish a defence mechanism meant at avoiding Ca^{2+} overload and, ultimately, Ca^{2+}-dependent cell death. This may provide a basis for P2X$_6$ up-regulation in CHF. Globally, these findings suggest that the interaction between TNF-α and the up-regulated P2X$_6$ receptor may represent a novel pathogenic mechanism in CHF.

In the present study, based on our previous results and on the demonstration that, besides ATP, UTP is also released from the heart during cardiac ischaemia

[12], we have investigated the effects of both adenine and uracil nucleotides on the viability of HL-1 cardiomyocytes, the only available cell line that spontaneously contracts *in vitro* and maintains a differentiated cardiac phenotype [12]. In line with our previous findings [20], we show that exposure of HL-1 cardiomyocytes to ATP or ADP induces cell death by both apoptosis and necrosis, an effect which was increased by pre-treatment of cells with TNF-α. On the contrary, uracil nucleotides (UTP, UDP and UDPglucose), utilized either alone or with TNF-α, did not induce apoptosis or necrosis '*per se*', but significantly reduced cardiomyocyte death induced by adenine nucleotides. We conclude that activation of uracil-preferring P2Y receptors in cardiomyocytes counteracts the deleterious effects mediated by ATP and TNF-α on myocardial cells. We also suggest that release of UTP during cardiac ischaemia and during development of CHF may be protective against myocardial damage. These findings may have important therapeutic implications and set the basis for the development of novel cardioprotective agents that specifically target uracil-preferring P2Y receptors.

Materials and methods

HL-1 cardiomyocytes

HL-1 cardiomyocytes were a kind gift of Professor W.C. Claycomb, LSU Health Sciences Center, New Orleans, LA, USA [20]. Cells were cultured in Complete Claycomb Medium supplemented with 10% foetal calf serum (FCS) (JRH Biosciences, UK) and Norepinephrine (100 μM, Sigma-Aldrich), following Professor Claycomb's instructions. Cells were seeded in six-well plates at various densities $(48–100–200 \times 10^3)$; on the next day, medium was replaced and after 8 hrs, HL-1 cells were pre-treated overnight with TNF-α (10 ng/ml) followed by addition of ATP (25–500 μM) or ADP (250–500 μM) or 3´-benzoyl adenosine 5´-triphosphate (BzATP, 100 μM), adenosine 5´-O-(3-thiotriphosphate) (ATPγS, 500 μM), 2-methylthioadenosine 5´-triphosphate (2MeSATP, 500 μM) and 2-methylthioadenosine 5´-diphosphate (2MeSADP, 500 μM) (Sigma-Aldrich). Uracil nucleotides (UTP, UDP, UDPglucose, 100 nM-500 μM, Sigma-Aldrich) and all antagonists [8-cyclopentyl-1, 3-dipropylxanthine (CPX 10 μM, Sigma-Aldrich), 5-amino-7-(β-phenylethyl)-2-(8-furyl)pyrazolo(4,3-epsilon)-1,2, 4-triazolo(1,5-c)pyrimidine (SCH58261, 100 nM, from Schering-Plough), 9-chloro-2-(2-furyl)-5-phenylacety-lamino(1,2,4)-triazolo(1,5-c)quinazoline (MRS1220, 100 nM, Sigma-Aldrich), 2´deoxy-N^6-methyl adenosine 3´,5´ diphosphate (MRS2179, 100 μM, Sigma-Aldrich), pyridoxal-phosphate-6-azophenyl-2´,4´-disulfonate (PPADS, 100 μM, Sigma-Aldrich), suramin (100 μM, Sigma-Aldrich), reactive blue 2 (RB2, 100 μM, Sigma-Aldrich) and Cangrelor (10 μM, The Medicines Company, Parsippany, NJ, USA)] were all added to cultures 30 min before adenine nucleotides. In selected experiments, 24 hrs after seeding, medium was replaced with Claycomb medium without FCS and Norepinephrine, followed by addition of TNF-α and adenine/uracil nucleotides. In these experiments, after pharmacological treatments cells were maintained in Claycomb medium without serum and norepinephrine; under these conditions, cells are more sensitive to cell death and therefore ATP was used at lower concentration (100 μM).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Extraction of total RNA and RT-PCR were performed as described [18], Control samples lacking reverse transcriptase were processed in parallel. All reagents were from Invitrogen (Milan, Italy). Amplifications were performed in a GeneAmp 9700 thermal cycler (Applied Biosystems) using primers designed with Oligo4 (see Table 1), as described [18].

For the expression profile of P2 receptors the following murine positive controls were used: trigeminal ganglia for P2X$_2$ and P2X$_3$, total brain for P2X$_1$, P2X$_4$, P2X$_5$, P2X$_7$, P2Y$_{13}$ and P2Y$_{14}$, heart for P2X$_6$, liver for P2Y$_4$, lung for P2Y$_2$ and N9 cells for P2Y$_1$, P2Y$_6$ and P2Y$_{12}$ [8, 21, 22].

Nuclear staining of adhering cells

After 24 hrs in culture, cells were fixed in 4% paraformaldehyde in PBS and nuclear chromatin stained by using the fluorescent dye Hoechst 33258 (Società Italiana Chimici), as described [23].

Flow cytometric evaluation of apoptosis

The percentage of apoptotic and necrotic cells in the total population (adhering+detached cells) was evaluated by means of hypotonic propidium iodide (PI) staining of DNA followed by flow cytometric analysis as described [18, 24, 25]. When utilized under hypotonic conditions, PI enters all cells and allows a direct measure of DNA fragmentation and of the ongoing cell death process. In selected experiments, induction of apoptosis was also confirmed by measuring

Table 1. RT-PCR primers for murine P2X and P2Y receptors: sequences, annealing temperatures, accession number and expected amplification product lengths

P2X and P2Y primers		T_A (°C)	Accession number	cDNA (bp)
$P2X_1$	S 5′-GTTTGGGATTCGCTTTGA-3′ A 5′-TCAGGAAGGGAAGTGTGG-3′	55.8	NM_008771	452
$P2X_2$	S 5′-GGTGGAGGATGGGACTTC-3′ A 5′-ATGGTGGGAATGAGACTG-3′	53.1	Y10473, AB094664, AB094663	498
$P2X_3$	S 5′-ACTTTGTGGGGTGGGTTT-3′ A 5′-GCTGCCATTCTCCATCTT-3′	55.6	NM_145526	767
$P2X_4$	S 5′-GGAATTGGGACTGGAAGG-3′ A 5′-GGAGTGGAGACCGAGTGA-3′	55.8	NM_011026	774
$P2X_5$	S 5′-CCAATCTCTACTGCCCCATC-3′ A 5′-TGTCTCGGTAAAACTCGC-3′	54.2	NM_033321	438
$P2X_6$	S 5′-GCTCAAGTCCAGGGCAGATG-3′ A 5′-CAGTCAGAGCCTTTCGTGTCC-3′	57.7	NM_0011028	482
$P2X_7$	S 5′-GGCATCCGTTTTGACATC-3′ A 5′-CTGGGGTCTTGGAACTTC-3′	54	NM_0011027	360
$P2Y_1$	S 5′-CCTGCGAAGTTATTTCATCTA-3′ A 5′-GTTGAGACTTGCTAGACCTCT-3	51.6	NM_008772	319
$P2Y_2$	S 5′-GCAGCATCCTCTTCCTCACCT-3′ A 5′-CATGTTGATGGCGTTGAGGGT-3′	60.2	NM_008773	503
$P2Y_4$	S 5′-CTTTGGCTTTCCCTTCTTGA-3′ A 5′-GTCCGCCCACCTGCTGATGC-3′	56.5	NM_020621	427
$P2Y_6$	S 5′-CGCTTCCTCTTCTATGCCAA-3′ A 5′-GTAGGCTGTCTTGGTGATGTG-3′	59.6	AF298899	480
$P2Y_{12}$	S 5′-CCGCTACCTGAAGACCACCA-3′ A 5′-GTTCGCCACCTTCTTGTCCTT-3′	55.1	NM_027571	641
$P2Y_{13}$	S 5′-CAGGGACACTCGGATGACA-3′ A 5′-CACCGCATAAAACAGAAGC-3′	55.4	NM_028808	577
$P2Y_{14}$	S 5′-GTCTCTGCCGTCATCTTCT-3′ A 5′-GGGTCCAGACACACATTG-3′	54.3	NM_133200	591

Annexin V binding to the outer membrane of apoptotic cells; in these experiments, isotonic PI was used in parallel, as described [25]. When employed at isotonic conditions, PI can indeed only permeate cells with damaged membranes, thus providing a direct measure of earlier membrane events associated to necrosis (ibidem).

Statistical analysis

All results are expressed as mean ± S.E.M. of three independent experiments. Statistical significance between groups was derived from one-way-ANOVA followed by Scheffe's F-test. A *P* value less than 0.05 was considered significant.

Results

HL-1 cardiomyocytes express P2 receptors responding to both adenine and uracil nucleotides

As a first step to the characterization of the effects of P2 receptors on the viability of HL-1 cells, we assessed the presence of all rodent P2 receptors by RT-PCR. Amplification products with the expected molecular weight for all known P2X receptors (with the only exception of $P2X_2$) and for $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{14}$ were obtained (Fig. 1). No specific
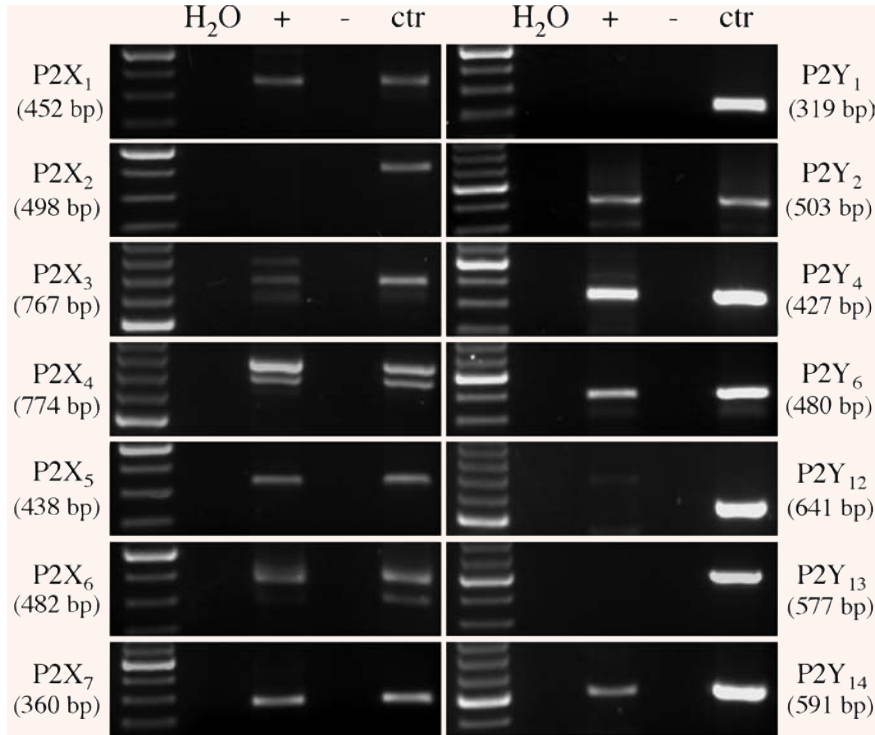
Fig. 1 Murine HL-1 cardiomyocytes express P2X$_{1,3,4,5,6,7}$ and P2Y$_{2,4,6,14}$. The presence of P2 receptors was assessed using *ad hoc* designed RT-PCR primers for all cloned murine P2 receptors. For each receptor, amplification products in parallel with MW standards are reported. Various tissues or cell lines were analysed in parallel as a positive controls: trigeminal ganglia for P2X$_2$ and P2X$_3$, total brain for P2X$_1$, P2X$_4$, P2X$_5$, P2X$_7$, P2Y$_{13}$ and P2Y$_{14}$, heart for P2X$_6$, liver for P2Y$_4$, lung for P2Y$_2$ and N9 cells for P2Y$_1$, P2Y$_6$ and P2Y$_{12}$. No products were detected in samples that did not undergo retrotranscription (–).

signals for P2Y$_1$, P2Y$_{12}$ and P2Y$_{13}$ were obtained; negative data were not due to technical problems, since specific amplification products for these receptors were detected in murine tissues or cell lines which express these receptors [8, 21, 22] here utilized as positive controls (Fig. 1).
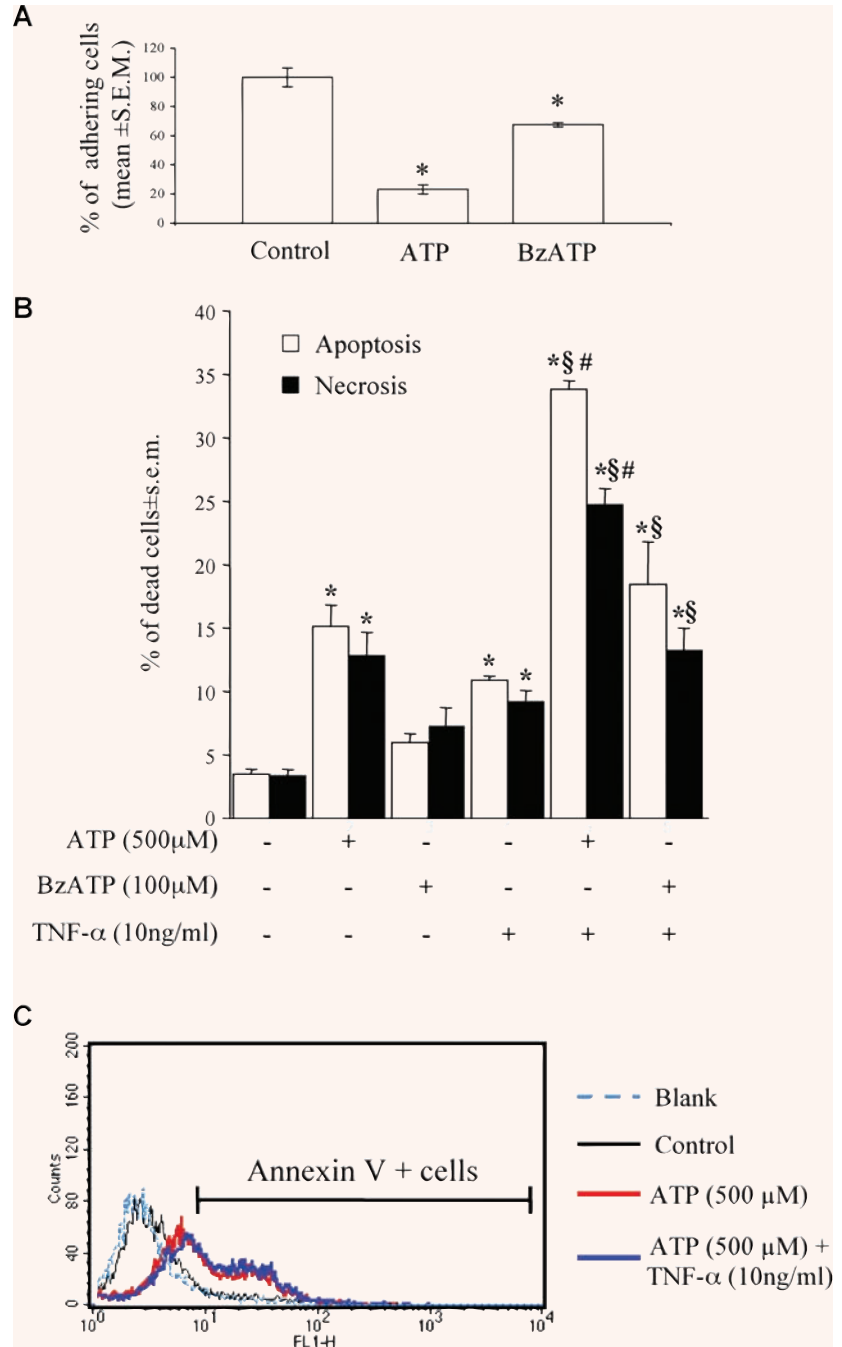
Exposure of HL-1 cardiomyocytes to adenine nucleotides induces cell death, which is exacerbated by TNF-α

In line with our previous findings [18], a 24 hr-exposure of HL-1 cardiomyocytes to ATP and to the relatively-selective P2X agonist BzATP resulted in a dramatic reduction of the number of cells adhering to the culture substrate (Fig. 2A). Cell number reduction by ATP and BzATP was due to induction of cell death by both apoptosis and necrosis, as shown by flow cytometric analysis of PI-stained nuclei (Fig. 2B). In a similar way to cardiac fibroblasts [18], the pro-inflammatory cytokine TNF-α exerted little effect '*per se*' on the viability of HL-1 cardiomyocytes, but dramatically potentiated the cell death induced by ATP, and, to a lesser extent, by BzATP (Fig. 2B). To further confirm

that the cell death induced by adenine nucleotides is indeed apoptosis, we evaluated another established marker of apoptosis, that is, Annexin V binding to the outer membrane of apoptotic cells, as previously described [25]. Exposure to adenine nucleotides produced a significant increase in the percentage of Annexin V (apoptotic)-positive cells, from about 8.00% in Control cultures to more than 35.00% after exposure to ATP (Fig. 2C). With the latter method, in the ATP+TNF-α condition, apoptosis was 47.00%; thus, potentiation of ATP-induced effects by TNF-α was less evident if compared to that observed at the nuclear level with hypotonic PI (Fig. 2B). This could be explained by hypothesising that TNF-α is indeed accelerating the progression of the ATP-induced cell death program downstream the cell membrane. At a membrane level, where apoptosis-induced changes precede nuclear fragmentation, potentiation of ATP effects by TNF-α is less visible, since Annexin V-positive cells are still alive and will undergo cell death at later time points.

Of a number of other adenine nucleotides tested, only ADP and ATPγS (but not 2MeSATP or 2MeSADP) were able to induce cardiomyocyte apoptosis and necrosis; also in this case, induction of cell death was potentiated by TNF-α (Fig. 3A).

Fig. 2 Cardiomyocyte death induced by ATP or BzATP alone or in combination with TNF-α. After 24 hrs treatment with either ATP (500 M) or the selective P2X-agonist BzATP (100 μM), cardiomyocytes were fixed, stained with the Hoechst 33258 dye and the number of adhering cells evaluated by fluorescence microscopy. Data are reported as % of control cell number (**A**). For evaluation of apoptosis and necrosis (**B**), cells were exposed to ATP or BzATP, alone or in combination with TNF-α (10ng/ml), as indicated. After 24 hrs, cells were collected and the percentage of apoptotic and necrotic cells measured by flow cytometric analysis of DNA fragmentation with Propidium Iodide. Data represent the mean ± S.E.M. of three independent experiments. *$P<0.05$ *versus* control, ($P<0.05$ *versus* TNF-α alone, and §$P<0.05$ *versus* ATP or BzATP alone, by one way ANOVA (Scheffe's F test). (**C**) Annexin V staining confirmed the induction of apoptotic cell death. Cells were exposed to ATP alone or in combination with TNF-α, as previously described. At the end of the incubation period, cells were collected, and stained with Annexin V to detect membrane signs of apoptotic death. Blank refers to unstained negative control cells, used to calibrate the instrument. The graph shows a representative experiments (percentages of Annexin V-positive cells: Control, 8.61%; ATP alone, 38.57%; ATP+TNF-α, 47.12%). Comparable results were obtained in four independent experiments.

Adenine nucleotides-induced cardiomyocyte death is mediated by activation of P2 receptors

To verify if the cell death effect reported above is actually due to activation of P2 receptors, HL-1 cardiomyocytes were exposed to ATP and TNF-α in the presence of various P2 receptor antagonists such as Reactive Blue 2 (RB2), PPADS and suramin [8, 26, 27]. Suramin was the most potent of the three tested antagonists in inhibiting the nuclear signs of apoptotic death induced by ATP and TNF-α; a lower,
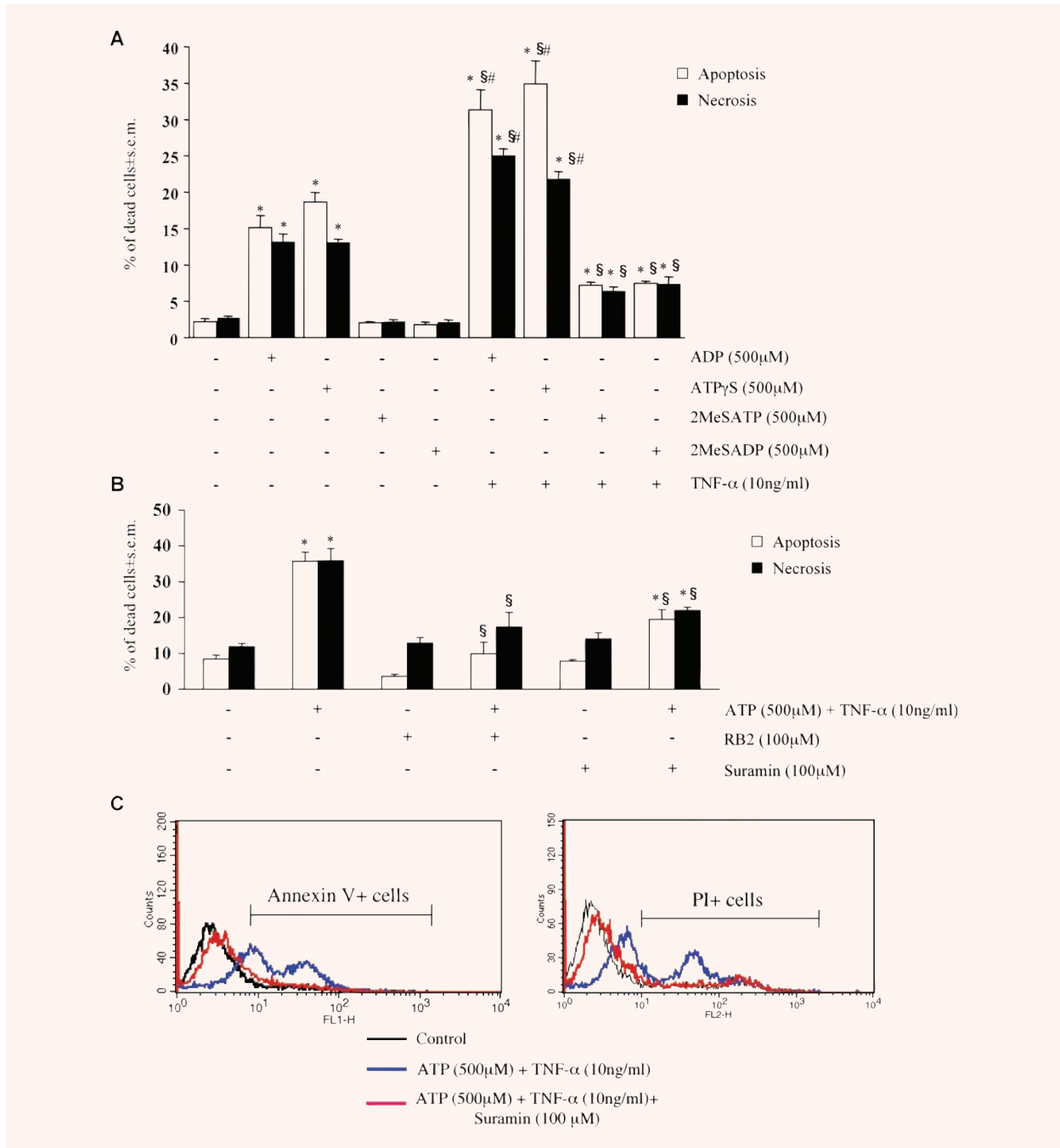
Fig. 3 Pharmacological characterization of adenine nucleotides-induced cardiomyocyte death. HL-1 cardiomyocytes were exposed to either ADP, ATPγS, 2MeSATP or 2MeSADP (all 500 μM) for 24 hrs, alone or in combination with TNF-α (10 ng/ml), as indicated (**A**), or to ATP (500 μM) and TNF-α (10 ng/ml), either alone or in combination with the P2 receptor antagonists RB2 or suramin, all utilized at a 100 μM concentration (**B**). For suramin, the original traces of a representative experiment are shown in C. The percentages of apoptotic and necrotic cells measured by flow cytometric analysis with hypotonic PI (**A**) or by Annexin V/isotonic PI (**B**) (see Materials and methods) are shown. In A and B, data represent the mean ± S.E.M. of three and four independent experiments, respectively. *$P<0.05$ *versus* control. In A, #$P<0.05$ *versus* TNF-α and §$P<0.05$ *versus* corresponding adenine nucleotide alone, and in B §$P<0.05$ *versus* ATP + TNF-α, by one way ANOVA (Scheffe's F test).

but statistically significant inhibition was observed with PPADS and RB2 (Table 2). Also in this case, the specificity of these effects was confirmed by the Annexin V method. In these experiments, isotonic PI (see Materials and methods) was used to measure necrotic cell death in the same samples. Figure 3B shows the results from four independent Annexin V/isotonic PI experiments confirming the protective effects of RB2 and suramin on both the apoptosis (white bars) and the necrosis (black bars) induced by ATP and TNF-α (for suramin, the original traces of one representative experiment are shown in Fig. 3C). Basically, suramin and RB2 were equivalent in reducing cell death at the membrane level, with RB2 showing a little more pronounced effect. Conversely, RB2 did not modify the cell death effects mediated by the P2X agonist BzATP (data not shown), suggesting that different P2 receptor subtypes may be involved in the cell death induced by different adenine nucleotides (see also below). We also tested MRS2179 and cangrelor which act as selective $P2Y_1$ and $P2Y_{12,13}$ receptor antagonists, respectively [26]. In line with the lack of expression of these three receptor subtypes (Fig. 1), none of these antagonists had any effect on the cell death induced by ATP and TNF-α (data not shown). Upon addition to cells, ATP is quickly degraded to adenosine, which can then activate the P1 receptors (subdivided into the A_1, A_{2A}, A_{2B} and A_3 subtypes [28]). Based on previous data suggesting important functional effects of these receptors in the cardiovascular system [29, 30], we next verified if P1 receptors could contribute to the detected effects by utilizing specific P1 receptor antagonists. In our experimental model, ATP-induced cytotoxicity was not counteracted by either 8-cyclopentyl-1,3-dipropylxanthine (CPX, which primarily antagonizes the A_1 and A_{2B} receptors [31–33]), or by the selective A_{2A} antagonist SCH58261, or by the selective A_3 antagonist MRS1220 [28] (data not shown), thus ruling out a role for these receptors in the detected effects.

As mentioned above, BzATP primarily behaves as a P2X receptor-agonist [4], whereas ATP activates both P2X and adenine-sensitive P2Y receptors [4]. Of the utilized P2 receptor antagonists, RB2 is relatively ineffective at P2X receptors and primarily acts as a $P2Y_{4,6,11}$ antagonist, PPADS antagonises $P2X_{1,2,3,5}$ and $P2Y_{4,6}$, whereas suramin effectively blocks $P2X_{1,2,3,5}$ and $P2Y_{2,6,11}$ [4, 34]. On the basis of the results reported above, we suggest that both

Table 2. Reduction of ATP+TNF-α-induced apoptotic cell death by P2 receptor antagonists

	Apoptosis (% of ATP+TNF-α set to 100% ± S.E.M.)
ATP+TNF-α	100.00±5.64
+ Suramin (100 µM)	33.52±6.85*
+ PPADS (100 µM)	49.3±2.1*§
+ RB2 (100 µM)	42.11±7.69*§

Murine cardiomyocytes were exposed to ATP (500 µM) in combination with TNF-α (10 ng/ml), together with the various P2 receptor antagonists (which were added 30 min before). At the end of the incubation period, the percentage of apoptotic death was evaluated by isotonic PI staining of nuclei (see Materials and methods), followed by flow cytometric analysis. ATP+TNF-α-induced apoptosis was set to 100 %. The three antagonists had no effect on cell survival when utilized alone (data not shown). Data represent the mean ± S.E.M of three independent experiments. *$P<0.05$ *versus* ATP+TNF-α, and §$P<0.05$ *versus* control, by one-way ANOVA (Scheffe's F test).

P2X and P2Y receptors mediate cardiomyocytic cell death by adenine nucleotides. Based on the expression profile of P2 receptors in HL-1 cells (Fig. 1), we also suggest that, among P2Y receptors, $P2Y_2/P2Y_4$, are the most likely candidates for induction of cell death. Among P2X receptors, $P2X_1$ and $P2X_5$ which are antagonised by both suramin and PPADS, may be involved in cell death induction; however, since P2X receptors can form heteromers characterized by different and unpredictable pharmacological profiles [4], in this case, a definite conclusion regarding the specific receptor subtypes involved cannot be drawn.

Uracil nucleotides do not affect cardiomyocytic viability and protect against cell death induced by adenine nucleotides

Since several of the P2Y receptors expressed by HL-1 cardiomyocytes also respond to uracil nucleotides (i.e. $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{14}$, Fig. 1), the effects of uracil agonists on HL-1 cardiomyocytes were also assessed. At variance from ATP, neither UTP (which activates $P2Y_2$ and $P2Y_4$) nor UDP nor UDPglucose (which activate the $P2Y_6$ and $P2Y_{14}$ receptor subtypes,

respectively [34]), had any effect *'per se'* on the viability of HL-1 cardiomyocytes, not even when assayed in presence of TNF-α (Fig. 4A). We then tested the possible effect of these ligands on adenine nucleotides-induced death. In a first set of experiments, uracil nucleotides were added to cells 30 min before addition of either ATP alone or ATP+TNF-α. As expected, exposure of HL-1 cardiomyocytes to ATP and TNF-α for 24 hrs resulted in dramatic cytotoxicity (see micrograph in Fig. 4C) and in highly significant induction of both necrosis and apoptosis (see cytogram in Fig. 4C) with respect to control healthy cells (Fig. 4B). Addition of either UDP (Fig. 4D), or UTP or UDPglucose (Fig. 4E) fully reversed cell death induced by ATP+TNF-α. As an example, in the experiment reported in Figure 4E, the number of necrotic and apoptotic cells in the ATP+TNF-α group was reduced from 22.8% and 28.8% to 6.5% and 6.2%, respectively, upon addition of UDPglucose. Results were fully confirmed also by evaluating Annexin V staining of apoptotic cells (Fig. 4F). Here again, Annexin V binding induced by ATP+TNF-α was prevented by UDP. A similar protective effect was observed against ADP-induced cell death. UDP significantly reduced both the apoptosis and necrosis induced by either ADP alone, or by ADP+TNF-α (Fig. 4G).

Both adenine nucleotides-induced death (sum of apoptosis and necrosis) and uracil nucleotides-mediated cytoprotection are concentration-dependent events. In particular, ATP displayed an EC_{50} value (*i.e.* concentration eliciting 50% of maximal effect) of 99.68 µM and 91.41 µM in the absence and presence of TNF-α, respectively (Fig. 5A; Table 3); thus, potentiation of cell death by TNF-α seems mainly due to increased effectiveness rather than to changes in the potency of adenine nucleotides (see also Table 3). Also the protective effects mediated by uracil nucleotides were strictly concentration-dependent, with IC_{50} values (*i.e.* concentrations needed to inhibit 50% of ATP-induced cell death) of 7.29 µM, 10.64 µM and 2.76 µM for UTP, UDP and UDPglucose, respectively (Figs 5B–D; Table 3). This suggests a rank order of potency of UDPglucose $\geqslant$ UTP $\geqslant$ UDP. A comparable rank order of potency for uracil nucleotides was obtained when the effects of UTP, UDP and UDPglucose were analysed separately against ATP-induced apoptosis or necrosis (data not shown). All experiments above have been performed by adding uracil nucleotides to cells 30 min before

adenine nucleotides. We next tested the time-dependence of uracil nucleotides-mediated protection. Addition of UDP together with (Fig. 6C) or 30 min after ATP (Fig. 6D) still fully protected cells against adenine nucleotide-induced death, suggesting the existence of a possible 'therapeutic' window for uracil nucleotides-mediated protection, and that cardiomyocytes do not need to be 'primed' by uracil nucleotides to become insensitive to ATP-induced death.

Discussion

Here we demonstrate that adenine and uracil nucleotides, which both in rodents [9, 35, 36], pig [37] and man [9, 12] are released in the blood stream during heart hypoxia, play strikingly different effects on cardiomyocyte viability. The novelty of the present results is twofold: first, we show a role for P2 receptors (both P2X and adenine-preferring P2Y receptors) in the cardiomyocyte death induced by adenine nucleotides (an effect which is exacerbated by TNF-α, a well-established pathogenetic factor in cardiac disease development), and, second, we demonstrate that uracil nucleotides fully protect against the deleterious effects mediated by adenine nucleotides and TNF-α. A combination of pharmacological and RT-PCR studies suggests that several uracil-preferring P2Y receptors expressed on cardiomyocytes likely contribute to these pro-survival effects. These receptors may thus represent new appropriate targets for innovative drug therapies aimed at cardiac protection during myocardial infarction and in CHF.

In the heart, hypoxic conditions induce ATP release [35, 36]. Functionally, released ATP has been involved in inotropy and hypertrophy of cardiomyocytes [38], and, as more recently demonstrated, in myocardial cell death (our previous data [18] and the present results). All these effects are likely mediated by the activation of specific P2 receptors on either cardiomyocytes or fibroblasts. Here we show that murine cardiomyocytes express a wide panel of P2X and P2Y receptors known to either exclusively respond to adenine nucleotides (P2X receptors), to both adenine and uracil nucleotides (P2Y$_2$, P2Y$_4$, P2Y$_6$) or to sugar nucleotides (P2Y$_{14}$ receptor). Such a large heterogeneity of P2 receptor expression is consistent with previous studies [18, 39–42], and suggests involvement of these receptors in multiple
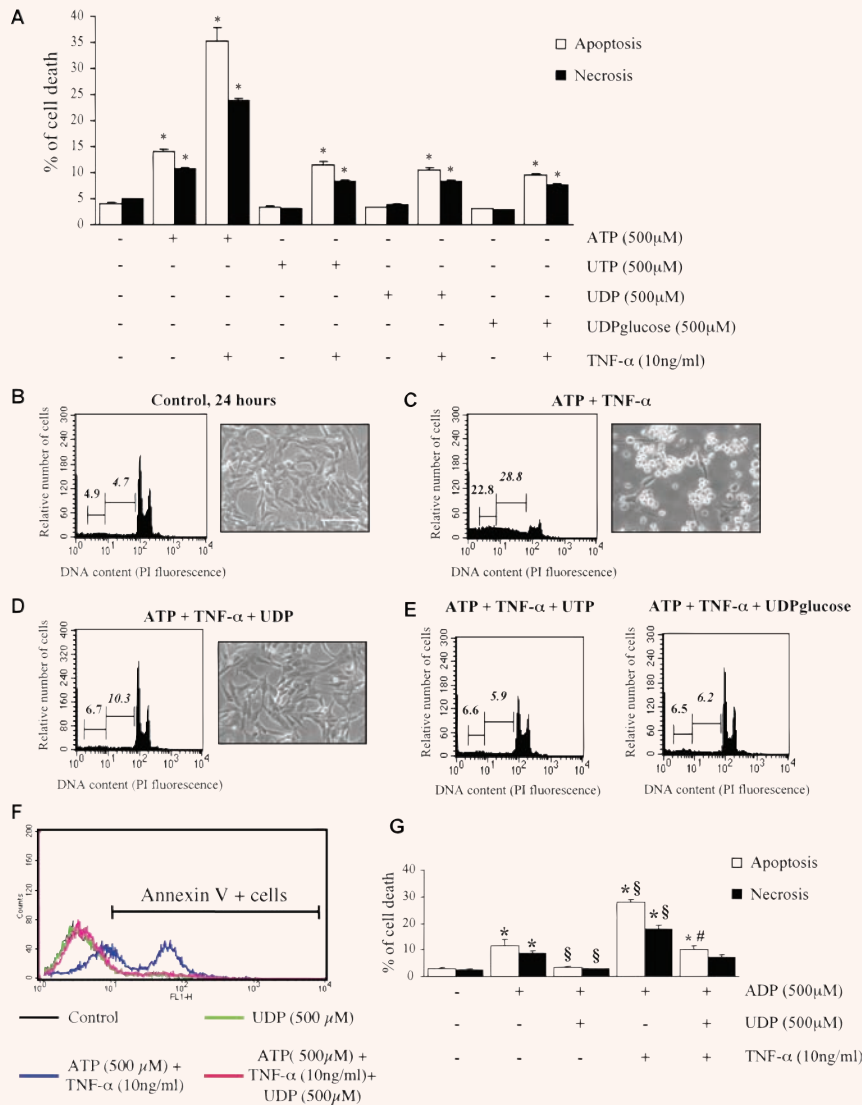
Fig. 4 Uracil nucleotides have no effect *'per se'* on cardiomyocytic viability, but protect cells from ATP and ADP-induced cytotoxicity. To assess the effect of uracil nucleotides on HL-1 cardiomyocytic viability, cells were exposed to UTP, UDP or UDPglucose (500 μM) alone or in combination with TNF-α, and apoptosis and necrosis determined after 24 hrs by flow-cytometry. Parallel cultures were treated with ATP, here utilized as a positive control (**A**). The effect of TNF-α alone is shown in Figure 2B. *$P < 0.05$ *versus* corresponding Control, by one-way ANOVA (Scheffe's F test). To assess the ability of uracil nucleotides to counteract cardiomyocyte death induced by ATP+TNF-α, cells were treated with either vehicle (**B**), or ATP+TNF-α (500 μM and 10 ng/ml, respectively) in the absence (**C**) or presence of UDP (100 μM, D), UTP (25 μM) or UDPglucose (10 μM) (**E**). In these experiments, uracil nucleotides were added to cells 30 min before adenine nucleotides. After 24 hrs, micrographs of adhering cells were taken (scale bar: 50 μm) and flow cytometric analysis was performed on the total cell population. In cytograms, first and second numbers on the left represent the percentage of necrotic and apoptotic cells, respectively. Results from a typical experiment are shown in (**B**–**E**). Similar data were obtained in five independent experiments. UDP similarly protected cells from the apoptosis and necrosis induced by either ADP alone (500 μM) or ADP and TNF-α (10 ng/ml) (**F**). Annexin V staining of cells confirmed the complete protection exerted by uracil nucleotides. The percentages of Annexin V-positive cells in this representative experiment (out of three) were: Control, 8.1%; UDP alone, 8.4%; ATP+TNF-α, 56.4%; ATP+TNF-α+UDP, 11.3%. (**G**) *$P < 0.05$ *versus* corresponding Control, §$P<0.05$ *versus* ADP alone, #$P < 0.05$ *versus* ADP+TNF-α, by one-way ANOVA (Scheffe's F test); the mean values ± S.E.M. of four independent experiments are shown.
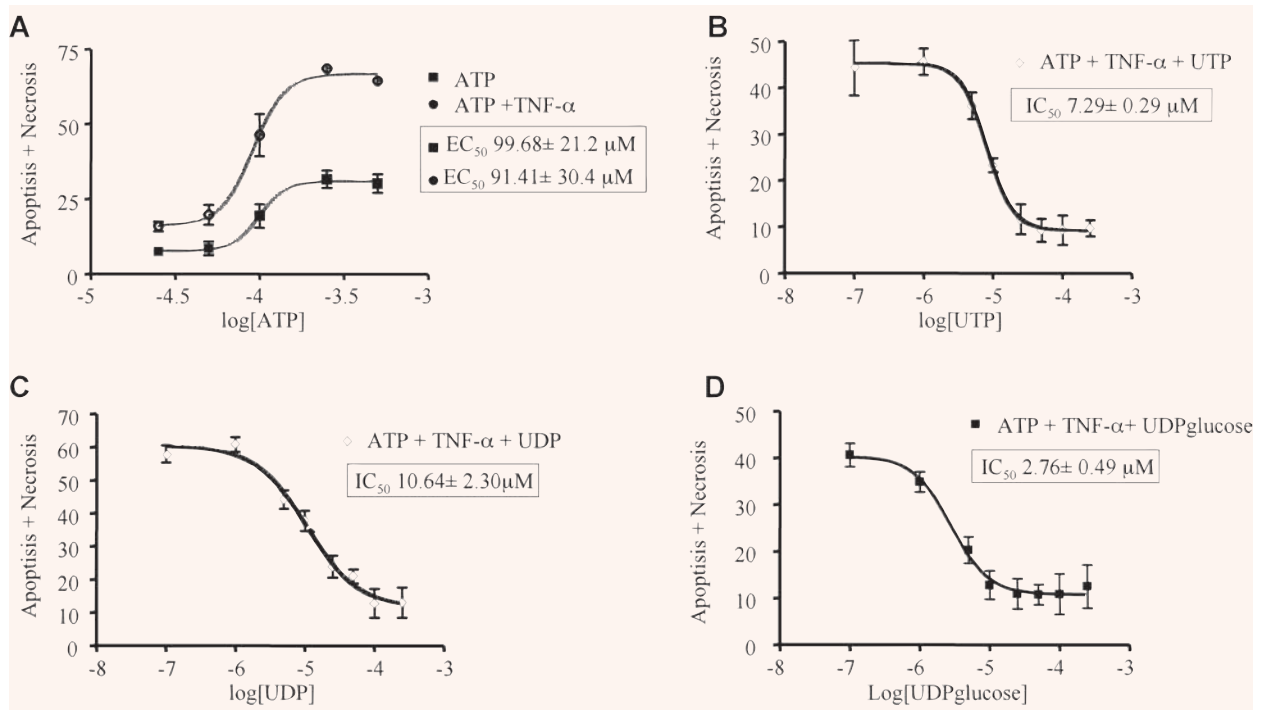
Fig. 5 Concentration dependence of ATP-induced cardiomyocytic death (apoptosis+necrosis) and of uracil nucleotides-mediated protection. HL-1 cardiomyocytes were exposed to graded concentrations of ATP (25–250 μM) alone or in combination with TNF-α (10 ng/ml, **A**). Cultures pre-treated with TNF-α and incubated with 250 μM ATP were exposed to graded (100 nM to 250 μM) concentrations of UTP (**B**), UDP (**C**), or UDPglucose (**D**). After 24 hrs, the percentage of cell death was evaluated by cytofluorimetric analysis of PI stained nuclei. Data represent the mean ± S.E.M. of three independent experiments. Curves were calculated using GRAPH PAD Prism 4.0 software. EC$_{50}$ values for induction of cell death by ATP or ATP+TNF-α and IC$_{50}$ values for uracil nucleotides protection are also shown in individual panels and summarized in Table 3.

functional effects. Specifically, our data suggest that activation of P2 receptors by adenine nucleotides in the presence of TNF-α results in apoptosis and necrosis. In a similar way to pig cardiac fibroblasts [18], only modest effects on HL-1 cardiomyocytes viability were detected when adenine nucleotides and TNF-α were utilized alone ([18] and the present data), suggesting that a synergistic interaction between ATP/ADP and TNF-α is needed to induce cell death. Effects induced by ATP and ADP are concentration-dependent, with EC$_{50}$ values in the μMolar range, which are consistent with the estimated nucleotide concentrations at the cell surface during cardiac ischaemia [12, 37], further confirming that the effects reported here may bear a pathophysiological significance. EC$_{50}$ values for ATP and ADP in induction of cell death were not significantly altered by TNF-α, suggesting that the higher degree of cell

death observed in the presence of the cytokine is likely due to increased effectiveness rather than increased affinity of nucleotides towards their receptors. Induction of cardiomyocyte apoptosis by adenine nucleotides is a receptor-mediated event, as also confirmed by data with the P2 receptor antagonists RB2, PPADS and suramin. Based on pharmacological data, we suggest that both P2X and P2Y receptors may contribute to induction of cell death in murine cardiomyocytes.

At variance from ATP, it is only recently that UTP has been shown to be released during heart ischaemia. The first demonstration was provided by Erlinge and coworkers in a pig cardiac ischaemia *in vivo* model [37]. UTP levels in the venous blood from the cardiac vein increased according to a biphasic pattern, with an increase 1 min after onset of cardiac ischaemia, and a second increase 1 min after reperfusion.

Table 3. Potency of adenine and uracil nucleotides in regulation of HL-1 cardiomyocytes viability

Nucleotide	Type of effect	EC$_{50}$ or IC$_{50}$ ± S.E.M.
ATP	Induction of apoptosis and necrosis	99.68±21.2 μM
ATP + TNF-α	Induction of apoptosis and necrosis	91.41±30.4 μM
UTP	Protection against cell death induced by ATP+TNF-α	7.29±0.29 μM
UDP	Protection against cell death induced by ATP+TNF-α	10.64±2.30 μM
UDPglucose	Protection against cell death induced by ATP+TNF-α	2.76±0.49 μM

Murine cardiomyocytes were exposed to graded concentrations of either ATP alone (25–500 μM), or ATP in the presence of the cytokine TNF-α (10 ng/ml), or UTP, UDP and UDP-glucose (100 μM to 250 μM). In experiments where uracil nucleotides were challenged for their ability to inhibit the effects induced by adenine nucleotides, an ATP concentration of 250 μM in the presence of 10 ng/ml TNF-α was utilized. In all experiments, effects were detected after 24 hrs. At the end of treatments, the percentage of cell death (apoptosis+necrosis) was evaluated by cytofluorimetric analysis of PI stained nuclei. Reported EC$_{50}$ and IC$_{50}$ values refer to experiments shown in Fig. 5 (for more details, see Fig. 5 legend and text).

In the same animals, total venous blood flow from the heart and episodes of ventricular fibrillation or tachycardia peaked at the same time points, suggesting that nucleotide release positively correlated with both cardiac blood flow and ventricular arrhythmia. Importantly, release of UTP during myocardial infarction has been more recently confirmed also in man [12]. There still is little information regarding the source of UTP release during heart ischaemia. Although both myocytes, endothelial cells and platelets may theoretically release uridine nucleotides into the blood stream, the only published evidence for significant release of UTP is from endothelial cells during changes in flow (shear stress) [43]. Released UTP may then act on myocardial P2 receptors; in line with our previous findings [18], several uracil-preferring P2Y receptors were indeed found by Wihlborg and coworkers [12] in the human heart (P2Y$_2$, P2Y$_4$, P2Y$_6$). These receptors are also expressed in the

murine cardiomyocyte model utilized here, thus supporting its validity for unveiling the functional roles of these receptors in mammalian heart. In the Wihlborg *et al.* study, UTP and UDP were also demonstrated to mediate inotropic effects likely *via* P2Y$_6$. On this basis, and based on previous findings demonstrating induction of vasoconstriction by UDP and UTP [44], these authors proposed that uracil nucleotides may play a similar role as angiotensin II in the development of cardiac disease, and that, in a similar way to angiotensin II receptor antagonists and angiotensin converting enzyme inhibitors, selective antagonists of UDP and UTP receptors may represent novel agents in the treatment of hypertension and heart failure *via* inhibition of peripheral resistance, inotropy and cardiac hypertrophy. Our results instead suggest that uracil nucleotides may also induce beneficial effects on cardiomyocytes. Both UTP and UDP and the sugar-nucleotide UDPglucose effectively counteracted cell death induced by adenine nucleotides and TNF-α on HL-1 cardiomyocytes. Protection by uracil nucleotides is concentration-dependent, with strikingly low IC$_{50}$ values (in the low μMolar range) against ATP and ADP-induced cell death. Pharmacological and expression data suggest that cardiomyocyte protection is mediated by multiple uracil-sensitive P2Y receptors (P2Y$_2$, P2Y$_4$, P2Y$_6$ and P2Y$_{14}$; in this respect, see also below).

The protective effects described here are consistent with the previous demonstration that UTP protects neonatal rat cardiomyocytes against injury induced by 120 min of hypoxia [16]. However, the possible functional antagonism between uracil and adenine nucleotides was not addressed in this study, and only UTP, but not UDP, effectively protected cardiomyocytes. P2Y$_2$ and P2Y$_4$ receptors, whose presence in neonatal cardiomyocytes was confirmed by immunofluorescence staining and western blot, are likely to mediate protection in this study, whereas a role for P2Y$_6$ was ruled out, based on the lack of effect of UDP. This protection profile seems different from that reported here, where all tested uracil nucleotides were found to be protective. Moreover, the effects described above [16] seems related to a preconditioning mechanism, since cells needed to be pre-exposed to UTP before hypoxia to show protection. Here, both UTP and other uracil nucleotides were able to effectively protect cells even when added after adenine nucleotides and TNF-α. Strikingly, our data suggest that the same receptors may even be
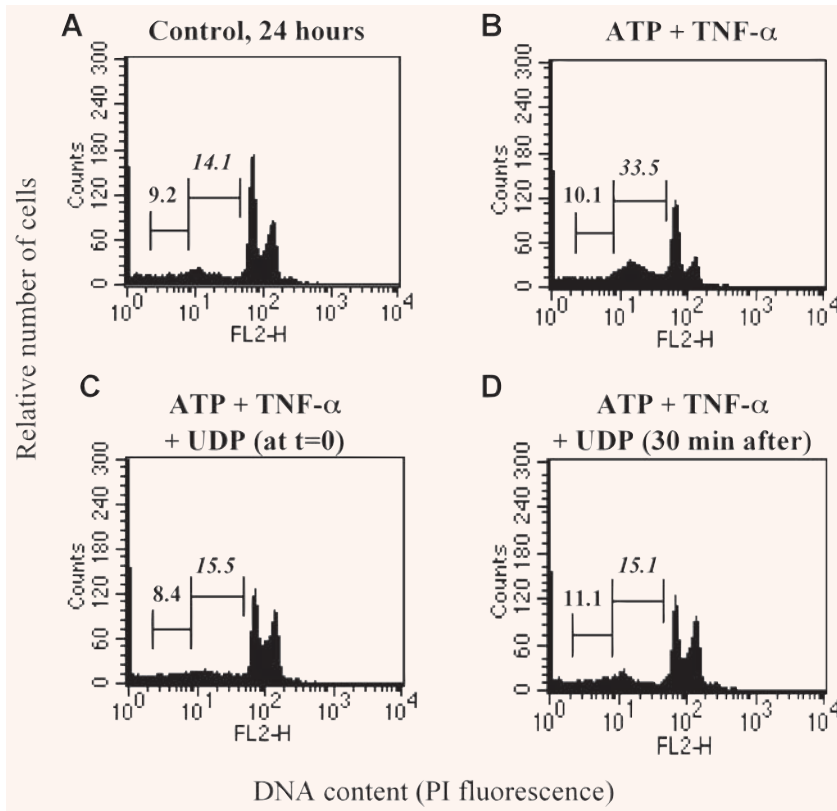
Fig. 6 Characterization of UDP-induced protection. HL-1 cardiomyocytes were exposed to either vehicle (Control, **A**), ATP (100 µM) and TNF-α (10 ng/ml) (**B**), as indicated, for 24 hrs. UDP (100 µM) was added to cultures either together with ATP (**C**) or 30 min after its addition (**D**). At the end of treatment, flow cytometric analysis was performed on the total cell population. In cytograms, first and second numbers on the left represent the percentage of necrotic and apoptotic cells, respectively.

involved in both cell death and cell protection. This applies in particular to the HL-1 P2Y$_2$ and P2Y$_4$ receptors, that in rodents respond to both ATP and UTP [8, 32]. It may be hypothesised that, depending upon the identity of the nucleotide (adenine *versus* uracil) binding to the receptor, different intracellular pathways are activated, eventually leading to either cell death (in the case of ATP) or cell protection (in the case of UTP). Alternatively, when the receptor is activated by one of its endogenous agonists (*e.g.* ATP) another agonist (UTP) can act as an antagonist. This would not be unexpected for P2Y receptors: for example, under specific conditions, the agonist ATP can act as an antagonist at the ADP-activated P2Y$_{12}$ receptor subtype [8]. This interesting issue will be investigated in HL-1 cardyomiocytes future studies.

The protective effects of UTP have been also very recently confirmed in rats upon induction of myocardial infarction [17]. In animals injected with UTP 30 min before infarction, the ratio of infarct size to area at risk was significantly smaller with respect to control untreated rats.

In conclusion, therapeutic targeting of pyrimidinergic receptors may represent a novel promising strategy for myocardial protection that certainly deserves further exploration.

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