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Uridine triphosphate (UTP) induces profibrotic responses in cardiac fibroblasts by activation of $P2Y_2$ receptors

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Abstract

Cardiac fibroblasts (CFs) play a key role in response to injury and remodeling of the heart. Nucleotide (P2) receptors regulate the heart but limited information is available regarding such receptors in CFs. We thus sought to determine if extracellular nucleotides regulate fibrotic responses (e.g., proliferation, migration and expression of profibrotic markers) of CFs in primary culture. UTP increased rat CF migration 3-fold ($p < 0.001$), proliferation by 30% ($p < 0.05$) and mRNA expression of profibrotic markers: alpha smooth muscle actin (α-SMA), plasminogen activator inhibitor-1 (PAI-1), transforming growth factor beta, soluble ST2, interleukin-6 and monocyte chemoattractant protein-1 (MCP-1) by 3.0-, 15-, 2.0-, 7.6-, 11-, and 6.1-fold, respectively ($p < 0.05$). PAI-1 protein expression induced by UTP was dependent on protein kinase C (PKC) and extracellular signal-regulated kinase (ERK), based on blockade by the PKC inhibitor Ro-31-8220 and the ERK inhibitor U0126, respectively. The rank order for enhanced expression of PAI-1 and α-SMA by nucleotides (UTPγS≫UDPβS≫ATPγS), the expression of $P2Y_2$ receptors as the most abundantly expressed P2Y receptor in rat CFs and a blunted response to UTP in $P2Y_2^{-/-}$ mice all implicate $P2Y_2$ as the predominant P2Y receptor that mediates nucleotide-promoted profibrotic responses. Additional results indicate that $P2Y_2$ receptor-promoted profibrotic responses in CFs are transient, perhaps as a consequence of receptor desensitization. We conclude that $P2Y_2$ receptor activation is profibrotic in CFs; thus inhibition of $P2Y_2$ receptors may provide a novel means to diminish fibrotic remodeling and turnover of extracellular matrix in the heart.

Keywords

ATP; cardiac fibroblasts; purinergic signaling; P2Y receptors; PAI-1; UTP

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Disclosures
None

Introduction

Heart failure is a major cause of morbidity and mortality in economically developed countries. Although heart failure is classically characterized by systolic dysfunction, i.e., decreased cardiac contraction, inadequate filling of the heart, diastolic dysfunction, is increasingly recognized as an important contributor to heart failure [1–3].

Increased deposition of extracellular matrix (ECM) in the heart, cardiac fibrosis, increases the stiffness of the myocardium, thereby contributing to impaired diastolic function. Fibrosis accompanies many types of cardiac pathology, including hypertensive heart disease, post myocardial infarction remodeling, diabetic cardiomyopathy and in aging [1,3–5]. Cardiac fibroblasts (CFs), the most numerous cell type in the heart, play a key role in the homeostatic maintenance of the ECM [6].

CFs are regulated by profibrotic and antifibrotic signals. Transforming growth factor-β (TGF-β) and angiotensin II (Ang II) are two profibrotic peptides that induce signaling events in CFs to increase ECM synthesis. TGF-β activates protein kinase receptors while Ang II signals through Gq-coupled AT_1 G-protein-coupled receptors (GPCRs) [4,7,8]. Offsetting such pro-fibrotic effects, signaling through cAMP and its downstream mediators induce antifibrotic responses [9,10].

Nucleotides (ATP, ADP, UTP and UDP) can be released into the interstitial space in response to stimuli that include mechanical stretch, chemical stress, platelet activation and cell death [11]. Extracellular nucleotides can then stimulate plasma membrane-localized nucleotide receptors: P2X receptors, which are ion channels and P2Y receptors, which are GPCRs [12]. P2X receptors preferentially interact with ATP while the 8 P2Y receptor subtypes are adenine nucleotide-preferring ($P2Y_1$, $P2Y_2$, $P2Y_{11}$, $P2Y_{12}$ and $P2Y_{13}$), uridine nucleotide-preferring ($P2Y_2$, $P2Y_4$ and $P2Y_6$) or responsive to sugar nucleotides ($P2Y_{14}$) [12]. Cell type-specific expression of particular P2Y receptors determines the responses to nucleotide stimulation. In cardiomyocytes, at least three subtypes of P2Y receptors can increase inotropy, regulate hypertrophic growth and modulate the response to pressure overload [13–17]. Limited data are available regarding P2Y receptors in CFs. We thus sought to determine if extracellular nucleotides regulate profibrotic responses of CFs and if so, the identity of the P2Y receptors that mediate such responses.

Materials and Methods

Reagents

UTP, UDP, ATPγS and Ro-31-8220 were purchased from Sigma. Antibody to PAI-1 was from BD Biosciences, α-SMA antibody was from Sigma, ERK antibodies were from Stressgen and GAPDH antibody was from Abcam. U0126 was from Tocris. Antibody to $P2Y_2$ receptors was from Alomone. UTPγS and UDPβS were a kind gift from Prof. D. Erlinge (University of Lund, Sweden).

Isolation and Culture of Adult Rat and Mouse CFs

CFs were isolated from adult Sprague–Dawley rats (250 – 300 g, male) or C57/BL6 wild-type or $P2Y_2^{-/-}$ mice (20 – 25 g, male), as previously described [18]. Briefly, CFs were separated from cardiac myocytes by gravity separation and grown to confluency on 10-cm cell culture dishes at 37°C with 90% air/10% CO_2 in growth media (DMEM/10% FBS/1% penicillin/1% streptomycin). All animals were cared for in compliance with the guiding principles of the American Physiological Society and as approved by the UCSD Institutional Animal Care and Use Committee.

[^{3}H]Thymidine Incorporation

[^{3}H]Thymidine incorporation was used to assess DNA synthesis. CFs (1.5×10^5 per well) were seeded into a 12-well culture plate and serum-starved overnight. One μCi (1 Ci = 37 GBq) of [^{3}H]thymidine/ml was added in combination with UTP or vehicle control and the cells were incubated for 24 or 48 h at 37°C. The cells were washed with cold PBS and 7.5% TCA and then dissolved in 0.5 M NaOH before liquid scintillation counting.

CF Migration

Migration of CFs was assayed by using the Boyden chamber method. The cells were maintained in serum-free conditions for 24 h and then suspended in serum-free DMEM at a density of 1×10^5 CFs/100 μl in uncoated chamber inserts. Basal migration was assessed by adding serum-free DMEM (600 μl) to the lower chambers. Cells were allowed to migrate for 16 h, then fixed in 10% formalin and stained with Hema 3 (Fisher Scientific). Cells on the upper surface of the membrane were mechanically removed with a cotton swab. Cells that migrated were counted from 3 different fields (0.1 mm^2/field)

Real-time Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated by using RNeasy (Qiagen) and cDNA was generated using the Superscript III cDNA synthesis system (Invitrogen) according the manufacturers' instructions. RT-PCR analysis was performed on a DNA Engine Opticon 2 (Biorad) using the qScript™ One-Step qRT-PCR kit (Quanta Biosciences). Primers for PCR amplification (Table 1) were designed based on the nucleotide sequences of the respective gene target and for P2Y receptors were from ref. [19]. When possible, each forward and reverse primer set was designed between multiple exons. Amplification efficiency of each primer pair was tested prior to analysis. Relative gene expression levels were determined using the ΔΔCT method [20] with 18S as the reference gene.

Immunoblot Analysis

Whole cell lysates were prepared in $NaCO_3$ buffer (pH 11) and homogenized by sonication. Equal amounts of protein (assayed using a dye-binding reagent, Bio-Rad) were separated by SDS/PAGE using 10% polyacrylamide precast gels (Invitrogen) and transferred to a poly(vinylidene difluoride) membrane with the iBlot system (Invitrogen). Membranes were blocked in PBS Tween (1%) containing 5% nonfat dry milk and incubated with primary antibody 18 h at 4°C. Bound antibodies were visualized using horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) and ECL reagent (Amersham Pharmacia). Bands were compared to molecular weight standards to confirm migration of proteins at the appropriate size. Quantitation of protein expression densitometry was performed using ImageJ software (NIH).

Flow Cytometry

FACS analysis was performed on a BD FACScan flow cytometer equipped with an argon laser capable of excitation at 488 nm. FITC fluorescence was detected with a 530/30 bandpass filter (FL-1). Amplifier gains and instrument voltage were not changed for the duration of each experiment. CFs were serum-starved in DMEM overnight and treated for 4 and 24 h with UTP, UTPγS, or Ang II. Cells were then detached with PBS with 5mM EDTA (pH 7.2), fixed in 1.5% formalin and permeabilized in ice-cold methanol.

CFs were washed in PBS containing 1% BSA, 0.05% NaN_3 and incubated for 1 h at room temperature with antibody against α-SMA, PAI-1, or mouse-isotype control antibody diluted in PBS/1% BSA. Cells were washed and incubated for 30 min with Alexa Fluor 488 donkey anti-mouse antibody (Invitrogen) and then washed and resuspended in PBS for FACScan

analysis. Fluorescence was acquired in log-scale via an FL-1 filter and data was analyzed and plotted with CellQuest (BD) and Weasel (WEHI).

Immunofluorescence Analysis

Cardiac ventricles were harvested, frozen and mounted on a cryostat to cut 10-μm sections. Sections were fixed in cold acetone, blocked with 4% BSA in 0.1% Tween and PBS, and incubated with primary antibodies (1:100) in 4% BSA/0.1% Tween/PBS. After incubation with Alexa-conjugated secondary antibody (Molecular Probes) (1:250), samples were mounted in Vectashield (Vector Laboratories) mounting media containing DAPI. Specificity of staining was determined by omission of the primary antibody. Images were obtained by using a Zeiss LSM510 Laser Scanning Confocal Microscope and Zeiss Image Examiner software.

Statistical Analysis

Calculations and statistics were performed using GraphPad Prism 5.0 software. Values are presented as mean ± S.E.M. ANOVA with Dunnett's post-test was used to compare quantitative RT-PCR relative expression data with untreated controls. Analysis of experiments with multiple comparisons was by ANOVA with Bonferroni's correction.

Results

UTP Induces Proliferation and Migration in Rat CFs

Proliferation and migration of CFs are key events in the fibrotic response of cardiac remodeling and extracellular nucleotides are known to have mitogenic effects in other cell types such as vascular smooth muscle cells [21]. Stimulation of serum-starved, quiescent rat CFs with 10 μM UTP increased [^{3}H]thymidine incorporation by 24 h (Fig. 1A). We observed a similar effect with 10 μM ATP at 24 h (data not shown). UTP also induced a 3-fold-increase ($p < 0.001$) in cell migration by 24 h, as assessed by the Boyden chamber method (Fig. 1B).

UTPγS Induces Transcription of Profibrotic Genes

The fibrotic response in the heart is modified by a large number of genes that regulate ECM homeostasis. Alpha smooth muscle actin (α-SMA) is a marker of the conversion of resting CFs to activated, phenotypically distinct myofibroblasts. Incubation of rat CFs with UTPγS (10 μM, 4 h) increased the expression of α-SMA mRNA 3.0-fold ($p < 0.001$) (Fig. 2). Plasminogen activator inhibitor (PAI-1) plays a key role in tissue fibrosis as a protease inhibitor that regulates matrix metalloproteinases [22]. UTPγS (10 μM, 4 h) increased PAI-1 mRNA expression 15-fold ($p < 0.001$) and mRNA expression of soluble ST2 (sST2), a receptor for interleukin-33, a prognostic marker in heart failure 7.6-fold ($p < 0.001$) (Fig. 2) [23]. In addition, 4 h UTPγS increased mRNA expression of the proinflammatory, profibrotic factors TGF-β and monocyte chemoattractant protein-1 (MCP-1) by 2.0- and 6.1-fold, respectively ($p < 0.01$ and $p<0.001$, respectively) (Fig. 2). UTP transiently stimulated profibrotic and proinflammatory markers in CFs. Even with the use of an ectonucleotidase-resistant analog, UTPγS, most gene expression was only significantly up-regulated at 4 h of treatment, with the exception of interleukin-6 (IL-6) which had a peak 11-fold increase ($p<0.01$) in mRNA levels after 8 h (Fig. 2). Desensitization of P2Y receptors rather than degradation of extracellular UTP thus likely accounts for the decrease in responses over the 24 h period of treatment, especially because CF treated with TGF-β or Ang II show increased profibrotic gene expression past 24 h (data not shown).

UTP Induces PAI-1 Protein Expression in a Dose- and Time-Dependent Manner but Response to UTP Desensitizes

We further characterized PAI-1 expression in UTP-treated CFs since its mRNA increase was the most prominent of all the markers we tested. UTP dose-dependently increased mRNA and protein expression of PAI-1 (Fig. 3A–C). PAI-1 protein expression increased within 2 h, peaked after 4 h and gradually decreased to basal levels after 24 h (Fig. 3D, E), consistent with the trend in PAI-1 mRNA expression after UTP stimulation. UTPγS produced a similar time-dependent pattern of PAI-1 protein expression (data not shown).

Flow cytometry was used as an additional approach to assess PAI-1 protein expression and also, that of α-SMA in rat CFs. Consistent with the data obtained by RT-PCR and Western blotting, we found that UTP increases PAI-1 expression at 4 h (Fig. 4A). However, by 24 h the enhanced PAI-1 expression reverts nearly to baseline levels even though Ang II produces sustained upregulation of PAI-1 expression (Fig. 4B). Furthermore, CFs are less responsive to UTP than to Ang II (Fig. 4A, B) and UTP does not seem to enhance α-SMA protein expression at either 4 or 24 h. The initial rise in α-SMA mRNA levels (Fig 2) thus does not lead to a later increase in protein expression (Fig. 4C, D). UTPγS elicited the same response as UTP in all time points (data not shown). Interestingly, the enhanced expression of α-SMA in response to Ang II is not observed after 4 h treatment but only at 24 h, suggesting that the increase in α-SMA production requires persistent signaling, which does not occur with stimulation by UTP.

UTP Induces PAI-1 Expression in an Erk-and PKC-dependent Manner

$P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors couple to Gq and increase diacylglycerol and intracellular Ca^{2+} leading to protein kinase C (PKC) activation [12]. Treatment of CFs with the PKC inhibitor Ro-31-8220 abolished UTP-induced PAI-1 expression (Fig. 5A, B). Consistent with the ability of P2Y receptors to increase extracellular signal-regulated kinase (ERK) signaling [12], the MAPK/ERK kinase (MEK) inhibitor U0126 decreased UTP-induced PAI-1 expression (Fig. 5A, B); such an effect may derive from transactivation of the EGF receptor or GPCR activation of ERK by Gβγ subunits [24]. UTP stimulated ERK-phosphorylation in CFs (Fig. 5C). The calcineurin inhibitor cyclosporine A did not affect UTP-induced PAI-1 response (Fig. 5A, B).

UTP-induced Effects Are Predominately Mediated by $P2Y_2$ Receptors

UTP can stimulate $P2Y_2$ and $P2Y_4$ receptors but in addition, UTP is hydrolyzed to UDP, which acts on $P2Y_6$ receptors [12]. To identify the P2Y receptor responsible for the effects of UTP that we observed on CFs we used ATP, UDP and the stable nucleotides ATPγS, UDPβS and UTPγS and found that the effects of UTPγS (10 μM) on α-SMA and PAI-1 mRNA expression were similar to those observed with UTP (Fig. 6A, B). By contrast, incubation of CFs with UDP (10 μM) or the stable agonist UDPβS (10 μM) did not increase α-SMA expression and increased PAI-1 to a lesser extent than did UTP (Fig. 6A, B). Expression of PAI-1 and α-SMA mRNA was not significantly increased by ATP or ATPγS (both 10 μM) (Fig. 6A, B). The rank order for nucleotide-promoted increase in PAI-1 and α-SMA mRNA expression (UTPγS≫UDPβS≫ATPγS) suggests that either $P2Y_2$ or $P2Y_4$ is the main receptor that mediates UTP-induced effects. $P2Y_2$ receptors are much more highly expressed than $P2Y_4$ receptors in CFs (Fig. 8A), thus implicating $P2Y_2$ as the predominant receptor sub-type that mediates response to UTP. ATP and ATPγS produce different effects than do UTP and UTPγS in terms of expression of α-SMA or PAI-1 (Fig. 6A, B). Since ATP and UTP are equipotent agonists for $P2Y_2$ receptors, more than one subtype of P2Y receptor may mediate the effects of ATP.

UTP-induced PAI-1 Expression and Cell Proliferation are Blunted in $P2Y_2^{-/-}$ Mice

To help define the role of $P2Y_2$ receptors in the response of CFs, we isolated CFs from wild-type and $P2Y_2^{-/-}$ mice and assayed ability of UTP to regulate PAI-1 expression and induce proliferation. Incubation with UTPμS (10 μM) prominently increased PAI-1 protein expression in CFs from wild-type mice, an effect completely blunted in CFs from $P2Y_2^{-/-}$ mice (Fig. 7A, B). These results indicate that the UTP-induced increase in PAI-1 expression is mediated by $P2Y_2$-receptors in CFs from mice and provide data that are complementary to those shown in the previous section regarding the role of $P2Y_2$ receptors in rat CFs. In addition, the stimulatory effect of UTP on CF proliferation is also blunted in $P2Y_2^{-/-}$ cells: UTPγS (10 μM) increased proliferation of CF from WT mice by almost 50% ($p<0.01$) at 24 h but had no proliferative effect on CFs from $P2Y_2^{-/-}$ mice (Fig. 7C, D). Proliferation occurred in WT CF at 24 h but not 48 h (Fig. 7C).

The $P2Y_2$ Receptor is the Predominant P2Y Receptor in Rat Cardiac Fibroblasts and is Detected on Cardiac Myocytes and Fibroblasts in Rat Left Ventricle

Quantification of mRNA for $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{12}$ revealed that the $P2Y_2$ receptors are the most highly expressed (>50-fold higher than $P2Y_4$ receptors, 3.5-fold more than the $P2Y_6$ and 11-fold more than the $P2Y_1$ receptors [$p < 0.001$]) in rat CFs (Fig. 8A). $P2Y_{12}$ receptors are expressed at very low levels. Immunoblotting detected a single band (with the appropriate size of $P2Y_2$ receptors) in CFs but not human platelets, a $P2Y_2$-null tissue (Fig. 8B). Immunohistochemistry and confocal microscopy revealed $P2Y_2$ receptor expression on cardiomyocytes in rat left ventricular tissue but also colocalized with fibroblasts, identified with a fibroblast-specific DDR-2 antibody (Fig. 8C).

Discussion

Cardiac remodeling following stress or damage to the myocardium is associated with increased morbidity and mortality [25]. Stiffening due to fibrosis is a key event in cardiac remodeling. The most effective pharmacological agents for heart failure, inhibition of the renin-angiotensin-aldosterone system and β-adrenergic receptor blockers, improve cardiac remodeling [25]. GPCR expression profiling has revealed that $P2Y_2$ receptors are expressed in the left ventricles at a level comparable to that of Angiotensin-1 and β1-adrenergic receptors, suggesting that the $P2Y_2$ receptors contribute to cardiac regulation and perhaps remodeling [26].

Three P2Y receptors ($P2Y_2$, $P2Y_6$ and a $P2Y_{11}$-like receptor) increase inotropy [13,17]. Moreover, UTP can increase hypertrophic growth of rat neonatal cardiomyocytes [14,16]. Neonatal rat cardiac myofibroblasts were recently shown to have $P2Y_1$-, $P2Y_2$-, $P2Y_4$-, $P2Y_6$- and $P2Y_{11}$-induced signaling through Gq, Gi and Gs [27]. Nishida et al described a role for $P2Y_6$ in modulating $G\alpha_{12/13}$ signaling and cardiac fibrosis in response to pressure overload in mice [15]. Those authors concluded that cardiac myocyte $P2Y_6$ receptors contribute to stretch-induced modulation of the ECM but did not consider receptors on CF. Other studies suggest that UTP has a protective role in ischemia-reperfusion injury; the receptors that mediate this protection may include $P2Y_2$ receptors [28,29]. ATP inhibits proliferation and modulates adrenergic-promoted growth of rat neonatal CFs but the nucleotide receptor(s) and mechanisms for this response are not known [30]. Our data that implicate $P2Y_2$ receptors in fibrotic response of CFs identify a cellular consequence of the increases in phosphoinositide hydrolysis and cellular Ca^{2+} promoted by cardiac $P2Y_2$ receptors [31].

Communication between CFs and cardiomyocytes has recently been shown to contribute to cardiac development and homeostasis. Release of nucleotides by cardiomyocytes can act in

an autocrine and/or paracrine manner to stimulate P2Y receptors on cardiomyocytes and also potentially receptors on CFs [32]. Our finding that $P2Y_2$ receptors on CFs induce TGF-β, sST2 and IL-6 expression, factors that are known to be involved in CF-cardiomyocyte crosstalk, identifies a receptor that mediates such autocrine/paracrine response and CF-cardiomyocyte crosstalk.

The current results show that UTP induces proliferation, migration and an increase in profibrotic gene expression in CFs. The use of pharmacological approaches and assessment of P2Y gene expression in rats and in $P2Y_2$-knockout mice strongly implicate a predominant role for $P2Y_2$ receptors in these profibrotic effects. Thus, our data demonstrate a previously unappreciated role for nucleotide receptors in promoting a profibrotic phenotype in CFs. Though UTP does not increase protein expression of α-SMA, a marker of phenotypic transformation to myofibroblasts, the increase in other fibrotic markers suggests that UTP contributes to the acute-phase response after injury of CF, perhaps by modulating formation and composition of cardiac ECM.

Although UTP and ATP are equipotent in stimulating rat $P2Y_2$ receptors [33], we find that ATP-stimulated increases in PAI-1 and α-SMA mRNA expression are less than those in response to UTP, thus implicating more than one receptor in ATP response. In mouse cardiomyocytes, which show a similar discrepancy for ATP and UTP, ATP acts via $P2Y_2$ and $P2Y_{11}$-like receptors [13]. Our finding that UTP acts predominantly via $P2Y_2$ receptors to increase PAI-1 mRNA and protein expression in CFs contrasts with data in rat vascular smooth muscle cells in which $P2Y_6$ receptors mediate PAI-1 induction [34].

P2Y receptors regulate the proliferation and ECM production by renal mesangial cells and exaggerated release of nucleotides and increase in P2Y-induced fibrotic responses can occur in a diabetic setting [35,36]. Moreover, decreased degradation of extracellular nucleotides in CD39-knockout mice results in more severe glomerular sclerosis after induction of diabetes mellitus [37]. ATP also contributes to hypoxia-induced increase in growth of lung fibroblasts and P2Y receptors are profibrogenic in hepatic stellate cells [38,39]. Our results for CFs and those for fibroblasts from kidney, lung and liver suggest that signaling via P2Y receptors is a general profibrotic mechanism.

PAI-1 was initially characterized as an inhibitor of the tissue-type and urokinase-type plasminogen activators (tPA and uPA) [40]. Plasmin formed after cleavage of plasminogen, the main substrate for tPA and uPA, regulates fibrin degradation but also matrix metalloproteinase (MMP) activation and TGF-β activation. In addition to the cleavage of plasminogen, tPA and uPA can activate several MMPs in renal fibroblasts, namely MMP-1, -2, -3, and -9 [41–43]. Moreover, PAI-1 is a critical modulator of kidney and liver fibrosis [37,44,45]. In the heart, PAI-1 increases in diabetes mellitus, with increasing age and contributes to cardiac fibrosis post myocardial infarction [22,46,47]. An acute rise in plasma PAI-1 levels after myocardial infarction is a strong prognostic indicator of mortality and development of heart failure [48,49]. Notably, we found that PAI-1 was dramatically upregulated in CFs in response to $P2Y_2$ receptor activation. The inhibitory role of PAI-1 on fibrinolysis and MMP activation suggests that the acute, transitory increase of PAI-1 in response to UTP may alter ECM dynamics.

In conclusion, the current results show a role for $P2Y_2$ receptor activation in the function of CFs. Our findings imply that $P2Y_2$ receptor inhibition is potentially a novel means to diminish fibrotic remodeling in the heart by reducing matrix production and deposition, release of PAI-1 and via MMP-promoted turnover of ECM.

Abbreviations

ADP	adenosine diphosphate
ATP	adenosine triphosphate
CFs	cardiac fibroblasts
DAPI	4',6-diamidino-2-phenylindole
ECM	extracellular matrix
ERK	extracellular signal-regulated kinase
GPCR	G-protein-coupled receptors
MEK	MAPK/ERK kinase
PAI-1	plasminogen activator inhibitor-1
PKC	protein kinase C
α-SMA	α-smooth muscle actin
sST2	soluble ST2
TGF-β	transforming growth factor-β
UDP	uridine diphosphate
UTP	uridine triphosphate

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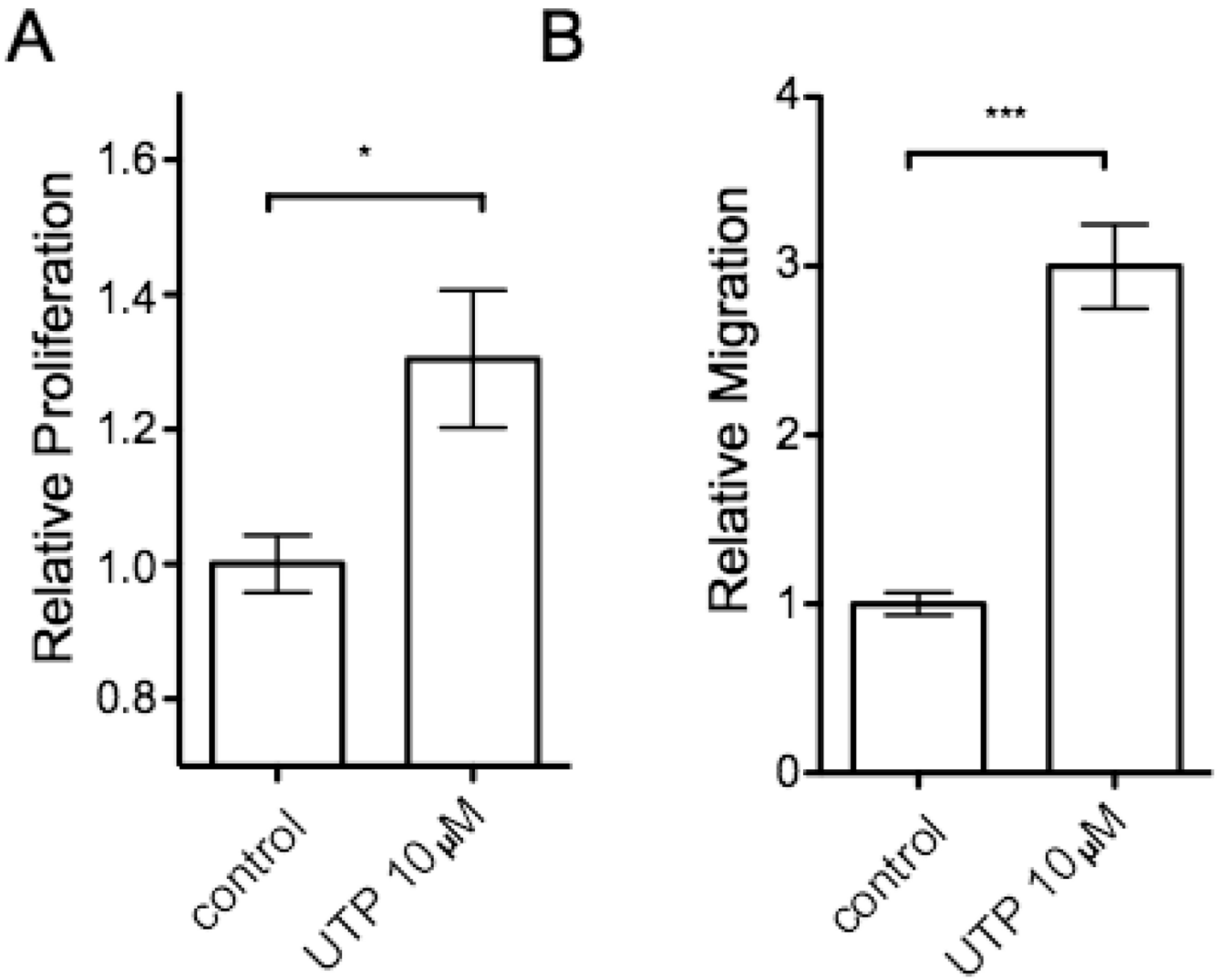

Figure 1. UTP induces proliferation and migration of rat cardiac fibroblasts

(A) Proliferation of passage 1 rat CFs serum-starved for 24h and stimulated with 10 μM UTP was examined by [^{3}H]thymidine incorporation. UTP increased [^{3}H]thymidine incorporation by 30% after 24 h. **(B)** Fibroblast migration was assessed by a modified Boyden chamber method in the absence (control) or presence of 10 μM UTP for 24 h. The data are shown as the fold-increase relative to control and are mean ± SEM of at least 3 independent experiments performed in triplicate and compared by using Student's t test. *, $p < 0.05$ and ***, $p < 0.001$ in response to UTP.

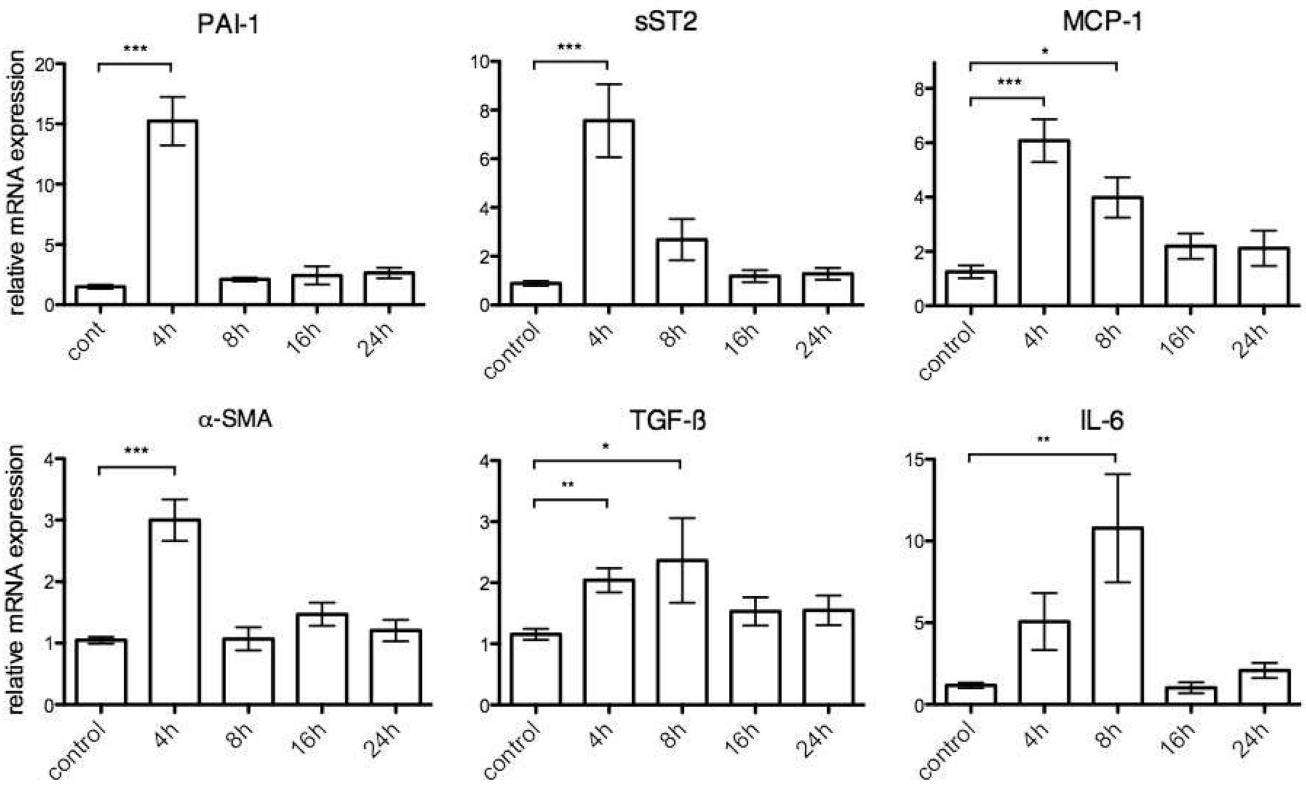

Figure 2. UTPγS induces transcription of profibrotic genes in rat cardiac fibroblasts

CFs were serum-starved for 24 h and then incubated with UTPγS (10 μM) for 4, 8, 16, or 24 h. Real-time RT-PCR was used to quantify PAI-1, α-SMA, sST2, TGF-β1, MCP-1 and IL-6; the data are normalized to 18S RNA. UTPγS (10 μM) at 4 h stimulated peak upregulation of profibrotic genes: PAI-1, α-SMA, sST2, TGF-β, MCP-1 by 15-, 3.0-, 7.6-, 2.0-, 6.1-fold, respectively. IL-6 expression peaked at 8 h by 11-fold. The data shown represent mean ± SEM of at least 3 independent experiments performed in triplicate and compared by using Student's t test. *, $p<0.05$; **, $p<0.01$ and ***, $p<0.001$ in response to UTPγS.

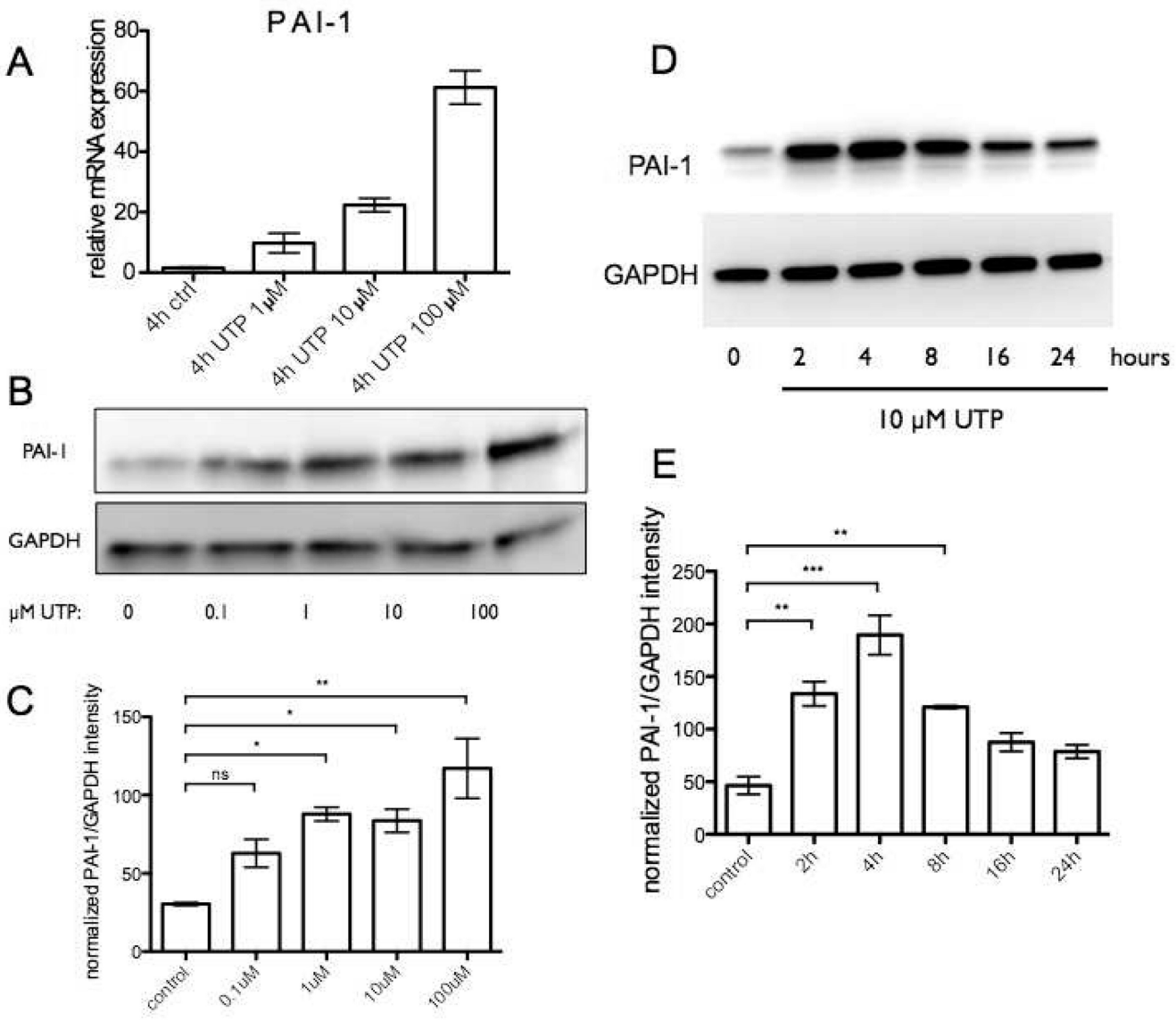

Figure 3. UTP induces PAI-1 protein expression in a dose- and time-dependent manner but response to UTP desensitizes.

(A–C) CFs were serum-starved for 24h and then stimulated with 0 – 100 µM UTP for 4h. Cells were assayed by real-time RT-PCR to quantify mRNA expression and immunoblot analysis to quantify protein expression of PAI-1. UTP dose-dependently increased PAI-1 mRNA expression **(A)** and protein expression **(B)**. **(C)** Quantification of PAI-1 protein from two independent Western blots is shown as ± SEM compared by using ANOVA with post-hoc multiple comparison tests. *, $p<0.05$ and **, $p<0.01$. **(D)** Time-dependent increase in PAI-1 protein expression was observed after 2 h, peaked after 4 h and decreased to baseline levels after 24 h. **(E)** Quantification is shown from two independent Western blots as ± SEM compared by using ANOVA with post-hoc multiple comparison tests. **, $p<0.01$ and ***, $p<0.001$.

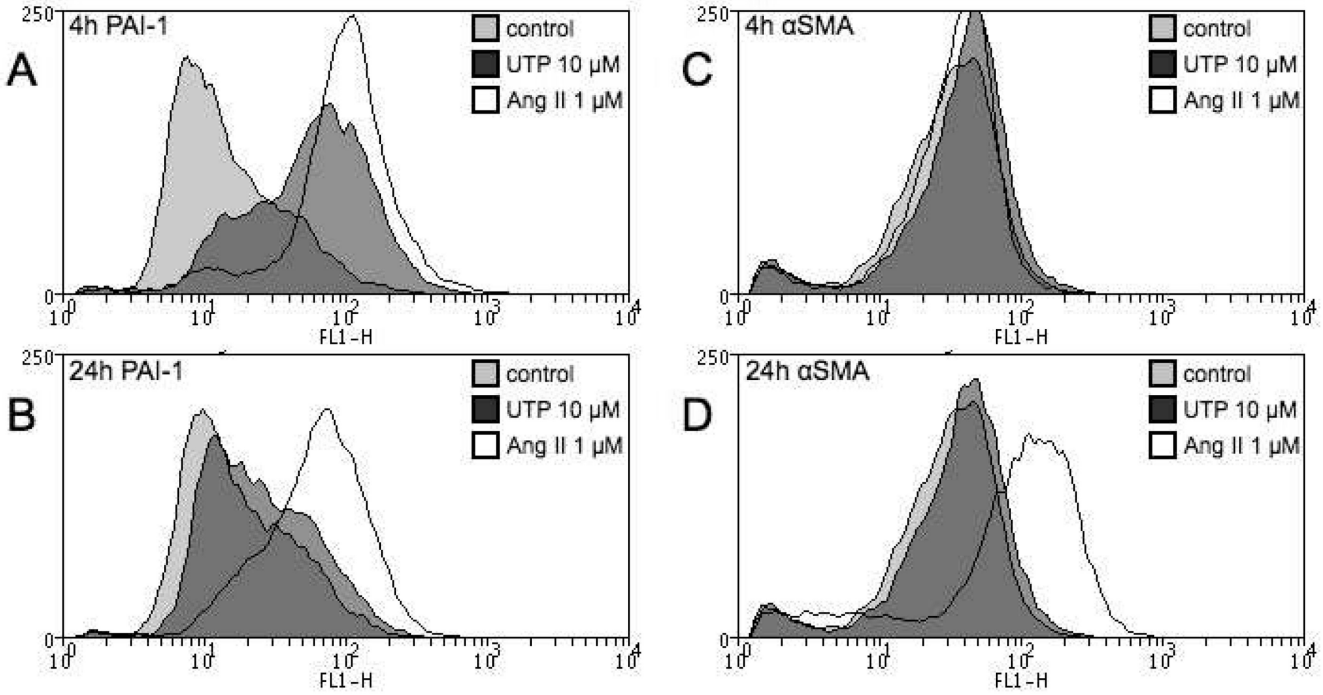

Figure 4. UTP has no effect on α-SMA protein expression of rat CFs

Cells were serum-starved for 24 h, treated with UTP (10 µM) or Ang II (1 µM) for 4 or 24 h and protein expression analyzed via flow cytometry. **(A)** 4 h UTP treatment increased PAI-1 expression in CFs but this increase reverts to basal levels by 24 h **(B)**. Ang II increases PAI-1 at 4 and 24 h **(A, B)**. UTP does not increase α-SMA at 4 h **(C)** or 24 h **(D)** but Ang II stimulation for 24 h significantly increases α-SMA protein expression **(D)**.

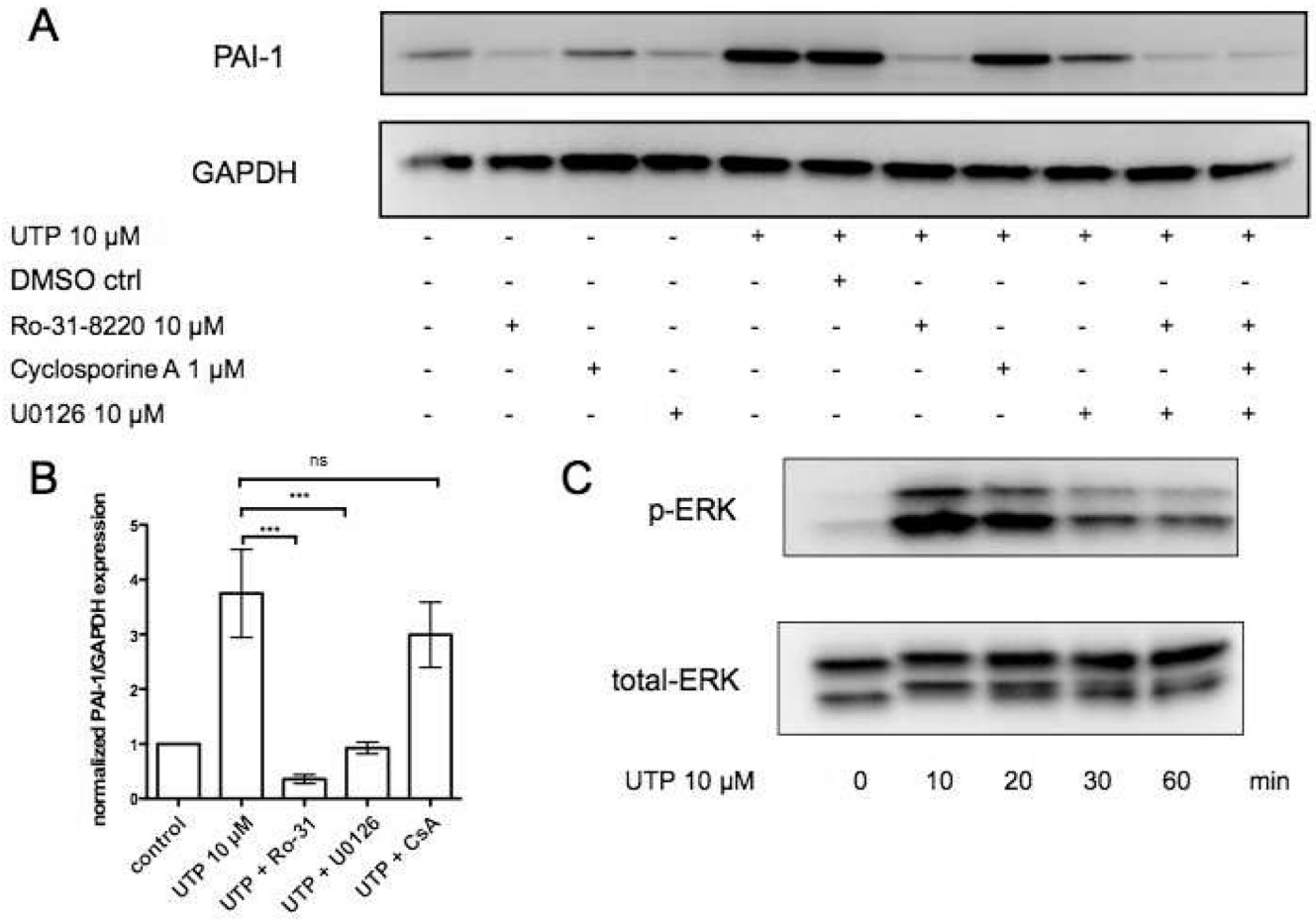

Figure 5. UTP induces PAI-1 expression in rat cardiac fibroblasts in a PKC and ERK-dependent manner

(A) CFs were serum-starved for 24h and then incubated in the presence or absence of: DMSO (vehicle control), Ro-31-8220 (PKC inhibitor), U0126 (MAPK/ERK kinase (MEK) inhibitor), or Cyclosporine A (calcineurin inhibitor) for 30 min. The cells were then stimulated with UTP (10μM, 4 h). Cells were lysed and assayed for PAI-1 protein expression. GAPDH was used to normalize for protein loading. Panel **(B)** shows quantification of the immunoblots from panel **(A)**. **(C)** ERK-phosphorylation and total ERK protein were assessed using immunoblots following stimulation with 10 μM UTP for 0, 10, 20, 30, 60 min. The data shown are mean ± SEM of at least 3 independent experiments performed in triplicate and compared by using ANOVA with post-hoc multiple comparison tests. ***, $p<0.001$.

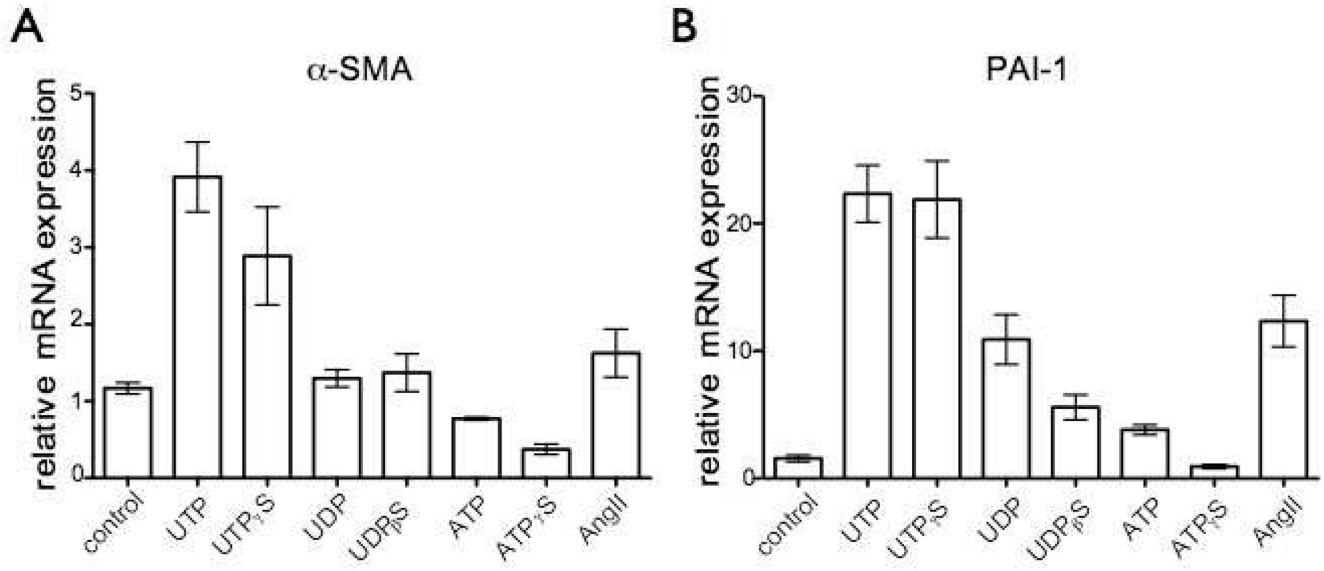

Figure 6. UTP-induced effects in rat cardiac fibroblasts are predominately mediated by $P2Y_2$ receptors

(A–B) CFs were serum-starved for 24 h and then incubated with UTP, UTPγS, UDP, UDPβS, ATP, ATPγS (all 10 μM) or angiotensin II (Ang II) for 4 h. Cells were assayed using real-time RT-PCR to quantify mRNA expression of α-SMA **(A)** and PAI-1 **(B)**. Incubation of CFs with UTP or the stable agonist UTPγS (10 μM) increased α-SMA and PAI-1 mRNA expression. UDP (10 μM) and the stable agonist UDPβS (10 μM) did not increase α-SMA expression; PAI-1 was increased but to a lesser extent than with UTP. ATP and ATPγS (both 10 μM) did not significantly increase α-SMA or PAI-1. The data shown are mean ± SEM of at least 3 independent experiments performed in triplicate and compared using ANOVA with post-hoc multiple comparison testing.

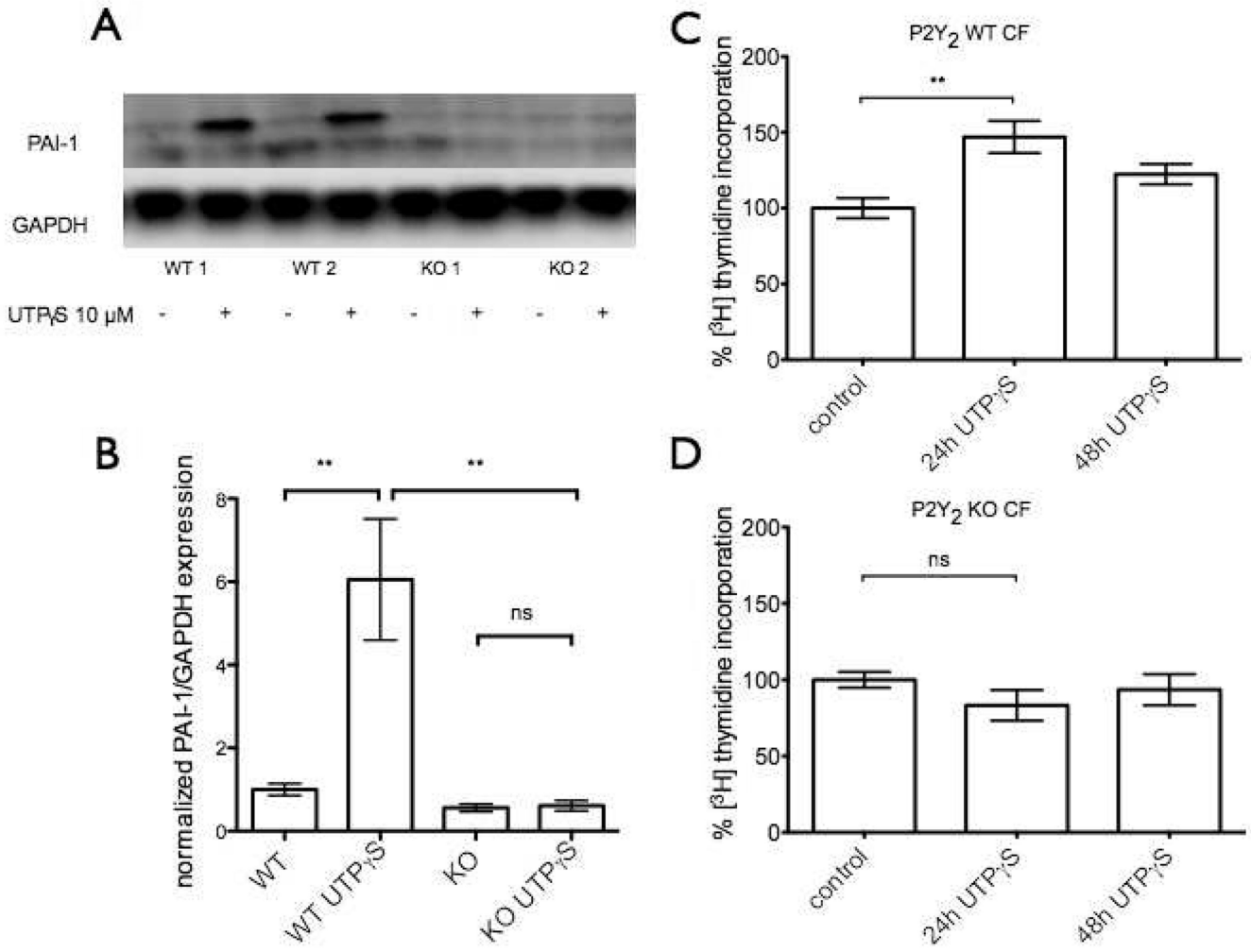

Figure 7. UTP-induced PAI-1 expression and cell proliferation are blunted in $P2Y_2^{-/-}$ mice

CFs from wild-type or $P2Y_2^{-/-}$ mice were serum-starved for 24 h and then incubated with UTPγS (10 μM) for 4 h. Cells were assayed by immunoblot analysis to quantify protein expression of PAI-1. **(A)** Immunoblotting shows that UTPγS increases PAI-1 protein expression in CFs from wild-type mice but not from $P2Y_2^{-/-}$ mice. Panel **(B)** shows quantification of the immunoblots from panel **(A)**. The data shown in **(B)** are the mean ± SEM of at least 3 independent experiments compared by using ANOVA with post-hoc multiple comparison tests. **, $p<0.01$. **(C)** CFs from wild-type mice show ~50% increase in proliferation in response to treatment with UTPγS (10 μM) for 24 h but not 48 h. **(D)** UTPγS is not proliferative in CFs from $P2Y_2^{-/-}$ at either time point.

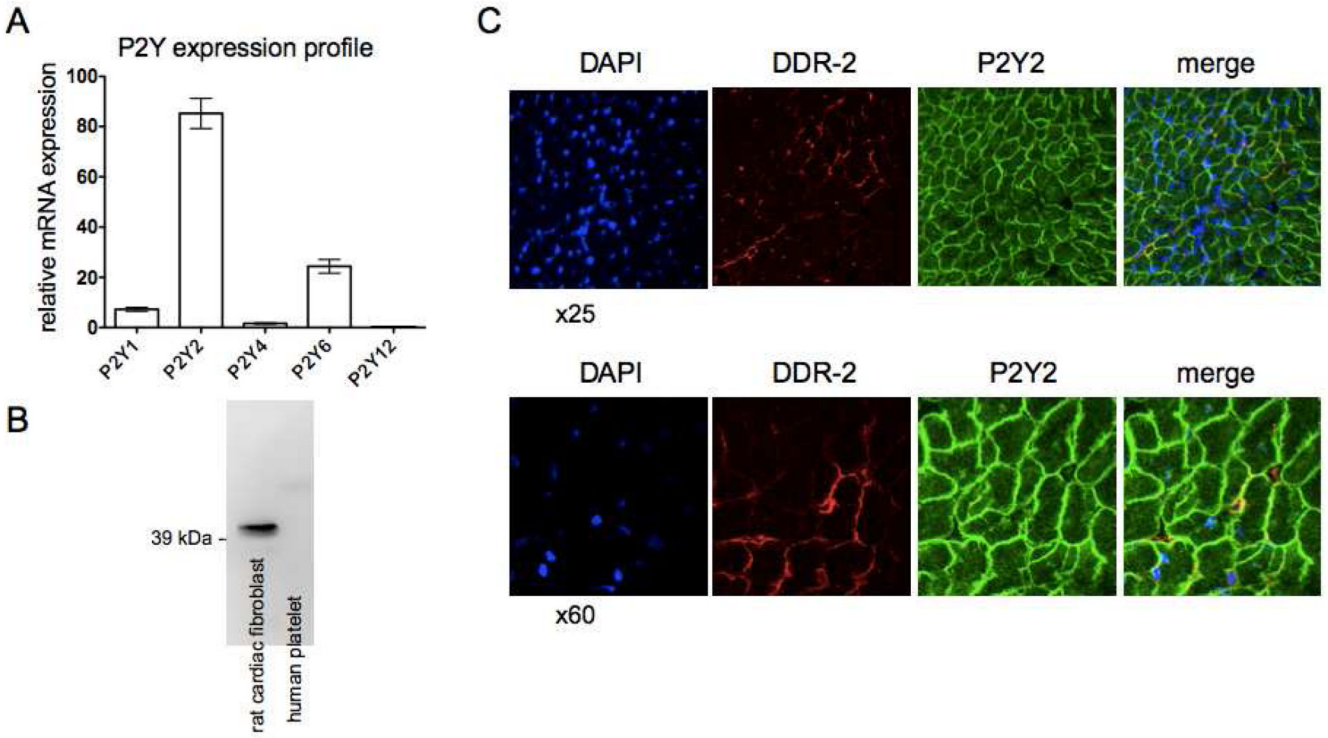

Figure 8. The $P2Y_2$ receptor is the predominant P2Y receptor expressed in cardiac fibroblasts

(A) Quantification of the mRNA for $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{12}$ reveals that the $P2Y_2$ receptors are the most highly expressed P2Y receptor subtype in rat CFs. $P2Y_2$ mRNA expression is 50-, 3.5-, and 11-fold higher than that of $P2Y_4$, $P2Y_6$ and $P2Y_1$ receptors, respectively ($p < 0.001$). **(B)** Immunoblotting with a $P2Y_2$ antibody detects $P2Y_2$ receptors in CFs but not human platelets. **(C)** Sections of left ventricles from rats were stained for DDR2 (red), $P2Y_2$ receptor (green) and nuclei with DAPI (blue). Cross-sectional images of cardiac myocytes and fibroblasts are shown.

Table 1

Oligonucleotides used for real-time RT-PCR

Gene	Forward, 5'–3'	Reverse, 5'–3'
18s	GTA ACC CGT TGA ACC CCA TT	CCA TCC AAT CGG TAG TAG CG
α-SMA	CAT CAG GAA CCT CGA GAA GC	TCG GAT ACT TCA GGG TCA GG
PAI-1	GGA GAA GCG AAA CAG GAG TG	TCC AGA AGG GGA TAT GTT GC
sST2	TTA CCC AGC CAG GAT GTT TC	CTA GGG GCT TGG CTT CTC TT
TGF-β1	CCT GGA AAG GGC TCA ACA	GTT GGT TGT AGA GGG CAA GG
IL-6	GCC TTC CCT ACT TCA CAA GTC C	CTG ACC ACA GTG AGG AAT GTC C
MCP-1	CTT CTG GGC CTG TTG TTC A	TTC CTT ATT GGG GTC AGC AC
$P2Y_1$	AAC CGT GAT GTG ACC ACT GA	TTC AAC TTG TCC GTT CCA CA
$P2Y_2$	TGC TGG GTC TGC TTT TTG CT	ATC GGA AGG AGT AAT AGA GGG T
$P2Y_4$	TCG ATT TGC AAG CCT TCT CT	CCA TAG GAG ACC AGG GTG AT
$P2Y_6$	TGC TGC TAC CCC CAG TTT AC	TGG CAT AGA AGA GGA AGC GT
$P2Y_{12}$	GGA TTC CCT ACA CCC TGA GC	ACC TTC CTG TCC TTT CTT CT