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UTP reduces infarct size and improves mice heart function after myocardial infarct via $P2Y_2$ receptor

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ABSTRACT

Pyrimidine nucleotides are signaling molecules, which activate G protein-coupled membrane receptors of the P2Y family. $P2Y_2$ and $P2Y_4$ receptors are part of the P2Y family, which is composed of 8 subtypes that have been cloned and functionally defined. We have previously found that uridine-5′-triphosphate (UTP) reduces infarct size and improves cardiac function following myocardial infarct (MI). The aim of the present study was to determine the role of $P2Y_2$ receptor in cardiac protection following MI using knockout (KO) mice, *in vivo* and wild type (WT) for controls. In both experimental groups used (WT and $P2Y_2^{-/-}$ receptor KO mice) there were 3 subgroups: sham, MI, and MI + UTP. 24 h post MI we performed echocardiography and measured infarct size using triphenyl tetrazolium chloride (TTC) staining on all mice. Fractional shortening (FS) was higher in WT UTP-treated mice than the MI group ($44.7 \pm 4.08\%$ vs. $33.5 \pm 2.7\%$ respectively, $p < 0.001$). However, the FS of $P2Y_2^{-/-}$ receptor KO mice were not affected by UTP treatment ($34.7 \pm 5.3\%$ vs. $35.9 \pm 2.9\%$). Similar results were obtained with TTC and hematoxylin and eosin stainings. Moreover, troponin T measurements demonstrated reduced myocardial damage in WT mice pretreated with UTP vs. untreated mice (8.8 ± 4.6 vs. 12 ± 3.1 $p < 0.05$). In contrast, $P2Y_2^{-/-}$ receptor KO mice pretreated with UTP did not demonstrate reduced myocardial damage. These results indicate that the $P2Y_2$ receptor mediates UTP cardioprotection, *in vivo*.

1. Introduction

Myocardial infarction (MI) and heart failure are leading causes of morbidity and mortality in humans. Considerable effort has been devoted to improving functional recovery and reducing the extent of infarction after ischemic episodes. A step in this direction is the discovery that the heart is significantly protected against ischemic injury if first preconditioned (PC) by a brief period of ischemia [1]. This PC effect is mimicked by several pharmacological agents including adenosine, a purine metabolite [2,3]. However, accumulating evidence has suggested that adenosine receptors are not the only mediators of PC [4–6], but that adrenoceptors [7], bradykinin receptors [8], opioid receptors [9], and ATP receptors [10] may also contribute to PC. Extracellular nucleotides such as ATP and UTP modulate cellular function by the activation of membrane-bound P2-receptors [11]. P2-receptors are expressed on the surface of almost all cells. The physiological effects of the purinergic signaling system are dependent on the release of extracellular nucleotides, the degradation by ectonucleotides, the type of P2 receptors expressed on the cells, their desensitization rates and their second messengers [12]. Although nucleotides (such as ATP and UTP) are mainly intracellular, they can be released into the extracellular fluids by various mechanisms. One is cell damage: both necrotic and apoptotic cells release ATP and other nucleotides that thus constitute 'danger signals' [13,14]. Once in the extracellular fluid, nucleotides can activate two families of receptors: metabotropic P2Y receptors that are coupled to G proteins and fast P2X ion channels. In agonist selectivity, the P2X receptors are more structurally restrictive than P2Y. P2X respond principally to ATP as the active ligand, whereas the P2Y receptors are activated by a group of five or more naturally occurring nucleotides, including ATP, UTP, ADP, UDP and UDP-glucose. The P2Y family comprises eight members encoded by distinct genes [15]. $P2Y_2$ and $P2Y_4$ receptors are activated by the uracil nucleotide, uridine 5′-triphosphate (UTP) [16,17]. While the

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effects of purine nucleosides and nucleotides in myocardial infarction and ischemia have been intensively studied [18,19], the role of pyrimidine nucleotides under hypoxic conditions has not been well explored [20], despite the level of both ATP and UTP being elevated as a result of ischemia [21]. We have previously found that UTP reduces infarct size and improves cardiac function following MI [22]. Recently, we published an article where we used a culture of neonatal cardiomyocyte from $P2Y_2$ and $P2Y_4$ double knockout. UTP protected these cells from hypoxia and therefore we concluded that UTP protection *in vitro* does not depend on $P2Y_2$ or $P2Y_4$ receptor activation but on the formation of tri/di-phosphate a product of UTP degradation by ectonucleotides [23]. The purpose of the present study was to determine the role of $P2Y_2$ receptors in UTP-mediated cardiac protection following MI and whether, *in vivo*, pyrophosphate offers similar protective effects as UTP. This work provides evidence *in vivo*, that the $P2Y_2$ receptor, not pyrophosphate, mediates UTP cardioprotection, as revealed by using $P2Y_2^{-/-}$ receptor knockout (KO) mice and wild type (WT) for controls.

2. Materials and methods

2.1. Animals and experimental protocol

Homozygous $P2Y_2^{-/-}$ receptor KO mice and the corresponding WT mice (C57BL/6) were a generous gift of Dr Jens Leipziger (Department of Physiology & Biophysics, Aarhus University, Aarhus, Denmark). The $P2Y_2^{-/-}$ receptor KO mice were originally produced by Dr Beverly H. Koller (University of North Carolina, Chapel Hill, USA). All experiments were carried out in accordance with the guidelines of the Animal Care and Use Committee of Tel Aviv University, with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. Two experimental groups were used: WT and $P2Y2^{-/-}$ receptor KO mice. There were 3 subgroups: (1) sham without left anterior descending coronary artery (LAD) ligation, (2) MI = LAD ligation, (3) injected with UTP (0.44 μg/kg) 30 min before MI into vein of tail. Echocardiography was performed at baseline 1 day before UTP injection. A second echo was done 24 h post MI. Blood samples were taken 24 h post MI before sacrificed. Troponin T and lactate dehydrogenase (LDH) activity in the serum were assessed.

2.2. Genotyping of $P2Y_2$ receptor knockout mice

Isolation of genomic DNA and subsequent PCR was performed as previously described [24,25]. Briefly, a 2 mm piece of the tail was placed into a tube, 500 μL of NaOH was added for 15 min at 95 °C. 50 μL Tris–buffer containing 10 mmol/L EDTA was included and vortexed for 30 min. A triple primer genomic PCR was used for the $P2Y_2$ mice genotyping. The two different forward primers had the following sequences:

5′-GTCACGCGCACCCTCTACTA-3′ (identifying the WT allele) and

5′-GGGGAACTTCCTGACTAGG-3′ (identifying the inserted neo-cassette in the disrupted allele). The reverse primer for both alleles had the following sequence:

5′-GTCGGGTGCACTGCCTTTCT-3′. The segment length of WT amplification was 551 bp long, and the corresponding to the knockout region was ~700 bp.

2.3. MI model — LAD ligation

Mice were anesthetized (with a mixture of 100 mg/kg ketamine and 10 mg/kg xylazine), intubated and ventilated with air. Ischemia was achieved by LAD ligation as we have previously described [26]. Mice in the sham group were operated without LAD ligation. The mice were ventilated until spontaneous breathing commenced.

2.4. Two-dimensional guided M-mode echocardiography

Animals were lightly anesthetized by inhaling isoflurane. Two-dimensional (2D) guided M-mode echocardiography was performed using an echocardiogram (Siemens 512, Sequoia) equipped with a 15 MHz linear transducer. The 2D mode in the parasternal long-axis view was used to monitor the heart. From this view, an M-mode cursor was positioned perpendicular to the interventricular septum and posterior wall of the left ventricle (LV) at the level of the papillary muscles. An M-mode image was obtained at a sweep speed of 100 mm/s. Diastolic and systolic left-ventricular wall thickness, left-ventricular end-diastolic dimensions (LVDD), and left-ventricular end-systolic chamber dimensions (LVSD) were measured. The percentage of left-ventricular fractional shortening (FS) was calculated as [(LVDD–LVSD)/LVDD] × 100 [27].

2.5. Assessment of infarct size

At 24 h after coronary ligation the mice were sacrificed and their hearts were removed. Midventricular heart sections 0.8 mm thick were put in a 1% solution of 2,3,5-triphenyl tetrazolium chloride (TTC) in phosphate buffer for 30 min at 37 °C. TTC stained the viable tissue with red while the necrotic tissue remained discolored. Sections were fixed overnight in 4% formaldehyde to enhance the contrast between stained and unstained tissue. The sections were then placed between two cover slips and digitally photographed using a Nikon coolpix 5000 camera, with a resolution of 1400 × 960 pixels and quantified by IMAGE J 5.1 software. The area of irreversible injury (TTC-negative) is presented as a percentage of the entire area of the section [28].

2.6. Biochemical analysis

Levels of troponin T were determined in the serum using Cardiac T 2017423, Roche kit (Indianapolis, USA). Levels of lactate dehydrogenase were determined in the serum using commercial Olympus OSR6126 (Center Valley, PA, USA).

2.7. Histopathology

Hearts were removed and fixed in 4% formalin, then embedded in paraffin. Transverse sections were stained with hematoxylin and eosin (H&E), (Pioneer Research, UK) to assess leukocyte infiltration. Immunohistochemistry stains included rat anti-mouse neutrophil antibody MCA771GA (1:3000, AbD Serotec, UK). Secondary antibodies for neutrophil staining consisted of biotinylated donkey anti-rat (1:500, Jackson ImmunoResearch Laboratories, PA, USA) followed by incubation with peroxidase-conjugated streptavidine (1:1000, Jackson ImmunoResearch Laboratories, PA, USA) and development with 3,3′-diaminobenzidine substrate. Histological analysis was performed on hearts from 5 mice (10 × fields each) using ImagePro PLUS software (Media Cybernetics, USA).

2.8. Western blotting

Heart tissue samples (20 mg) were homogenized in lysis buffer and quantified for protein levels using a commercial assay (Bio-Rad). Proteins (60 μg/sample) were separated using sodium dodecyl sulfate (SDS) polyacrylamide gel (10%) under denaturing conditions and electrotransferred onto nitrocellulose (Bio-Rad) for 1 h at 100 V. Membranes were blocked with 5% nonfat milk in Tris–

buffered saline with 0.1% Tween 20 (TBST) for 1 h at room temperature. Primary antibodies were used at a 1:1000 concentration in TBST with 5% nonfat milk overnight at 4 °C. The p-c-Jun antibody was obtained from Santa Cruz Biotechnology (sc-822). The actin antibodies were obtained from PharMingen (San Diego, CA, USA). Immunodetection of actin with monoclonal anti-actin antibody was performed as an internal control. Dye 800 secondary antibodies were added at a concentration of 1:15000 for 1 h at room temperature (Goat Anti-Mouse IRDye 800, LI-COR Biosciences, NE, USA). Detection was carried out with Li CORE odyssey. Quantification of signals was carried out with the Odyssey program.

2.9. Chemicals

All chemicals were purchased from Sigma-Chemicals (St. Louis, USA) unless mentioned otherwise.

2.10. Statistical analysis

Results were expressed as means ± standard error (S.E). Values during stabilization period were defined as 100%. A statistical difference between the groups was assessed by analysis of variance (ANOVA) with post hoc Bonferroni test and significance was accepted at $p < 0.05$.

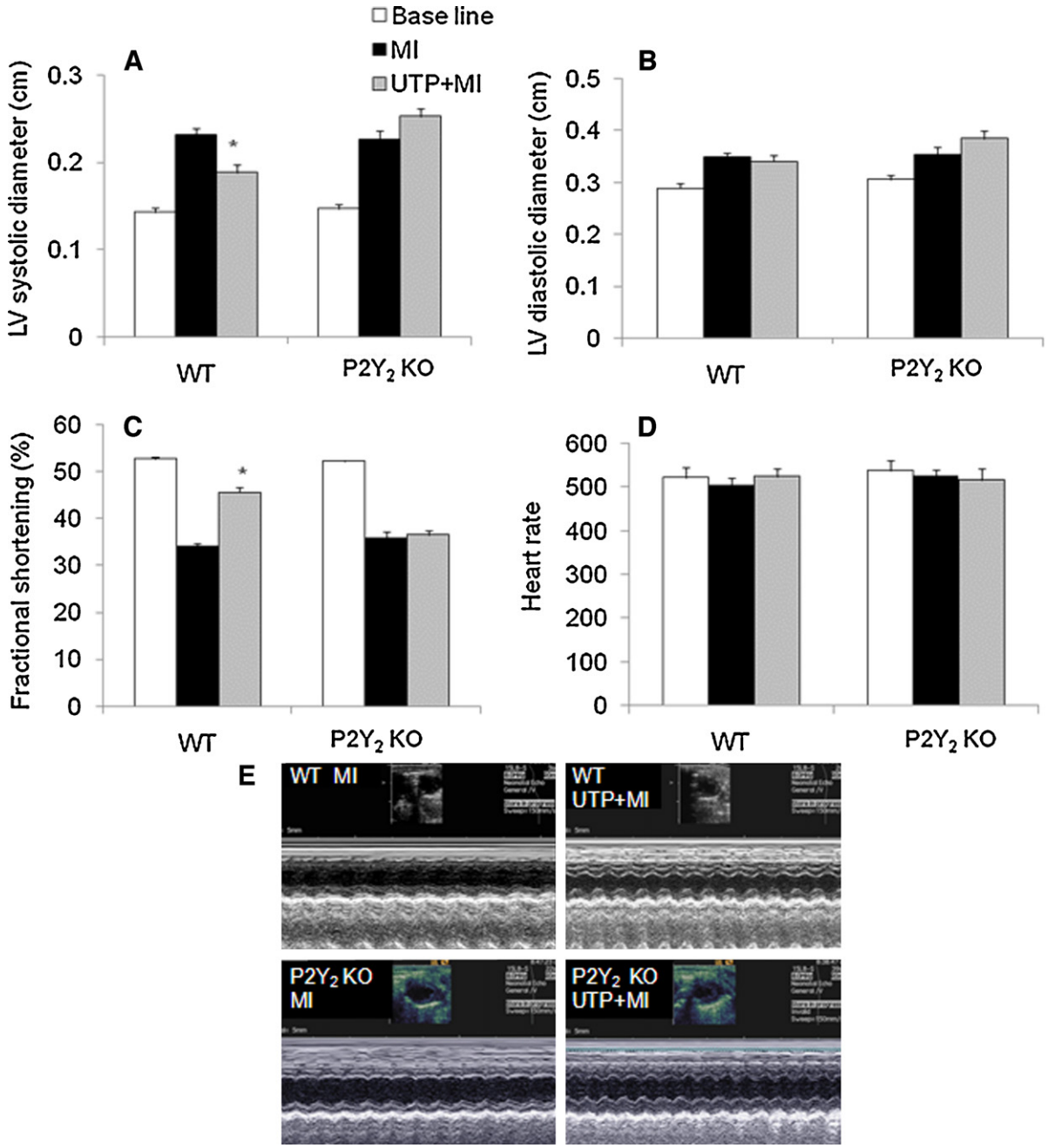

Fig. 1. Echocardiographic studies 24 h after MI of WT and $P2Y_2^{-/-}$ receptor KO mice. These studies demonstrated LV end systolic diameter (LVSD) and LV end diastolic diameter (LVDD) of base line and infarcted hearts compared with the 30 min UTP pretreated mice (A and B). In addition, fractional shortening (FS) of base line and infarcted hearts were compared with UTP pretreated mice (C), and heart rate at baseline and post MI of control (saline treated) and UTP treated (D). Representative M mode pictures are presented in (E). Values represent means ± SE, $n = 11$ mice in each group. $^*p < 0.001$ vs. MI.

3. Results

To determine the role of $P2Y_2$ receptors in cardioprotection following MI, we used two experimental groups: $P2Y_2^{-/-}$ receptor KO and WT mice for control. The mortality rate following MI was 25% in both WT and $P2Y_2$ KO mice at 24 h. Only mice that survived the operation and were alived 24 h following ischemia were included in this study.

3.1. Assessment of LV remodeling post-MI: echocardiography

At baseline, there were no significant differences between all groups in the echocardiographic measurements of cardiac dimensions or function. Induction of MI resulted in pronounced increased left ventricular end-systolic diameters in the WT mice compared to the UTP pretreated mice ($p < 0.001$). On the other hand there was no significant difference between the $P2Y_2^{-/-}$ receptor KO mice subjected to MI with and without UTP treatment. UTP treatment was beneficial in WT mice and significantly increased fractional shortening compared to the untreated ones ($p < 0.001$). No differences in cardiac function following MI were observed in $P2Y_2^{-/-}$ receptor KO mice pretreated with UTP compared to saline-pretreated (Fig. 1A–C). All tested groups had a similar heart rate (Fig. 1D).

3.2. Biochemical markers of ischemic damage

3.2.1. Troponin T

Compared with the sham group. The level of troponin T, a specific marker denoting damage in the heart muscle, was significantly elevated in WT and $P2Y_2^{-/-}$ receptor KO mice post MI. UTP pretreatment in WT mice significantly lowered troponin T levels in the serum ($p < 0.05$). UTP pretreatment in $P2Y_2^{-/-}$ receptor KO mice did not change troponin T levels in the serum (Fig. 2A).

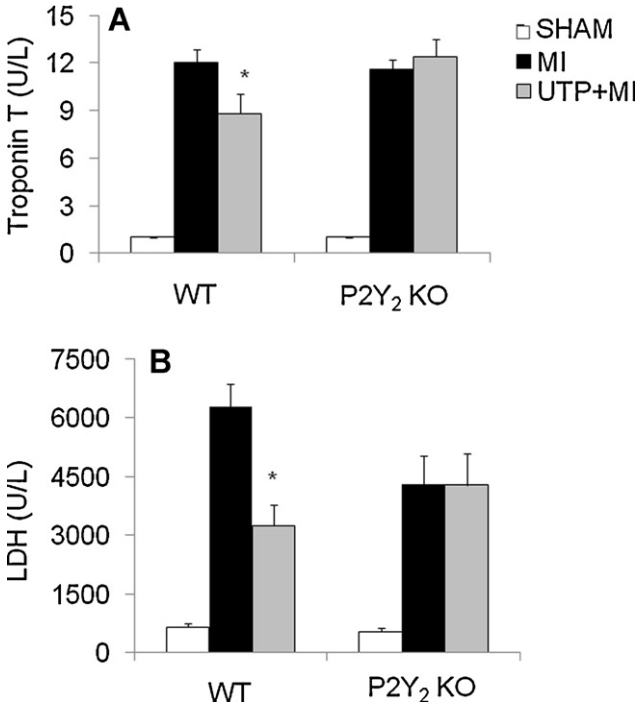

Fig. 2. Measurements of biochemical markers in UTP pretreated WT and $P2Y_2$ KO mice were compared with untreated. The release of troponin T and LDH to the serum 24 h post LAD ligation. Values are means ± SE. $n = 11–15$ hearts in each group. $^*p < 0.05$ vs. MI.

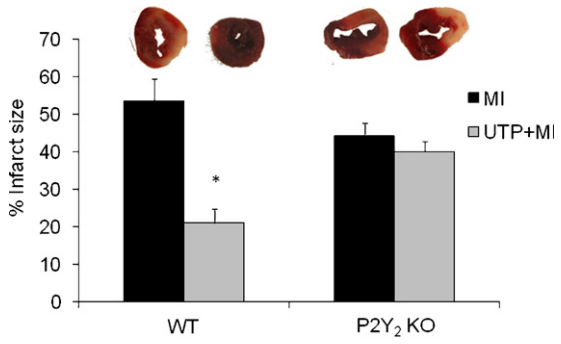

Fig. 3. The effect of left anterior descending coronary artery (LAD) ligation on the percent of irreversible injury was determined by scanning the images of mice hearts ventricular sections with triphenyl tetrazolium chloride. Representative images of the two different groups, revealing various degrees of myocardial ischemia (white to red zones after TTC staining). Hearts were subjected to 24 h of LAD ligation. A significant size of damaged tissue was noted in the myocardium of both MI groups. WT mice pretreated with UTP had significantly smaller infarct size. However, no differences in infarct size were found between $P2Y_2^{-/-}$ receptor KO mice pretreated with UTP. This figure represents the percent of irreversible injury from the total area of the section at 24 h post-LAD ligation. Values represent means ± SE. $n = 8$ hearts in each group. $^*p < 0.001$ vs. MI.

3.2.2. Lactate dehydrogenase

LDH activity in the serum, assessed 24 h post infarction increased significantly in MI mice (WT and $P2Y_2^{-/-}$ receptor KO) compared with the sham operated mice. UTP pretreatment in WT mice significantly reduced LDH levels in the serum ($p < 0.001$). UTP pretreatment in $P2Y_2^{-/-}$ receptor KO mice did not change LDH levels in the serum (Fig. 2B).

3.2.3. Irreversible ischemic damage

Following LAD ligation, infarct size was significantly smaller in WT mice pretreated with UTP compared to the untreated, subjected to MI 24 h after infarction ($p < 0.001$). There were no significant differences between $P2Y_2^{-/-}$ receptor KO mice pretreated with UTP and those subjected to MI without UTP treatment (Fig. 3).

3.2.4. Acute inflammatory cells infiltrated the myocardium

24 h after ischemia the infarcted myocardium loses its regular structure and there is significant infiltration of inflammatory cells. To determine the extent of immune cells infiltration into the heart, the tissues were excised 24 h post MI and evaluated by hematoxylin and eosin (H&E) histology staining (Fig. 4). In the sham groups (WT, and $P2Y_2^{-/-}$ receptor KO mice) neutrophils did not infiltrate the hearts. At 24 h post MI, neutrophils were observed in all infarcted tissues. $P2Y_2^{-/-}$ receptor KO mice pretreated with UTP showed neutrophil infiltration. However, in WT mice pretreated with UTP neutrophil infiltration the heart was significantly lower.

To be more specific, we performed immunohistochemistry staining using anti-mouse neutrophils recognizing polymorphic 40 kDa antigens expressed by polymorphonuclear cells (Fig. 5A). The immunostaining of neutrophils demonstrated significant neutrophil infiltration (25-fold higher) in WT and $P2Y_2^{-/-}$ receptor KO mice hearts 24 h post MI compared to the sham groups. The $P2Y_2^{-/-}$ receptor KO mice pretreated with UTP demonstrated significant neutrophil infiltration contrasted with the WT mice pretreated with UTP where only minor infiltration was observed (Fig. 5B).

3.2.5. Phospho-c-Jun protein expression

Fig. 6 is a representation of the levels of phospho-c-Jun (p-c-Jun) in hearts of WT and $P2Y_2$ receptor KO mice, pretreated with UTP or saline before MI. Low levels of p-c-Jun expression were observed in the hearts of all sham groups. A significant elevation in the expression of p-c-Jun as a result of MI was noted in WT and

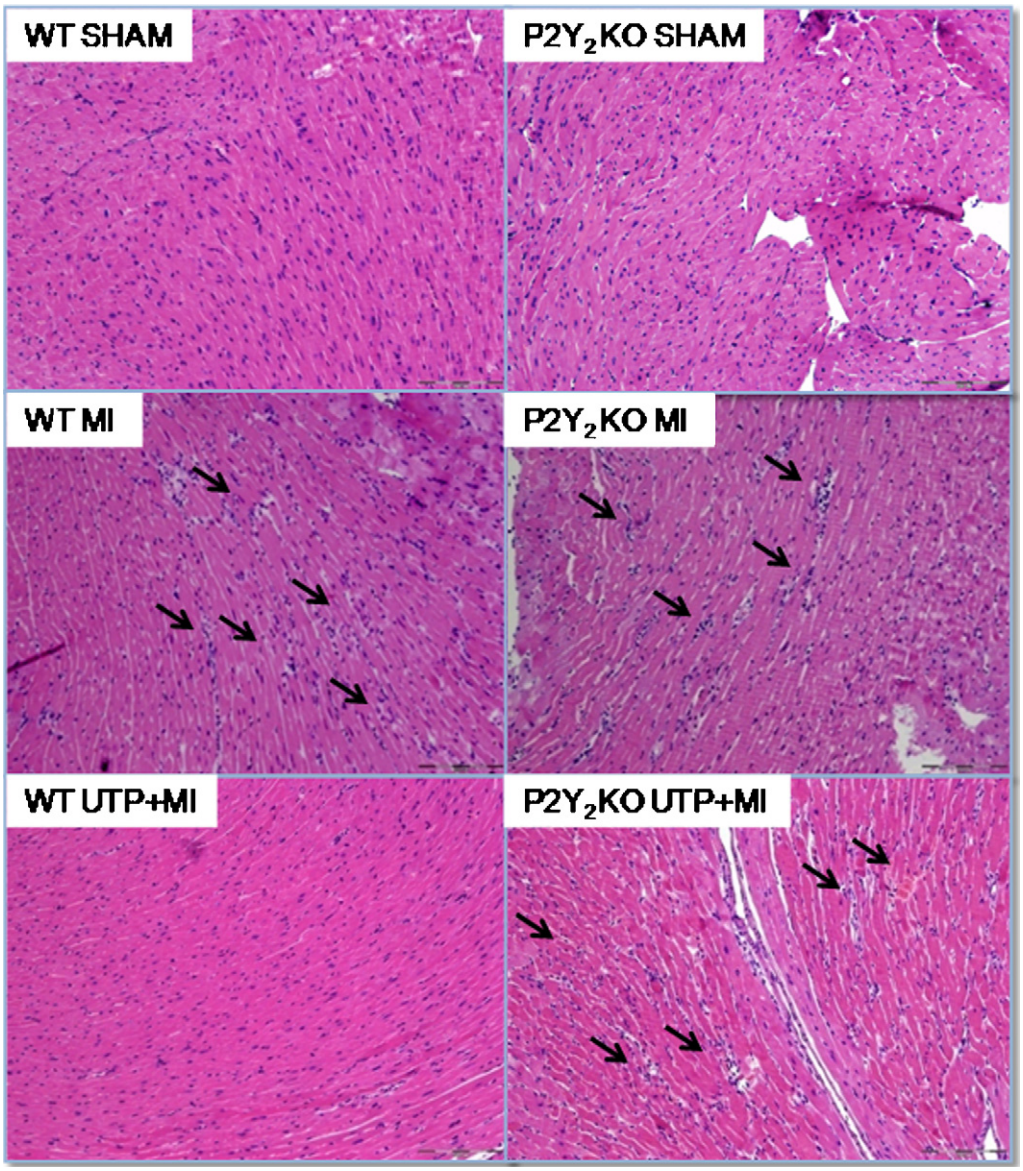

Fig. 4. Histologic study of hearts 24 h post MI: Hematoxylin and eosin staining showed infiltration to the hearts 24 h post MI of WT and $P2Y_2^{-/-}$ receptor KO mice. There was massive infiltration in $P2Y_2^{-/-}$ receptor KO mice pretreated with UTP compared to UTP-pretreated WT mice in which there was no leukocyte infiltration.

$P2Y_2^{-/-}$ receptor KO mice. A significant reduction in the expression of p-c-Jun in WT mice pretreated with UTP compared to the MI ($p < 0.05$). There was no difference in p-c-Jun expression between $P2Y_2^{-/-}$ receptor KO mice pretreated with UTP or saline before MI.

3.2.6. Pyrophosphate

We have recently shown that treatment with tri/di- phosphate is responsible for cardioprotection against hypoxic damage in cardiac cell culture [23]. In this work, we were interested in learning whether tri/di- phosphate protects the heart from ischemia, *in vivo.*

We tested several concentrations of pyrophosphate treatment prior to MI (0.44 μg/kg–2 mg/kg). Pyrophosphate was administered 30 min prior to MI via i.v. There was no improvement in FS, as tested by echocardiography, infarct size measured with TTC staining and biochemical markers (LDH and troponine T), following MI after pyrophosphate administration (Fig. 7A–D).

4. Discussion

Nucleotides, such as ATP and UTP, act as extracellular signaling molecules by the activation of membrane-bound P2- receptors. The fact that virtually all cells possess P2-receptors underlines a likely physiological significance of these receptors [29]. Evidence is accumulating suggesting an important role for the purinergic system in cardiovascular regulation [12,30–34]. It stimulates vasoconstriction and vasodilatation [30], the growth of vascular smooth muscle cells and endothelial cells [35], angiogenesis, it is involved in vascular remodeling [32], stimulates platelet aggregation, regulates coagulation [34], inflammation [33] and several aspects of cardiac function [31]. This system is involved in blood pressure regulation, the development of MI and heart failure [12]. We have previously shown that UTP reduces infarct size and improves cardiac function following MI in wild type animals using the same mode of permanent LAD ligation [22]. It is possible that UTP administration prior to the induction of ischemia vasodilated the vascular bed, mainly collaterals, affording an improved blood flow to the heart and consequently lowering the necrotic area as demonstrated by TTC measurements, and cardiac function [20,22]. We have also shown that UTP protected mitochondrial activity as seen by better membrane potential resulted the increased resistance to hypoxia [36]. In the present study we used KO mice to determine the role of $P2Y_2$ receptors in cardiac protection

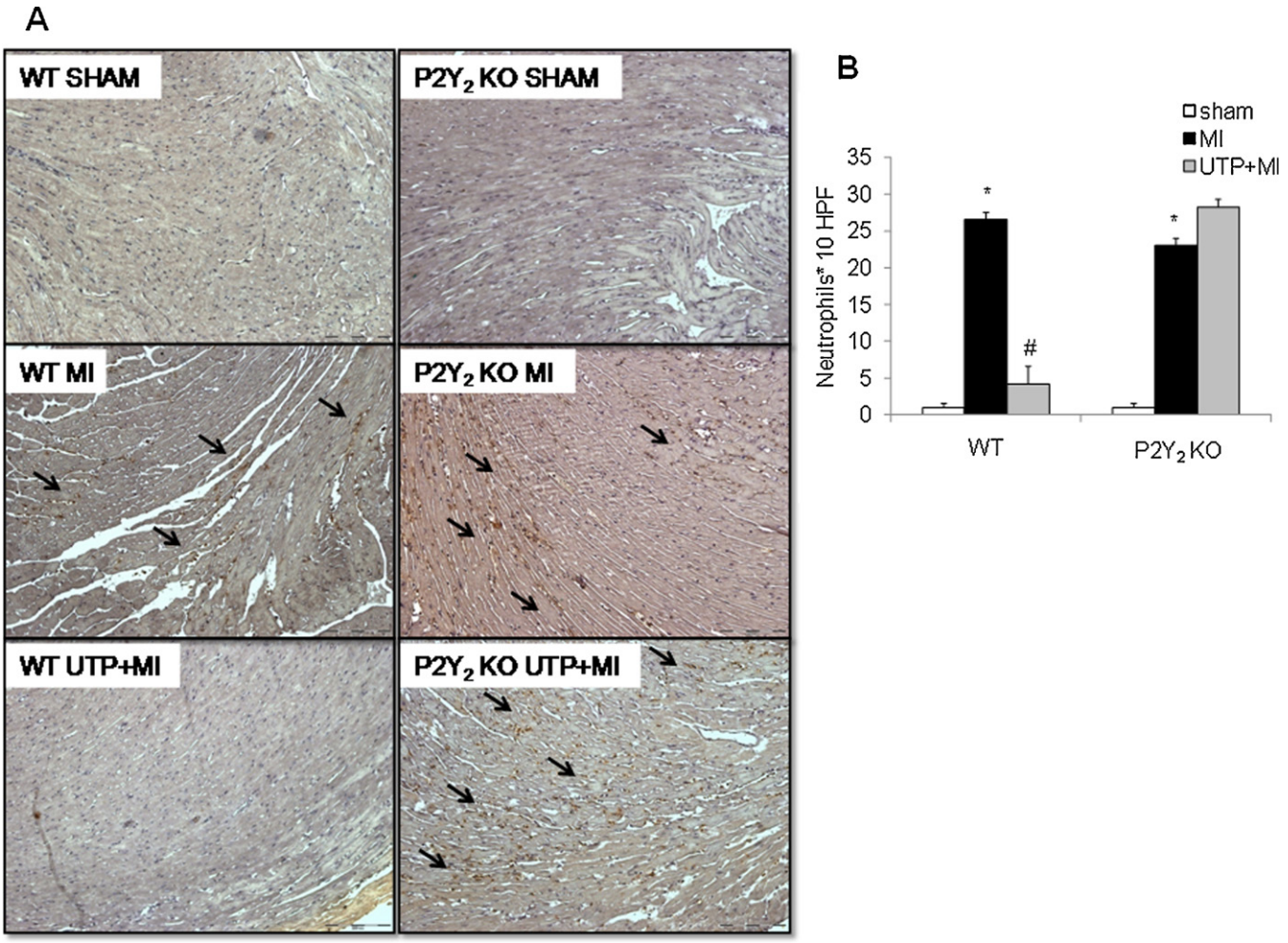

Fig. 5. Immunohistochemistry for neutrophils with rat anti-mouse neutrophil antibody (MCA771GA) showed a high level of neutrophil infiltration in MI groups (WT and $P2Y_2$ receptor KO mice) compared with the sham groups. In WT mice pretreated with UTP, minor infiltration was observed. On the other hand $P2Y_2$ KO mice showed a high level of neutrophil infiltration (A). (B) Quantification of immunohistochemistry staining for neutrophils by counting 10 randomly selected fields/slide ($n = 4$/group). Data are mean ± SE. Significant neutrophil infiltration was observed in WT and $P2Y_2$ KO mice hearts 24 h post MI, compared to the sham groups. WT mice pretreated with UTP had significantly lower neutrophil infiltration (indicated by arrows).*$p < 0.05$, sham control vs. MI. #$p < 0.05$ MI vs. MI mice pretreated with UTP. HPF = High Power Field.

following MI and tested functional, biochemical and histological parameters. UTP protected the heart of WT mice following MI but did not protect the $P2Y_2^{-/-}$ receptor KO mice, suggesting that UTP cardiac protection *in vivo* is mediated via $P2Y_2$ receptors.

P2 receptors are copiously expressed in the fetal human heart [37] as well as in the adult human heart [38,39]. The $P2Y_2$ receptor is the most abundantly expressed receptor with very low levels of the $P2Y_4$ receptor in the human heart. However in adult mice hearts only the $P2Y_2$ receptors are expressed [40]. Previously [23], we have focused on an *in vitro* study of UTP protection from hypoxia using $P2Y_2^{-/-}$ receptor and $P2Y_4^{-/-}$ receptor KO of neonatal cultured cardiomyocyte mice. The results indicated that UTP protected cardiomyocytes in the absence of each of the receptors. The present *in vivo* study, using 4-month-old adults shows that UTP protection is mediated via the $P2Y_2$ receptor, since $P2Y_2^{-/-}$ receptor KO abolished the protective effect of UTP. FS was higher in WT mice pretreated with UTP than in the untreated MI groups. However, FS of $P2Y_2^{-/-}$ receptor KO mice subjected to MI was not affected by UTP treatment. Similar results were obtained with TTC and hematoxylin and eosin stainings. Moreover, troponin T measurements demonstrated reduced myocardial damage in WT mice pretreated with UTP vs. untreated mice. In contrast, $P2Y_2^{-/-}$ receptor KO mice pretreated with UTP did not show reduced myocardial damage, as seen by the levels of troponin T.

Inflammation is an important factor in the pathogenesis of ischemic myocardial injury, suggesting that immune cells are involved in cardiac dysfunction. Various approaches that minimize neutrophil recruitment to the heart, improved heart function and recovery after myocardial ischemia. There are two cellular components contributing to the production of inflammatory cytokines in the heart. Cytokines are released by immune cells that infiltrate the heart and the myocytes themselves produce a variety of inflammatory cytokines and cardiac-suppressive factors [41]. c-Jun, a 39 kDa basic-region leucine zipper protein and prototypic member of the activating protein-1 (AP1) family of transcription factors, is activated by multiple stimuli including inflammatory cytokines [42,43]. Luo et al. [44] provided evidence that c-Jun regulates cytokine-inducible components of the complement system and that the knockdown of c-Jun inhibits neutrophil infiltration into the myocardium post ischemia reperfusion. Moreover, the knockdown of c-Jun reduces infarct size in post-ischemia reperfusion. Here, we provide novel evidence, that UTP reduces c-Jun activation and reduces neutrophil infiltration into the myocardium post MI. c-Jun activation was significantly reduced in WT mice pretreated with UTP prior to MI, hence lowering neutrophil infiltration into the myocardium. There was no change in c-Jun activation and massive neutrophil infiltration into the myocardium post MI in $P2Y_2^{-/-}$ receptor KO mice pretreated with UTP. This indicated that UTP

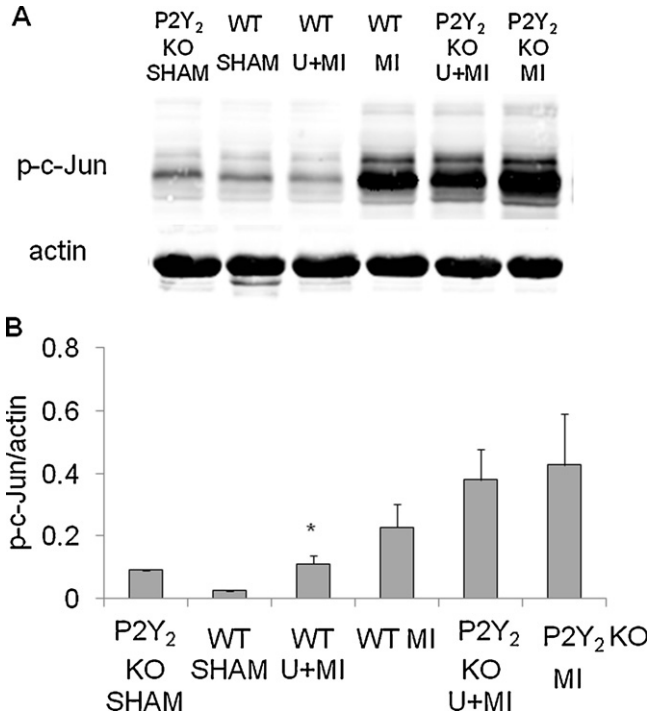

Fig. 6. Western blot analyses of phosphorylated c-Jun (p-c-Jun) in WT and $P2Y_2^{-/-}$ receptor KO mice hearts. Cell extracts were subjected to immunoblotting with antibodies to c-Jun phosphorylated at Ser63. This figure is representative of Western blot analyses of 3 similar separate experiments. (A) Pictures of Western blotting. (B) Quantification of Western blotting. $^*p < 0.05$ MI vs. MI mice pretreated with UTP.

reduces c-Jun activation via the $P2Y_2$ receptor leading to reduced neutrophil infiltration.

In our previous study we have shown that UTP protected neonatal double KO ($P2Y_2$ and $P2Y_4$) cardiomyocytes from hypoxia. We thus concluded that UTP protection *in vitro* does not depend on $P2Y_2$ or $P2Y_4$ activation but on the formation of pyrophosphate-a product of UTP degradation by ectonucleotides [23]. In the present study, we applied various concentrations of pyrophosphate (0.44 μg/kg–2 mg/kg) to mice before MI and found no beneficial effects on cardiac function, infarct size and enzyme levels. Administration of pyrophosphate prior to the induction of MI failed to improve cardiac function and reduce infarct size. This discrepancy could be either because pyrophosphate could not reach the heart as a result of liver barrier in the *in vivo* experiment, or because the experiments *in vivo* were done on adult mice whereas the experiments *in vitro* were done on neonatal animals.

The interpretation of the apparent pinpoint mutation in knockout technology requires caution and careful extrapolation to normal tissue of the experimental data. The $P2Y_2^{-/-}$ receptor KO mice were viable, their body weight and myocardial mass to body weight ratio were similar to those of the wild type, without signs of developmental abnormalities or hypertrophy. It is likely, however, that the deletion of an important gene activates the adaptive processes enabling normal development and function. We cannot exclude the possibility that, in addition to long-term adaptation to the absence of the $P2Y_2$ gene, acute compensatory mechanisms were also activated in our experimental conditions. Despite these limitations, deletion and over expression of genes using the knockout and transgenic technologies enabled us to investigate their function.

In conclusion, we provide novel evidence that the $P2Y_2$ receptor mediates UTP cardioprotection, *in vivo*. UTP's protection

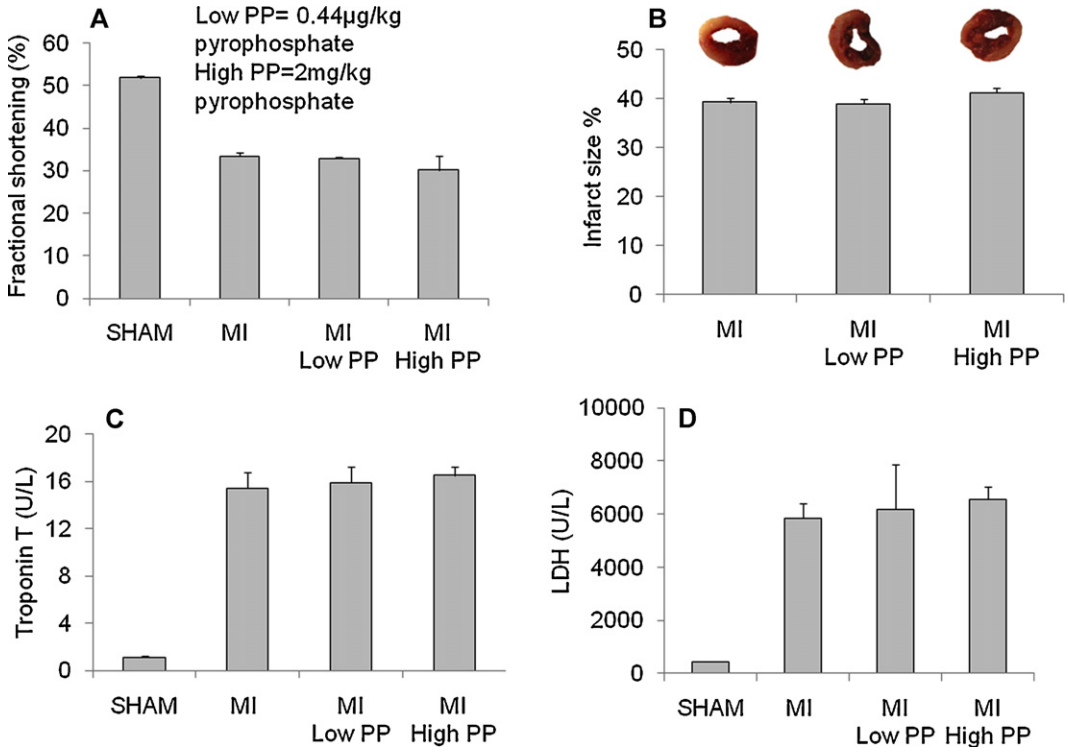

Fig. 7. The effect of pyrophosphate on mice hearts post MI. (A) Echocardiographic studies 24 h after MI showed that pretreatment with pyrophosphate (0.44 μg/kg or 2 mg/kg) had no effect on LV function; FS of mice pretreated with pyrophosphate was the same as the saline pretreated group. Values are means ± SE. $n = 4$ in each group. (B) Infarct size as measured with TTC staining showed no improvement with pretreatment with pyrophosphate compared to MI. Infract size was similar in all the tested groups. Values are means ± SE. $n = 4$ in each group. (C and D) Measurements of biochemical markers in pyrophosphate pretreated prior MI mice. Release of troponin T and LDH to the serum 24 h post LAD ligation. Values are means ± SE. $n = 4$ hearts in each group.

decreases inflammation to the heart and reduces the activation of c-Jun. Therapeutic targeting of pyrimidinergic receptors for protection against ischemic myocardial damage may be more effective than targeting the purinergic receptors. This is because the breakdown product of UTP and UDP, i.e. uridine, unlike adenosine, has limited ancillary pharmacologic effects [38]. Thus, UTP may be used in preference to ATP as a $P2Y_2$ receptor agonist, because it does not form cardiovascular active metabolites, such as adenosine. Therefore, the possible therapeutic application of such a potent and sustained effect in patients at risk of myocardial ischemic damage is very promising and justifies further exploration.

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