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Uridine-5′-triphosphate (UTP) reduces infarct size and improves rat heart function after myocardial infarct

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ABSTRACT

We have previously found that uridine 5′-triphosphate (UTP) significantly reduced cardiomyocyte death induced by hypoxia via activating $P2Y_2$ receptors. To explore the effect of UTP following myocardial infarction (MI) *in vivo* we studied four groups: sham with or without LAD ligation, injected with UTP (0.44 μg/kg i.v.) 30 min before MI, and UTP injection (4.4 μg/kg i.v.) 24 h prior to MI. Left ventricular end diastolic area (LVEDA), end systolic area (LVESA) fractional shortening (FS), and changes in posterior wall (PW) thickness were performed by echocardiography before and 24 h after MI. In addition, we measured different biochemical markers of damage and infarct size using Evans blue and TTC staining. The increase in LVEDA and LVESA of the treated animals was significantly smaller when compared to the MI rats ($p < 0.01$). Concomitantly, FS was higher in groups pretreated with UTP 30 min or 24 h (56 ± 14.3 and $36.7 \pm 8.2\%$, $p < 0.01$, respectively). Ratio of infarct size to area at risk was smaller in the UTP pretreated hearts than MI rats (22.9 ± 6.6, $23.1 \pm 9.1\%$, versus $45.4 \pm 7.6\%$, respectively, $p < 0.001$). Troponin T and ATP measurements, demonstrated reduced myocardial damage. Using Rhod-2-AM loaded cardiomyocytes, we found that UTP reduced mitochondrial calcium levels following hypoxia. In conclusion, early or late UTP preconditioning is effective, demonstrating reduced infarct size and superior myocardial function. The resulting cardioprotection following UTP treatment post ischemia demonstrates a reduction in mitochondrial calcium overload, which can explain the beneficial effect of UTP.

1. Introduction

Considerable effort has been devoted towards improving functional recovery and reducing the extent of infarction after ischemic episodes since coronary heart disease remains a major worldwide threat. It has been found that the heart was significantly protected against ischemic injury if it was first preconditioned (PC) by a brief period of ischemia [16]. This PC effect is mimicked by several pharmacological agents including adenosine, a purine metabolite [6,14].

Purine and pyrimidine nucleotides have widespread and specific extracellular signaling actions in the regulation of a variety of functions in many tissues. According to pharmacological evidence, mechanisms of signal transduction and

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molecular structure, there are two principal families of P2-receptors: P2X receptors which are ion channels; and P2Y-receptors, which belong to the group of G protein-coupled receptors [1]. Various subtypes of P2Y receptors are activated not only by ATP and its derivatives, but also by other naturally occurring nucleotides. For example, the $P2Y_2$ and $P2Y_4$ receptors are activated by the uracil nucleotide uridine 5′-triphosphate (UTP) [5,10]. Both are preferentially coupled to G_q and therefore activate phospholipase C_β, leading to the formation of inositol trisphosphate (IP_3), intracellular Ca^{2+} ($[Ca^{2+}]_i$) elevation and production of diacylglycerol (DAG). DAG activates protein kinase C (PKC) and mitogen-activated protein kinases [19]. Whereas the effects of purine nucleosides and nucleotides in myocardial infarction and ischemia have been intensively studied [2,20], the role of pyrimidine nucleotides under hypoxic conditions has not been well explored [18], although the level of both ATP and UTP were found to be elevated as a result of ischemia [7]. Using rat cell culture we found that UTP protected cardiomyocytes from hypoxic damage through the activation of $P2Y_2$ receptors [26].

The purpose of this study was to further explore the effects of UTP on the infarcted heart *in vivo*. This work provided evidence that extracellular UTP plays a significant role in protecting the ischemic heart against injury, as demonstrated by mechanical and biochemical markers. Using isolated cardiomyocyte cultures we found that this process is partially mediated through prevention of mitochondrial calcium overload during ischemia.

2. Materials and methods

2.1. Animals

Hearts of male Wistar rats (250–300 g) aged 2–3 months were used. All animals in this study received humane care according to the guidelines set forth in The Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 86-23, Revised 1996).

2.2. Left anterior descending (LAD) artery ligation

At the age of 12 weeks rats were anesthetized (mixture of 8 mg/100 g ketamine, 5 mg/100 g xylazine), intubated, and ventilated with a Harvard Rodent Ventilator Model 383 (respiratory rate: 50/min, respiratory volume: 2.5 mL). A rectal thermocouple was used to continuously monitor body temperature, which maintained at 37 °C using a heating-pad. A left thoracotomy in the third intercostal space was performed to expose the heart. The location of the left descending coronary artery was identified and then occluded with a 6-0 silk suture. Occlusion was confirmed by monitoring the pallor of the region at risk, and an electrocardiogram was used to observe changes such as widening of QRS and ST-T segment elevation. Sham-operated rats underwent similar surgery without occlusion of the coronary artery. The thorax was closed immediately after surgery and rats were returned to their cages. They were given water and standard chow and housed in a climate-controlled environment on a 12-h light/12-h dark cycle [15]. No death occurred in response to LAD occlusion, or drug injection.

2.3. Experimental protocol

Four main groups were tested: (1) sham without LAD ligation ($n = 15$), (2) LAD ligation ($n = 15$), (3) injected with UTP (0.44 μg/kg i.v.) 30 min before MI ($n = 25$), (4) UTP injection (4.4 μg/kg i.v.) 24 h prior to MI ($n = 25$). Fig. 1 describes the experimental protocol. Several different concentrations of UTP were tested (0.044–44.4 μg/kg). We chose the most effective concentration for our experimental protocol.

2.4. Hemodynamic measurements

Animals were anaesthetized with ketamine and xylazine, and ventilated as mentioned earlier. The right carotid artery was exteriorized and a polyethylene tube (PE 50) was inserted into the left ventricular cavity and connected to a pressure transducer filled with heparinized saline (Mennen Medical, Inc., Clarence, NY) using AT-CODAS Software (Dataq Instr. Inc., Akron, OH). Left ventricular systolic pressure (LVSP), end-diastolic pressure (LVEDP) and heart rates were monitored before, after UTP treatment and post MI [15].

2.5. Echocardiography

Animals were lightly anesthetized by the inhalation of isoflurane. Two-dimensional (2D) guided M-mode echocardiography were performed using an echocardiogram (Philips, Sonos 5500, USA) equipped with a S12 on the rats MHz phased-array transducer. The parasternal long-axis of the hearts was viewed in a 2D image. The M-mode cursor was then positioned perpendicular to the interventricular septum and posterior wall of the LV at the level of the papillary muscles. An M-mode

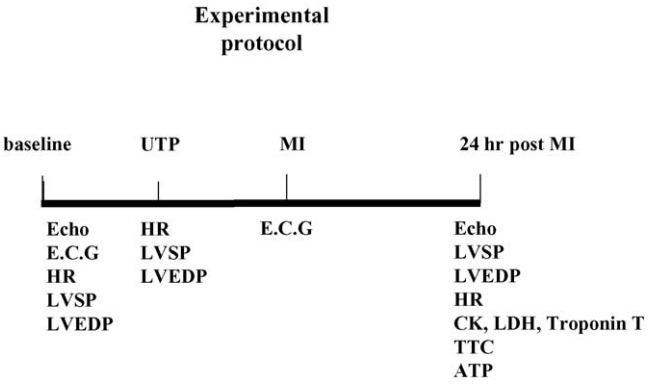

Fig. 1 – *Experimental protocol:* E.C.G. and echocardiography were performed at baseline 1 day before UTP injection. After 30 min or 24 h of UTP treatment MI was preformed. A second echo was done 24 h later. Heart rate, left ventricular systolic and diastolic pressure before and after the induction of MI and UTP treatment were monitored. Blood samples were taken 24 h post MI before scarified. The irreversible ischemic injury was determined in the excluded hearts by Evans blue, TTC staining and ATP level analysis.

image was obtained at a sweep speed of 100 mm/s. Diastolic and systolic LV wall thickness, LV end-diastolic dimensions (LVEDA), and LV end-systolic chamber dimensions (LVESA) were measured. The percentage of LV fractional shortening (LV%FS) was calculated as [(LVEDA − LVESA)/LVEDA] × 100. Echocardiography was performed one day before and 24 h post LAD ligation [9].

2.6. Measurement of irreversible ischemic injury

At 24 h of coronary ligation, 1% of Evans blue solution (5 mL), was infused into the abdominal vena cava to delineate the ischemic area at risk of the left ventricle. The heart was excised and cross-sectioned from the apex to the atrioventricular groove into four specimens of 0.8 mm in thickness with the use of a stereoscope. The two middle heart slices were incubated in 2,3,5-triphenyltetrazolium chloride (TTC) solution (1%) for 30 min in phosphate buffer at 37 °C. Sections were fixed overnight in 4% paraformaldehyde for contrast enhancement between stained and unstained tissue. TTC stained the viable tissue with red, while the necrotic tissue remained discolored. 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 with IMAGE J 5.1 software. The area of irreversible injury (TTC-negative) is presented as a percentage of the area at risk [25]. Posterior wall thinning was an average of 5 points measured in each slice.

2.7. Biochemical analysis

Levels of creatine kinase (CK) were determined in the serum using commercial kits (Boehringer Mannheim) 24 h following MI. For troponin T evaluation, Cardiac T 2017423, Roche kit was used.

2.8. Measurement of ATP concentration

Myocardial tissue was harvested in 1 mL cold 5% trichloroacetic acid. The cell extract was used to measure ATP content by the luciferin-luciferase bioluminescence kit (ATP Bioluminescence Assay Kit CLSH, Boehringer, Mannheim).

2.9. Cell culture

Rat hearts (2–3 days old) were removed under sterile conditions and washed three times in PBS to remove excess blood cells. The hearts were minced and then gently agitated in RDB, a solution of proteolytic enzymes (Life Science Research Inst.), prepared from a fig tree extract [22]. After treatment with RDB, cell viability was found to be 98%, in contrast to 85% in trypsin-treated monolayer cultures. The RDB was diluted 1:100 in Ca^{2+}- and Mg^{2+}-free PBS at 25 °C for a few cycles of 10 min each, as described previously [21]. Dulbecco's modified Eagle's medium, supplemented with inactivated 10% horse serum (Biological Industries) and 0.5% chick embryo extract, was added to supernatant suspensions containing dissociated cells. The mixture was centrifuged at 300 × *g* for 5 min. The supernatant phase was discarded, and the cells were resuspended in the same medium. The suspension of the cells was diluted to 1.0×10^6 cells/mL, and 1.5 mL of the suspension was placed in 35-mm plastic culture dishes on collagen/gelatin-coated coverglasses. The cultures were incubated in a humidified atmosphere of 5% CO_2, 95% air at 37 °C. Confluent monolayers exhibiting spontaneous contractions were developed in culture within 2 days. All experiments were performed between days 5 and 7 in culture.

2.10. Hypoxic conditions

Cardiomyocyte cultures 5–7 days old were washed from the medium with glucose-free PBS containing 5 mM HEPES at pH 7.4 before exposing the myocytes to the hypoxic conditions at 37 °C. The hypoxic condition consisted of 120 min in a hypoxic incubator in which the atmosphere was replaced by the inert gas argon (100%) in glucose-free media [21]. The hypoxic damage was characterized at the end of the hypoxic period by morphological and biochemical evaluation. Continuous monitoring of $[Ca^{2+}]_i$ during hypoxia was realized in a special barrier well, where cells were protected from oxygen by a laminar counterflowing layer of argon gas as previously described [26]. The coverglasses with cultured cells were placed at the bottom of the well. This chamber was mounted on a specially modified Zeiss inverted epifluorescence microscope.

2.11. Mitochondrial Ca^{2+} measurements

Mitochondrial calcium ion concentration was estimated from Rhod-2 fluorescence with the ratio method (the SAMPLE program) described elsewhere [23]. Cardiac cells grown on coverslips were exposed to Rhod-2-AM (10 μM), dissolved in PBS, at 4 °C, for 120 min then were washed and transferred to 37 °C for additional 120 min. Rhod-2 fluorescence, representing mitochondrial calcium, was elicited by excitation at 540 nm and emission was measured at a wavelength of 590 nm [12].

2.12. Statistical analysis

Results are expressed as means ± standard deviation of the mean (S.D.). Values during stabilization period were defined as 100%. A statistical difference between the groups was assessed by analysis of variance (ANOVA) with repeated measurements. If differences were observed, values were compared using Student's *t*-test. $p < 0.05$ was considered significant.

3. Results

3.1. Hemodynamic measurements

We examined the effect of UTP on the rat heart rate and left ventricular systolic and diastolic pressures prior to - and 5 min following UTP injection. UTP had no effect on heart rate or blood pressure compared to control animals. LAD occlusion caused an elevation in the heart rate (314 ± 19 bpm versus 276 ± 21 bpm in sham animals) that was lowered when the rats were pretreated with UTP 30 min or 24 h before ischemia (241 ± 48 and

Table 1 – Hemodynamic measurements

Treatment	Heart rate (bpm)	LVSP/LVEDP (mm Hg)
Control	276 ± 21	118 ± 27
UTP 30 min	283 ± 18	116 ± 25
UTP 24 h	279 ± 17	116 ± 5
MI	314 ± 19*	120 ± 3
MI + UTP 30 min	241 ± 48	114 ± 11
Mi + UTP 24 h	286 ± 17	118 ± 27

Heart rate and LVSP/LVEDP at baseline and post MI of control (saline treated), UTP treated 30 min or 24 h before MI (0.44 μg/kg, 4.4 μg/kg, respectively) were measured using intracardiac cannula. End diastolic pressure was stable throughout the experiment was 0–7 mm Hg without statistical difference between the tested groups. Values are mean ± S.D. of six animals.
* $p < 0.05$ vs. control animals.

286 ± 17 bpm, respectively). The intracardial left ventricular systolic and diastolic pressures before and after the induction of MI and UTP treatment did not significantly change (Table 1).

3.2. *Assessment of LV function post-myocardial ischemia*

There were no significant differences between groups in the echocardiograph measurements of cardiac structure or function at baseline. Induction of MI resulted in more pronounced increased left ventricular end-systolic and end-diastolic area (LVESA and LVEDA) compared with the UTP pretreated rats ($p < 0.01$, Fig. 2A and B). The infarcted rats also demonstrated significantly reduced fractional shortening (28.4 ± 5.4%), compared to the rats post MI pretreated 30 min or 24 h with UTP (56.0 ± 14.3 and 36.7 ± 8.2%, respectively, Fig. 2C). Changes in posterior wall thinning were significantly higher ($p < 0.05$) in the infarcted hearts (17.9 ± 7.0%) compared to UTP pretreated hearts, at 30 min or 24 h before MI (1.45 ± 0.58, 2.04 ± 0.82%, respectively, Fig. 2D).

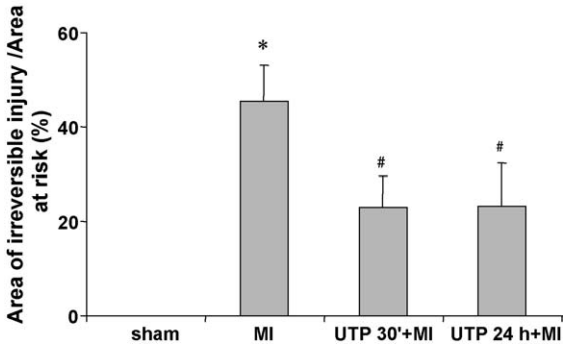

Fig. 3 – Effect of pretreatment with UTP on myocardial infarction size after LAD occlusion. The area of irreversible injury (TTC-negative) is presented as a percentage of the area at risk (Evans blue unstained). Values are means ± S.D. *$p < 0.001$ vs. sham, #$p < 0.01$ vs. MI.

3.3. *Irreversible ischemic damage*

At 24 h after MI, the area at risk of the left ventricle (Evans blue unstained) was similar in all groups, *i.e.*: untreated infarcted hearts, UTP injections 30 min or 24 h before MI (54.3 ± 6.4, 50.5 ± 6.8, 49.7 ± 9.3%, respectively). However, the infarcted area (TTC unstained) of the area at risk was markedly reduced after UTP treatment (45.4 ± 7.6, 22.9 ± 6.6, 23.1 ± 9.1%, respectively, $p < 0.001$, Fig. 3). The posterior wall thickness of all treated animals was similar to sham rats (3.08 ± 0.11 mm for sham animals, and 2.97 ± 0.40/2.88 ± 0.43 mm for 30 min or 24 h UTP treated rats), in contrast to those after MI, where the wall was significantly thinner (2.07 ± 0.09 mm, $p < 0.05$, Fig. 4).

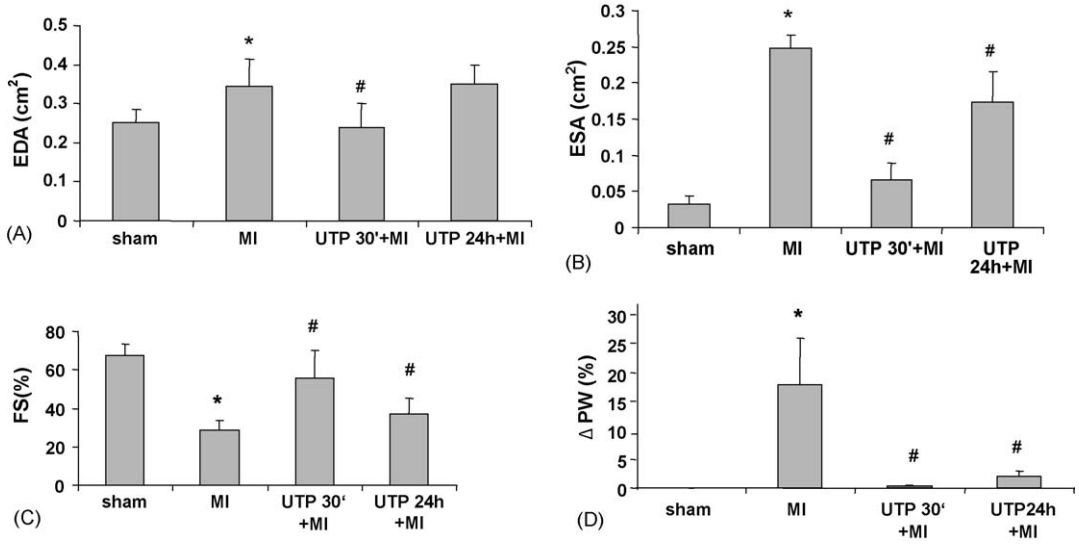

Fig. 2 – Echocardiographic studies 24 h after MI. These studies demonstrated LVESA and LVEDA of sham and infracted hearts compared with the 30 min or 24 h UTP pretreated rats (A and B). In addition, fractional shortening (ΔFS) in all infracted animals (C), and changes in the left ventricular posterior wall thickness (ΔPW) (D) were done. Pretreatment with UTP 30 min or 24 h before MI, decreased the changes in the posterior wall and fractional shortening when compared to the MI hearts ($p < 0.01$). Values represent means ± S.D., $n = 15–25$ hearts in each group.

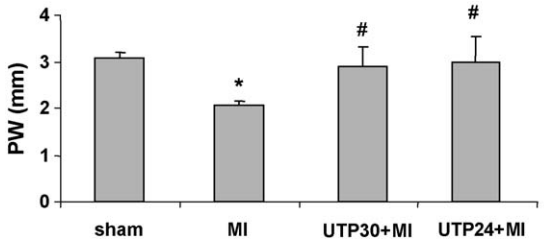

Fig. 4 – Posterior wall (PW) thickness of UTP treated animals. Posterior wall (PW) thickness determined by scanning the images of rat heart ventricular sections with triphenyltetrazolium chloride (TTC staining). Values are means ± SD. $^{*}p < 0.01$ vs. sham, $^{\#}p < 0.05$ vs. MI.

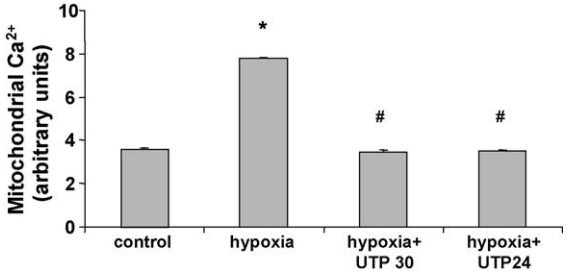

Fig. 6 – Effect of pretreatment with UTP on mitochondrial Ca^{2+} overload in cardiomyocytes subjected to hypoxia. Cardiomyocytes were treated with 50 μM UTP 30 min or 24 h before subjection to 2 h of hypoxia. During the hypoxia, cardiomyocytes were loaded with Rhod-2-AM, and Ca^{2+} level was determined. Values are means ± S.D. $^{*}p < 0.001$.

3.4. *Biochemical markers of ischemic damage*

3.4.1. *Creatine kinase (CK)*

Serum CK activity increased in all groups 24 h after LAD occlusion, but in the infarcted animals it was two-fold higher than in both UTP 30 min or UTP 24 h treated groups (960 ± 189 units/L versus 469 ± 82, 550 ± 182 units/L, $p < 0.05$, Fig. 5A).

3.4.2. *Troponin T*

The level of troponin T, a specific marker denoting damage in the heart muscle, was detected in rats post MI compared with the sham operated rats (6.97 ± 0.50 ng/ml versus 0.02 ± 0.003 ng/ml). UTP pretreatment either immediately before (early) or 24 h before (late) MI, significantly lowered troponin T levels in the serum (5.75 ± 1.22, 4.09 ± 1.00 ng/ml, respectively, $p < 0.05$, Fig. 5B).

3.4.3. *ATP*

The level of ATP was examined in the area of the infarct. The ATP level in rats post MI was significantly lower than in sham animals (0.53 ± 0.43, 9.62 ± 2.41 nmol/mg protein). In the early and late treated groups the ATP level in the damaged area was higher than the MI-nontreated rats (2.86 ± 1.01 or 5.58 ± 1.23 nmol/mg protein, respectively, Fig. 5C).

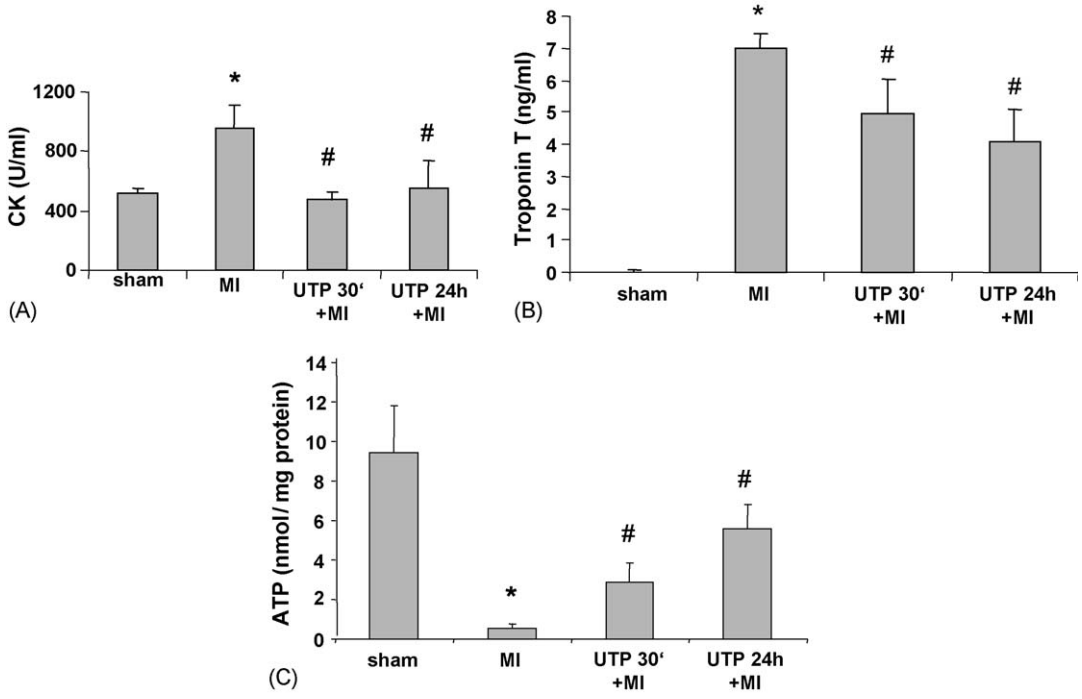

Fig. 5 – Measurements of biochemical markers in UTP pretreated MI rats. Release to the coronary effluent of creatine kinase (CK) (A), troponin T (B). The ATP level in the infarct area (C) after pretreatment with UTP 30 min or 24 h before LAD occlusion. Values are means ± SD. $^{*}p < 0.001$ vs. sham, $^{\#}p < 0.01$ vs. MI.

3.5. *Mitochondrial calcium in cardiomyocytes*

In order to examine the intracellular mechanism of UTP function on cardiac protection we used the mitochondrial calcium probe Rhod-2 [12]. The level of cardiomyocyte mitochondrial calcium observed in cells following 2 h hypoxia was high, compared to controls (Fig. 6). However, cardiomyocytes which were treated with 50 μM UTP for 30 min or 24 h prior to hypoxia demonstrated a two-fold reduction in mitochondrial calcium level.

4. Discussion

We have previously shown that activation of UTP receptors protects newborn rat cardiomyocytes against hypoxic damage and significantly reduces the cell death caused by hypoxia via activation of $P2Y_2$ receptors [26]. In the present study, we extend this observation and show that prior treatment with UTP reduces the ischemic damage to the rat heart using an *in vivo* model. Myocardial functions as well as biochemical parameters of damage were improved by prior administration of UTP. Rats treated with UTP had a smaller infarct size, increased muscle posterior wall thickness and superior diastolic and systolic functions, as compared to the sham operated animals. Biochemical tests (LDH, CK enzymes, troponin T and ATP levels) demonstrated the same protective effects.

Short treatment with UTP before MI (30 min) showed some advantage in functional parameters of the heart in comparison to long exposure to the agonist (24 h). However, biochemical parameters of heart damage did not indicate a significant difference. It is well known that PC agents such as adenosine lower the heart rate and consequently the cellular metabolism, thus decreasing the myocardial oxygen demand [13]. The decreasing heart rate affected by UTP resulted in superior post-MI tolerance, because the hearts were not as tachycardic as the control, non-treated hearts. It seems that lowering of the energy demands by slowing the heart rate after the MI had long-term effects in decreasing the whole ischemic insult as seen by ATP levels, infarct size and eventually cardiac function.

The $P2Y_2$ purinoceptors are likely a common subtype potently stimulated by ATP and UTP. It was found that ATP and UTP provoked an increase in coronary flow in a dose-dependent manner [18]. It has been reported that UTP has a vasodilatory effect, via the activation of specific P2Y purinoceptors [27]. It is possible that UTP administration prior to the induction of MI 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. UTP attenuated the posterior wall thinning process as demonstrated by both TTC staining and echocardiography. There is a difference between the measurements of posterior wall thinning using these measurements. In the former method (TTC staining), the assessment was an average of several points measured, while in the latter method (echocardiography), the analysis was based on one measurement per heart. Neither measurement is extremely accurate but has been mentioned as they reveal a trend in the remodeling process. According to the literature, troponin T is used as a specific marker of myocardial damage, while the enzyme CK is less specific and sensitive [3]. In addition, opening the chest caused skeletal muscle CK release, which elevated the background of these enzymes but not of troponin T.

In ischemic heart, calcium overload has been shown to play an important role in causing a dysfunction of the cells [8], and thereby the mitochondrial calcium surplus is produced which thus further impairs the cellular functions [17]. We have previously shown that activation of a UTP receptor prevented intracellular calcium elevation as a result of hypoxia and maintained an increased level of ATP in cultured cardiomyocytes. Suramin, a $P2Y_2$ receptor antagonist, impeded these protective effects [26]. In the present study, we further established the intracellular protective effects, as evidenced by preventing mitochondrial Ca^{2+} overload following hypoxia in cardiomyocyte cultures. Both immediate and prolonged UTP treatment prevented mitochondrial calcium elevation, thereby affording improved mitochondrial function. These data supported the recent finding of Belous et al. [4], who described a hepatocyte mitochondrial $P2Y_2$-like receptor linking cytosolic UTP to mitochondrial calcium uptake. It was found that UTP inhibits mitochondrial Ca^{2+} uptake, which may promote respiratory chain activity and ATP production. In addition, we have already demonstrated that UTP induced Ca^{2+} release through a mechanism that involves IP_3. Jaconi et al. reported that IP_3 directs Ca^{2+} flow between the mitochondria and the sarcoplasmic reticulum [11]. Thus, it is possible that inhibition of mitochondrial Ca^{2+} uptake by UTP is mediated through the regulation of intracellular IP_3 levels. Both immediate and late effects of UTP led to similar cardioprotection.

Although the precise mechanism for the inhibition of myocardial ischemic injury remains unclear, it appears that both modes of its administration played a crucial role in ameliorating the ischemic damage. Therapeutic targeting of pyrimidinergic receptors for protection against ischemic myocardial damage may be more effective than targeting the purinergic receptors. The breakdown product of UTP and UDP, *i.e.* uridine, unlike adenosine, has limited ancillary pharmacologic effects [24]. Thus, UTP may be used in preference to ATP as a $P2Y_2$ and $P2Y_4$ 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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