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Involvement of UTP in protection of cardiomyocytes from hypoxic stress¹

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Abstract

Massive amounts of nucleotides are released during ischemia in the cardiovascular system. Although the effect of the purine nucleotide ATP has been intensively studied in myocardial infarction, the cardioprotective role of the pyrimidine nucleotide UTP is still unclear, especially in the cardiovascular system. The purpose of our study was to elucidate the protective effects of UTP receptor activation and describe the downstream cascade for the cardioprotective effect. Cultured cardiomyocytes and left anterior descending (LAD)-ligated rat hearts were pretreated with UTP and exposed to hypoxia–ischemia. In vitro experiments revealed that UTP reduced cardiomyocyte death induced by hypoxia, an effect that was diminished by suramin. UTP caused several effects that could trigger a cardioprotective response: a transient increase of $[Ca^{2+}]_i$, an effect that was abolished by PPADS or RB2; phosphorylation of the kinases ERK and Akt, which was abolished by U0126 and LY294002, respectively; and reduced mitochondrial calcium elevation after hypoxia. In vivo experiments revealed that UTP maintained ATP levels, improved mitochondrial activity, and reduced infarct size. In conclusion, UTP administered before ischemia reduced infarct size and improved myocardial function. Reduction of mitochondrial calcium overload can partially explain the protective effect of UTP after hypoxic–ischemic injury.

Keywords

calcium; cardioprotection; cardiac cell culture; heart; hypoxia; ischemia; nucleotides; preconditioning; pyrimidines

Introduction

Despite intensive research and progress in medicine, myocardial infarction and heart failure remain the leading causes of death in the Western world. Myocyte loss, both necrotic and

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apoptotic, is a feature of many heart pathologies (Kajstura et al. 1996). Due to the fact that adult cardiomyocytes possess minimal capacity to reenter the cell cycle (Anversa and Kajstura 1998), the reduction of myocyte loss through suppression of cell death pathways represents a beneficial strategy to prevent myocardial infarction and heart failure.

More than 20 years have passed since the initial description of the phenomenon of ischemic preconditioning, whereby episodes of intermittent sublethal ischemia and re-perfusion confer resistance against a subsequent lethal episode of myocardial ischemia (Murry et al. 1986). Many experiments have since been done to elucidate the mechanism by which a short episode of ischemia makes the heart tolerant to prolonged ischemia. It was found that adenosine and other internal and external agonists can mimic ischemic preconditioning and protect the heart from prolonged ischemia and cell injury via the activation of specific cell surface receptors (Sommerschild and Kirkeboen 2000). This process is called pharmacologic preconditioning.

Purines and pyrimidines have widespread and specific extracellular signaling actions in the regulation of a variety of functions. In 1978 Burnstock proposed that specific extracellular nucleoside (P1) or nucleotide (P2) receptors mediate the physiologic effects of adenosine and ATP, respectively (Burnstock 1978). Receptors that mediate the extracellular actions of purines and pyrimidines are divided into 2 major families, one family of ligand-gated receptors (P2X) and another family of G protein-coupled receptors (P2Y), comprising 15 subtypes in all. In contrast to P2X receptors, P2Y receptors are activated not only by ATP or its derivatives, but also by other naturally occurring nucleotides. The P2Y₂, P2Y₄, and P2Y₆ receptor subtypes have high affinity for uracil nucleotides such as UDP and UTP, which mediate signaling actions through these unique pyrimidine receptor (Jacobson et al. 2009; von Kügelgen and Wetter 2000).

Massive amounts of nucleotides are released during ischemia and hypoxia in the cardiovascular system (Erlinge et al. 2005). Whereas the effect of purine nucleotides (e.g., ATP) in myocardial infarction has been intensively studied (Abbracchio and Burnstock 1998), the role of pyrimidine nucleotides under hypoxic conditions has not been explored as intensively. In this study we characterize and elucidate the protective effects of pretreatment with UTP against detrimental factors of hypoxia–ischemia in 2 systems: an in vitro model, in which the cells were exposed to 120 min of hypoxia, and an in vivo model, in which the left anterior descending (LAD) artery in rat hearts was ligated. The findings revealed that UTP pretreatment remarkably reduced lactate dehydrogenase (LDH) release, reduced infarct size, maintained morphologic structure, maintained ATP levels, and improved mitochondrial function.

Materials and methods

Cell culture

Rat hearts (2–3 days old) were removed under sterile conditions and washed 3 times in phosphate-buffered saline (PBS) to remove excess blood cells. The hearts were minced and then gently agitated in RDB, a solution of proteolytic enzymes (Life Sciences Research Institute, Ness-Ziona, Israel) prepared from a fig tree extract (Shneyvays et al. 1998). The RDB was diluted 1:100 in Ca²⁺- and Mg²⁺-free PBS at 25 °C for several cycles of 10 min each, as described previously (Safran et al. 2001). Dulbecco's modified Eagle's medium, supplemented with inactivated 10% horse serum (Biological Industries, Kibbutz Beit Haemek, Israel) 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 × 10⁶ cells/mL, and 1.5 mL of the suspension was placed in 35-

millimetre plastic culture dishes on collagen/gelatin-coated coverglasses. The cultures were incubated in a humidified atmosphere of 5% CO₂, 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.

[Ca²⁺]_i measurements

Intracellular free calcium ion concentration ([Ca²⁺]_i) was estimated from indo-1 fluorescence with the ratio method (the SAMPLE program) described elsewhere (Fixler et al. 2002; Shneyvays et al. 2001).

Hypoxic conditions

Myocyte cultures 5–7 days old were washed from the medium with glucose-free PBS containing 5 mmol/L Hepes at pH 7.4 before exposing the myocytes to the various 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 (Safran et al. 2001; Shneyvays et al. 2000). The hypoxic damage was characterized at the end of the hypoxic period by morphologic and biochemical evaluation. Continuous monitoring of [Ca²⁺]_i during hypoxia was realized in a special barrier well, in which cells were protected from oxygen by a laminar counterflowing layer of argon gas as previously described (Yitzhaki et al. 2005). 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.

Assays of intracellular ATP level

After hypoxia, control and experimental cells were harvested in 1 mL cold 5% trichloroacetic acid. The cell extract was used for the measurement of ATP content with a CL SII luciferin–luciferase bioluminescence kit (Boehringer) following the manufacturer's protocol. The values were expressed as nanomoles per milligram of protein (nmol/mg) (Yitzhaki et al. 2005).

Experiments with purinergic and pyrimidinergic ligands

UTP at concentrations of 3–50 μmol/L was applied to the cell cultures for 15 min after 15 min preincubation with various antagonists (Sigma). The inhibitors LY294002 (30 μmol/L) and 5-hydroxydecanoate (5HD) (300 μmol/L), the Ca²⁺ chelator 1,2-bis(*o*-aminophenoxy)ethane *N,N,N',N'*-tetraacetic acid (BAPTA) (5 μmol/L), and the P2Y antagonists suramin (300 μmol/L), pyridoxal-5'-phosphate-6-azophenyl-2',4'-disulfonate (PPADS) (10 μmol/L), and reactive blue 2 (RB2) (50 μmol/L) were dissolved in PBS. U0126 (10 μmol/L) was dissolved in DMSO.

Release of LDH

Protein content and LDH activity were determined according to El Ani et al. (1994). Briefly, 25 μL supernatant was transferred into a 96-well dish, and the LDH activities were determined with an LDH-L kit (Sigma), as described by the manufacturer. The product of the enzyme was measured spectrometrically at 30 °C at a wavelength of 340 nm as described previously (Shneyvays et al. 2000). The results were expressed relative to the control (fold change) in the same experiment. Each experiment was done in triplicate and was repeated at least 3 times.

Western blot analysis

After treatment with UTP, cardiomyocytes were washed with ice-cold PBS and harvested into an Eppendorf tube with ice-cold radioimmunoprecipitation assay (RIPA) buffer (50

mmol/L Tris-HCl, pH 7.4, 1% NP-40 (nonyl phenoxyl-polyethoxylethanol), 0.25% sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA, 0.1% SDS). The harvested outcome was then sonicated (3×5 s) in ice, and clarified by centrifugation (6 min at 12 000 rpm (10 000g)) in an Eppendorf microcentrifuge. Next, 100 μ L of the cell lysate was removed and stored at -20°C until required. Protein concentration was determined by using the Bradford protein assay, with bovine serum albumin as the standard. Aliquots of the cell lysate (50 μ g) in RIPA buffer were diluted with sample buffer (2:1, respectively) and heated at 100°C for 5 min. The sample protein was separated by SDS-PAGE (10% acrylamide gel) using a Bio-Rad Mini-Protein II system (2 h at 100 V). Proteins were transferred into nitrocellulose membranes with a Bio-Rad Trans-Blot system (2 h at 200 V). After transfer, the nitrocellulose membrane was routinely monitored by transiently staining the membranes with Ponceau S stain before application of the primary antibody. Then, the membrane was washed (3×5 min) with Tris-buffered saline (TBS) and blocked for 1 h at room temperature in blocking buffer (TBS, 5% (w/v) skimmed milk powder, 0.05% Tween 20). Blot was then incubated in blocking buffer overnight at 4°C with the appropriate primary antibody against phosphorylated extracellular signal-regulated kinase (p-ERK) (Tyr204) or against p-Akt (Thr308) (1:1000 dilution). The primary antibody was removed and the blot was extensively washed 3 times for 5 min in TBS-Tween 20. Blot was then incubated for 1 h at room temperature with the suitable secondary antibody coupled to horseradish peroxidase (1:10 000 dilutions) in blocking buffer. After removal of the secondary antibody by extensively wash, blot was developed using an enhanced chemiluminescence detection system and quantified by densitometry using ImageJ software. In these experiments, the uniformity of protein loading was confirmed by measuring the total amount of protein, with first antibody against desmin (1:1000 dilutions) as described above.

Western blotting of P2Y receptors was performed by using the following protocol. Aliquots (70 μ g) from each protein sample were loaded on 11% SDS polyacrylamide gels and blotted onto nitrocellulose membranes. Filters were saturated with TBS in 10% milk for 1 h at room temperature to avoid nonspecific binding of the primary antibody, and then incubated overnight at 4°C with anti-P2Y₂ (1:200) or anti-P2Y₄ (1:300) antibodies. Each P2Y receptor was also tested in the presence of neutralizing peptides (ratio 1:1 between peptide and antiserum). Blots were then washed in TBS (1 mmol/L Tris-HCl, pH 8.0, 15 mmol/L NaCl, 0.1% Tween 20, final concentration) and incubated for 1 h with anti-rabbit immunoglobulin (Ig)G coupled to fluorescein isothiocyanate (FITC). Immunoreactions were analyzed with enhanced chemiluminescence.

Mitochondrial [Ca^{2+}] measurement

Mitochondrial [Ca^{2+}] was estimated from rhod-2 fluorescence with the ratio method (the SAMPLE program) described elsewhere (Yitzhaki et al. 2006). Cardiac cells grown on coverslips were exposed to rhod-2 AM dye (10 μ mol/L) dissolved in PBS at 4°C for 120 min, and they were then washed and transferred to 37°C for an 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 (Jo et al. 2006).

Preparation of heart slices for histochemical staining

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 US National Society for Medical Research and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. The LAD artery in rat hearts was occluded as previously described (Yitzhaki et al. 2006). Twenty-four hours after ligation, hearts were excised and immediately cross-sectioned from the apex to the atrioventricular groove into 4 specimens of 0.8 mm thickness using a

stereoscope. Heart slices from the left ventricular papillary muscle area were washed with NaCl 0.85% and put between 2 coverglasses. The pieces were frozen in a 2800 Frigocut N cryostat (Reichert-Jung) and then put on a disk, using Tissue-Tek, 15 min before cutting. Slices of 5 μm were cut from the frozen pieces using Feather 35S microtome blades. The slices were then pasted and kept on SuperFrost Plus glass slides at 20 °C for histochemical staining.

LAD artery ligation

Rats were anesthetized (mixture of ketamine (8 mg/100 g body weight) and xylazine (5 mg/100 g)), intubated, and ventilated with a Harvard rodent ventilator model 683 (respiratory rate 50 breaths/min, respiratory volume 2.5 mL). A rectal thermocouple was used to continuously monitor body temperature, which was maintained at 37 °C by 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 (Morgan et al. 2004). No death occurred in response to LAD occlusion or drug injection.

Experimental protocol

Four main groups were tested: (i) sham-operated without LAD ligation ($n = 15$), (ii) LAD ligation ($n = 15$), (iii) injected with UTP (0.44 $\mu\text{g/kg}$ i.v.) 30 min before myocardial infarction (MI) ($n = 25$), (iv) UTP injection (4.4 $\mu\text{g/kg}$, i.v.) 24 h before MI ($n = 25$). Several different concentrations of UTP were tested (0.044–44.4 $\mu\text{g/kg}$). We chose the most effective concentration for our experimental protocol.

Staining

The propidium iodide assay is based on vital binding of propidium iodide to the nuclei of cells whose plasma membranes have become permeable because of cell damage. The assay was performed according to Nieminen et al. (1992). For counterstaining we used Hoechst 33342 (10 $\mu\text{mol/L}$), which stains the nuclei of all cells.

Lysosome staining was performed with neutral red, a widely used marker of membrane-bound compartments with an acidic lumen (lysosomes and several other acidic compartments). Living cardiomyocytes were incubated for 30 min in PBS containing 4 $\mu\text{g/mL}$ neutral red (Molecular Probes).

Cytochrome *c* oxidase

Heart slices were fixed with 0.5% formaldehyde solution in PBS for 5 min, washed in PBS, and placed in an incubation medium containing PBS with 1 mg/mL diaminobenzidine (DAB), 1 mg/mL cytochrome *c* (type III), and 85 mg/mL sucrose (Shneyvays et al. 2003). The slices were incubated in the dark at 37 °C for 1–1.5 h. They were then rinsed with distilled water and coverslipped with glycerol for microscopy and detection of cytochrome *c* oxidase (COX).

NADH:ubiquinone oxidoreductase and succinate dehydrogenase

NADH:ubiquinone oxidoreductase and succinate dehydrogenase (SDH) staining was performed using a cytochemical method, based on the reduction of nitroblue tetrazolium to

diformazan. Cell culture plates were washed in PBS and incubated with PBS containing 67.5 mg/mL succinate and 1 mg/mL nitroblue tetrazolium for 2 h at 37 °C (Shneyvays et al. 2003). Cardiomyocytes were fixed with 4% formaldehyde for 10 min and coverslipped with glycerol for microscopy.

Statistics

Results were expressed as means $\pm$ SD. Data were analyzed by analysis of variance with application of a post hoc Tukey–Kramer test. Statistical significance was determined as $p < 0.05$.

Results

Results in cell culture

Effect of UTP on cardiomyocytes after hypoxia—To investigate the protective effects of UTP in the attenuation of myocyte injury during prolonged (120 min) hypoxia, cultured cardiomyocytes were incubated with various concentrations of UTP (3–50 μ mol/L) for 15 min before hypoxia. As shown in Fig. 1A, the level of LDH release from the cardiomyocytes to the medium was elevated as a result of 120 min of hypoxia ($25.55\% \pm 2.84\%$) compared with that of control cells ($4.16\% \pm 0.48\%$). Treatment with 50 μ mol/L UTP, which was found to be the most efficacious lower dose, reduced LDH elevation ($6.06\% \pm 0.61\%$). UTP in normoxic conditions did not alter the LDH released to the medium (data not shown). On the other hand, the related derivatives uridine and UDP (50 μ mol/L) did not exhibit a protective effect against hypoxia in treated cardiomyocytes compared with control cells (data not shown). This ruled out the P2Y₆ receptor as a site of action of protective uracil nucleotides. These results suggest that UTP pretreatment diminishes acute hypoxia-induced cardiomyocyte injury.

To investigate the time-dependence of UTP in protecting cardiomyocytes, the cells were pretreated with 50 μ mol/L UTP for 1 h and then exposed to 120 min of hypoxia after incubation in full medium for 15 min to 72 h. As revealed by LDH release (Fig. 1B), UTP protected the cells from hypoxia compared with hypoxic cells ($55.25\% \pm 16.29\%$), even when treated 48 h before hypoxia ($33.02\% \pm 3.43\%$), but not when treated 72 h before ($58.81\% \pm 2.85\%$). There was no change in the protective effect between the cells that were washed after 1 h treatment or incubated with UTP during the entire prolonged treatment (48 and 72 h) (data not shown). These results suggest that UTP activates a long, persistent protective mechanism (a second window of protection).

Involvement of P2 receptors in UTP-induced cardioprotection—To verify whether the protective effect caused by UTP is mediated via P2 receptors, cardiomyocytes were pretreated for 15 min with P2 receptor antagonists (RB2, PPADS, or suramin) before UTP treatment and hypoxia. Pretreatment with suramin or PPADS, but not with RB2, inhibited the protective effect of UTP, as determined by LDH released to the medium (Table 1), indicating the involvement of P2Y₂ receptors and (or) P2Y₄ receptors in UTP-induced protection of cultured cardiomyocytes.

To confirm the presence of P2Y₂ and P2Y₄ receptors on neonatal cardiomyocyte membranes, immunostaining was performed with specific antibodies. We found that both receptors were present in significant amounts on the cell membrane (data not shown).

The protective effect of UTP was also demonstrated through cardiomyocyte morphology. After hypoxia the cells were fixed and stained with hematoxylin and eosin, which showed typical irreversible necrotic damage of the cardiomyocytes: vacuoles and disorder of the

myofilaments, pyknotic nuclei, and edematous areas in the cytoplasm and around the nucleus. In addition, hypoxic conditions increased the accumulation of the lysosomotropic vital dye neutral red in cytoplasmic granules and around the nucleus and increased the size of the lysosomes and their color intensity (Fig. 2). The damage to cultured cells treated with UTP before hypoxia was greatly reduced. Cell loss (percentage of cell death) was presented as the number of dead cells (propidium iodide-stained) as a percentage of the total number of cells (Hoechst 33342-stained). Cell loss was $40\% \pm 5\%$ after 120 min of hypoxia. Activation of the UTP receptors attenuated cell loss caused by hypoxia to $18\% \pm 2\%$; control untreated cells had $3.1\% \pm 0.2\%$ cell loss.

Effect of UTP on cytosolic ATP levels after hypoxia—To evaluate the protective effect produced by extracellular UTP, we measured the total cytosolic ATP content after hypoxia. The results showed that the ATP level decreased dramatically after 2 h exposure to hypoxia compared with that of normoxic cultures (2.92 ± 0.17 vs. 20.12 ± 2.31 nmol/mg) (Fig. 3). Pretreatment of cardiomyocytes with $50 \mu\text{mol/L}$ UTP prevented the dramatic decrease of ATP levels (16.62 ± 4.62 nmol/mg) in cultures subjected to hypoxia. Protection against ATP depletion did not occur when cells were pretreated with the P2 receptor antagonist PPADS (1.84 ± 1.31 nmol/mg) or suramin (2.56 ± 0.87 nmol/mg) before exposure to UTP, whereas treatment with RB2 did not prevent the protective effect of UTP (15.88 ± 1.9 nmol/mg) (Fig. 3), indicating that the protective effect of UTP is mediated via P2 receptors.

Effect of UTP on $[\text{Ca}^{2+}]_i$ levels during hypoxia—To investigate the effect of UTP pretreatment on $[\text{Ca}^{2+}]_i$ levels during hypoxic conditions, we continuously monitored the $[\text{Ca}^{2+}]_i$ level during hypoxia with a specially designed chamber in which oxygen was excluded from the cells by a laminar counterflowing layer of the inert gas argon. The level of $[\text{Ca}^{2+}]_i$ began to elevate, along with the cessation of beating activity, approximately 10–12 min after the initiation of hypoxia (Fig. 4A). When the cardiomyocytes were pretreated with UTP ($50 \mu\text{mol/L}$) for 15 min and then subjected to hypoxia 30 min later, a delay in $[\text{Ca}^{2+}]_i$ elevation was observed, and spontaneous contractions lasted for 30–40 min after the initiation of hypoxia (Fig. 4B). Similar pretreatment with UTP occurring 24 h before the hypoxia maintained spontaneous contractility even after 40 min of hypoxia (Fig. 4C). These results demonstrate that UTP pretreatment is able to prevent an increase in $[\text{Ca}^{2+}]_i$ during hypoxia.

Next, we examined cardiomyocyte mitochondrial calcium using the mitochondrial calcium probe rhod-2 (Jo et al. 2006). The level of mitochondrial calcium observed in cardiomyocytes after 2 h hypoxia was high compared with that of controls (Fig. 5). In cardiomyocytes that were treated with $50 \mu\text{mol/L}$ UTP 30 min or 24 h before hypoxia, however, mitochondrial calcium increase under hypoxic conditions was prevented.

Effect of UTP receptor activation on $[\text{Ca}^{2+}]_i$ —To investigate the signal transduction pathway of the cardioprotection against hypoxic stress produced by UTP, we examined several effects that might trigger a cardioprotective response. First, we measured the $[\text{Ca}^{2+}]_i$ levels by using indo-1 as a fluorescent probe. As shown in Fig. 6A, treatment with $50 \mu\text{mol/L}$ UTP induced transient acceleration of the beating rate and a transient elevation of the baseline $[\text{Ca}^{2+}]_i$, which lasted 30–40 s before normal beating activity was restored. Application of PPADS ($10 \mu\text{mol/L}$) or RB2 ($50 \mu\text{mol/L}$) prevented $[\text{Ca}^{2+}]_i$ elevation (Figs. 6B and 6C). Suramin ($300 \mu\text{mol/L}$), which acts as an antagonist of P2Y receptors among other P2 sub-types, did not prevent $[\text{Ca}^{2+}]_i$ elevation after activation of the UTP receptors (Fig. 6D).

A possible pathway of UTP receptor signaling leading to an increase in $[Ca^{2+}]_i$ is activation of phospholipase C (PLC), which mediates inositol 1,4,5-trisphosphate (IP_3) production and calcium release from IP_3 receptors (Puceat and Vassort 1996). Figures 6E and 6F show that application of 2 $\mu\text{mol/L}$ U73122 (an inhibitor of PLC activity) or 50 $\mu\text{mol/L}$ 2-aminoethoxydiphenyl borate (2APB) (an IP_3 receptor inhibitor) abolished Ca^{2+} elevation after activation of UTP receptors.

To correlate the $[Ca^{2+}]_i$ elevation after UTP treatment to the cardioprotective effect, we treated the cells with 2 $\mu\text{mol/L}$ U73122 or with 50 $\mu\text{mol/L}$ 2APB before UTP and hypoxia. LDH levels were measured after the hypoxia and revealed that neither of the 2 inhibitors diminished the protective effects produced by UTP. Other evidence to support the exclusion of $[Ca^{2+}]_i$ elevation involvement in these protective effects is that UDP and uridine also caused $[Ca^{2+}]_i$ elevation but not cardioprotection (measured by LDH) (data not shown).

Activation of mitogen-activated protein kinases after UTP treatment—It is well established that activation of various survival kinases can protect cardiomyocytes against stress conditions after pharmacologic preconditioning (Hausenloy and Yellon 2006; Zaugg and Schaub 2003). It was previously reported that UTP activates Akt and ERK cascades in neural cells (Arthur et al. 2006) and Akt in renal mesangial cells (Huwiler et al. 2002), which may promote cell survival.

To investigate the signal transduction pathway of UTP that protected cardiomyocytes against hypoxic stress, we examined whether UTP treatment before the hypoxic conditions induces a change in mitogen-activated protein kinase (MAPK) activation (i.e., Akt and ERK1/2). Therefore, we treated the cells with 50 $\mu\text{mol/L}$ UTP for 1–40 min. MAPKs activation occur via phosphorylation of specific amino acids, which were detected by Western blotting using specific antibodies. As shown in Fig. 7A, UTP caused an immediate and significant increase in the ERK phosphorylation level. UTP increased ERK phosphorylation in a time-dependent manner, starting after 1 min and lasting over 40 min, with peak activation at 5 min (680% relative to control level). Upon treatment with 10 $\mu\text{mol/L}$ U0126 (a highly selective inhibitor of both MEK1 and MEK2), which blocks the ERK pathway, the UTP-induced ERK phosphorylation was completely abolished (2% compared with control level) (Fig. 7B).

We also found that UTP induced Akt phosphorylation in a time-dependent manner, which started after 2 min and lasted over 20 min, with peak activation occurring at 5 min. Blocking this phosphorylation with LY294002 (a specific phosphatidylinositol 3 (PI3)-kinase inhibitor, 30 $\mu\text{mol/L}$) abolished UTP-induced Akt phosphorylation (data not shown).

Next, we examined whether UTP-induced ERK and Akt phosphorylation is related to the cardioprotection against hypoxic stress produced by UTP. Therefore, we treated cultured cardiomyocytes with 10 $\mu\text{mol/L}$ U0126 and 30 $\mu\text{mol/L}$ LY294002 for 15 min, and included 50 $\mu\text{mol/L}$ UTP for another 15 min before hypoxia. As shown in Fig. 8, U0126 and LY294002 treatment did not abolish the protective effect induced by UTP (5.5% and 7.6% vs. hypoxia alone). In addition, U0126 and LY294002 in hypoxic conditions had no protective effect like that induced by UTP, as revealed by LDH release compared with hypoxic conditions alone. These data indicate that ERK and Akt activation induced by UTP is not related to the cardioprotection.

In vivo results

Pretreatment with UTP reduces ischemic damage—To investigate the effect of UTP on the infarcted hearts in an in vivo model, rats were treated with UTP (0.44 $\mu\text{g/kg}$ i.v.) 30 min or 24 h before MI. The area at risk and the infarcted area at risk in the left

ventricle of rat hearts were measured 24 h after MI by using Evans blue and 2,3,5-triphenyltetrazolium chloride (TTC) staining, respectively. TTC solution stains functional mitochondria in red, while nonactivated mitochondria appear as white areas. As revealed in the results, the area at risk in the infarcted animals was similar in all groups, that is, untreated infarcted hearts and the UTP-injected group. The infarcted area (TTC unstained) within the area at risk, however, was markedly reduced compared with that of the control ($30.4\% \pm 2.6\%$) after UTP treatment 30 min or 24 h before MI ($10.9\% \pm 1.3\%$ and $11.1\% \pm 3.1\%$, respectively, $p < 0.001$) (Fig. 9).

UTP preserves ATP levels in LAD-ligated hearts—To evaluate the cytosolic ATP levels in the LAD-ligated hearts, we harvested the risky myocardial area. The cell extract was used to measure ATP content with the luciferin–luciferase bioluminescence kit. The in vivo experiments were consistent with results obtained in the cultured cardiomyocytes (Fig. 3), demonstrating that pretreatment with UTP partially prevented the dramatic decrease in intracellular ATP. The ATP level in the infarcted area of the rat heart post-MI was significantly lower than that in sham animals (0.53 ± 0.43 vs. 9.62 ± 2.41 nmol/mg). In the UTP-treated group, the ATP level in the damaged area was higher than that in the untreated MI rats (2.86 ± 1.01 nmol/mg) (Fig. 10).

UTP preserves mitochondrial complex activity in LAD-occluded rat hearts—To investigate the effects of UTP on mitochondrial complex activity in LAD-occluded rat hearts, sections of 10 μm from infarcted hearts, which were pretreated with UTP 30 min before the LAD occlusion, were frozen and mitochondrial complexes were stained for NADH:ubiquinone oxidoreductase (complex I), SDH (complex II), or COX (complex IV). The microscopic observation of mitochondria in the infarcted area showed significant damage at the mitochondrial I and II complexes, which was visualized as white unstained areas (Fig. 11) (staining results for complex I are not shown). However, pretreatment with UTP decreased the injury dramatically by maintaining their normal activity (as indicated by areas of blue staining). In addition, the efficacy of UTP treatment in mitochondrial protection in infarcted hearts was observed using COX staining. In slices of the infarcted hearts, large unstained areas were seen, but sham-operated animals and UTP-treated hearts exhibited normal COX activity, which appeared as a deep yellow color, indicating an improvement in mitochondrial respiratory complex IV activity (Fig. 11B). For comparison, the protective effect of UTP on SDH activity in cultured cardiomyocytes subjected to hypoxic conditions is demonstrated (Fig. 11A).

Discussion

In this work we have demonstrated that UTP protected and significantly reduced cell death in cultured rat cardiomyocytes and in LAD-ligated hearts that displayed hypoxic–ischemic damage. This effect of UTP has been demonstrated by biochemical and morphologic criteria. The difference between UTP-treated and untreated cells that were exposed to hypoxic conditions was remarkable. In untreated cultured cardiomyocytes, loss of striations in myofibrils, high lysosomal activity, pyknotic nuclei, and edematous areas in the cytoplasm and around the nucleus were observed. In the in vivo model, untreated LAD-ligated hearts showed nonactive mitochondria as white areas by TTC staining. On the other hand, cells treated with UTP, even when treated 24 h before the hypoxic conditions, exhibited strands of well-organized striated myofibrils running in various directions, and the myocytes possessed 1 or 2 nuclei, which were very similar to those observed with normoxic conditions. The in vivo model showed partially maintained ATP levels, improved mitochondrial activity, and reduced infarct size in UTP-treated animals.

One of the initial stages during our exploration of the protective effect of UTP was the detection of UTP receptors on rat cultured cardiomyocytes. By using specific antibodies to P2Y₂ and P2Y₄ receptors we were able to show that these receptors are highly expressed in these cells. It is worth mentioning that P2Y₄ receptors could not be detected in adult myocytes (Hou et al. 1999).

Several researchers have reported that the P2Y₂ receptor is preferentially inhibited by suramin, whereas RB2 and PPADS are antagonists for P2Y₄ receptors (Ralevic and Burnstock 1998). Our results imply that despite its central physiologic role in cardiac cells, intracellular Ca²⁺ elevation is not one of the critical elements in the cell signaling pathway required for UTP-induced cardioprotection. It is possible that activation of at least 2 distinct receptors elicits different downstream signaling pathways. We conclude that the stimulation of the P2Y₂ receptor and possibly the P2Y₄ receptor by UTP in heart initiates signaling pathways that delay irreversible cardiomyocyte injury and protect myocytes against hypoxic damage, whereas stimulation of the P2Y₄ receptor causes [Ca²⁺]_i elevation, which is probably irrelevant to classic cardioprotection.

The mechanism by which UTP induces cardioprotection is not known. Yet UTP has been shown to activate P2Y receptors (i.e., P2Y₂ and P2Y₄) and this by itself provides an indication of its mode of action. Recent studies on UTP signaling have shown that these receptors are coupled through a G_{q/11} protein to the IP₃ pathway, which causes Ca²⁺ elevation (Podrasky et al. 1997). Using Ca²⁺-free medium, or the L-type Ca²⁺ channel blocker verapamil, we were able to show that in normoxic conditions, UTP elevated the [Ca²⁺]_i from internal sources. Nevertheless, this pathway of Ca²⁺ elevation is probably not involved in myocyte protection from hypoxia, since using different inhibitors of Ca²⁺ elevation (U73122, 2APB) did not hamper the protective effect. Another piece of evidence is that other uridine derivatives (UDP, uridine), although causing a transient elevation of [Ca²⁺]_i, did not protect the cells from hypoxia. Moreover, suramin, which reduced the cardioprotective effect of UTP, had no influence on [Ca²⁺]_i elevation.

During ischemia, massive amounts of adenosine, UTP, and other nucleotides are released in the cardiovascular system. The adenosine receptors are thought to be the dominant players responsible for the induction of pharmacologic preconditioning, which enhances the resistance of the heart to ischemic conditions. One can suggest that both UTP and adenosine may act in a similar mode to promote cardiomyocyte survival under ischemic conditions. If this is the case, UTP may also contribute to the preconditioning effect. Indeed, pretreatment with UTP 24 h before exposing to hypoxic conditions led to reduced myocytes damage and preserved cell contractility. Like adenosine, the cardioprotective effect induced by UTP may be associated with protection of the mitochondrial respiratory chain, keeping ATP production level high by maintaining membrane potential and activating mitochondrial K_{ATP} channels. We also found that cardiomyocytes that were treated with 50 μmol/L UTP 30 min or 24 h before hypoxia demonstrated a prevention of mitochondrial calcium level increase under hypoxic conditions. This reduction may provide an excellent explanation for keeping ATP production level high by maintaining mitochondrial membrane potential (Yitzhaki et al. 2007).

It is well established that activation of different survival kinases can protect cardiomyocytes against stress conditions after pharmacologic preconditioning (Hausenloy and Yellon 2006; Zaugg and Schaub 2003). We found that UTP pre-treatment induced ERK and Akt phosphorylation. Blocking these pathways by U0126 and LY294002, respectively, had no effect on this protective effect. We will continue to investigate other survival kinases that may be activated by UTP pretreatment and may contribute in a cooperative way to the cardioprotection.

Finally, when thinking about therapeutic applications, targeting pyrimidinergic receptors for protection against ischemic myocardial damage has an advantage compared with targeting purinergic receptors. Unlike adenosine, the breakdown product of UTP, uridine, has limited ancillary pharmacologic effects (Vassort 2001). There is a general belief that ATP is the preferred agonist of P2 purinoceptors. At the same time, ATP is degraded to adenine nucleotides and nucleosides, so that the response of cardiomyocytes would be an integration of the various effects of these multiple compounds generated by the different surrounding tissues (Vassort 2001). UTP therefore may be used in preference to ATP as a P2Y₂ and P2Y₄ receptor agonist because it does not form cardiovascular active metabolites such as adenosine. Mazzola et al. (2008) confirmed this assumption by demonstrating different effects of these nucleotides on cardiomyocyte viability. Whereas exposure of the HL-1 cardiomyocytes to ATP induced apoptosis and necrosis, UTP counteracted the deleterious effects of ATP (Mazzola et al. 2008). Arthur et al. (2006) have shown that ATP causes a robust activation of phospholipase C via activation of G_q in newborn rat cardiomyocytes, and it would be expected to cause hypertrophic responses like other factors that active G_q-coupled receptors, such as α -adrenergic agonists, endothelin, and angiotensin II during long-term exposure (Pham et al. 2003). However, ATP was reported to be inhibitory to hypertrophic responses (Zheng et al. 1996). On the contrary, UTP, an alternative agonist to ATP at P2Y₂ receptors, was as effective as ATP in stimulating PLC and caused hypertrophic responses in rat cardiomyocytes (Pham et al. 2003). As we have shown in this study, short time exposure to UTP stimulates both a 'classic' and a persistent long-term mechanism of protection (second window of protection). The involvement of UTP receptors in mediating cardioprotection reflects the biological redundancy in life-saving signals of P2Y₂ and P2Y₄ purinoceptors, although the absence of selective antagonists restricts our understanding of these signal transduction pathways and the elucidation of the importance of the individual receptors.

In conclusion, therapeutic targeting of pyrimidinergic receptors for protection against ischemic myocardial damage may be more effective than targeting the purinergic receptors. The possible therapeutic application of such a potent and sustained effect in patients at risk of myocardial ischemic damage is therefore very promising and justifies further exploration.

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Fig. 1.

Concentration- and time-dependent effects of UTP on cultured cardiomyocytes subjected to hypoxia. (A) Six-day-old rat cardiomyocytes were treated for 15 min with various concentrations of UTP (3–50 $\mu\text{mol/L}$). The cells were then washed twice and subjected to hypoxia for 2 h in glucose-free PBS at 37 °C. The amount of lactate dehydrogenase (LDH) released to the medium was determined and compared with the total activity of control homogenate (100%). (B) Cardiomyocytes were treated for 15 min or 24 h with 50 $\mu\text{mol/L}$ UTP before hypoxia. Other groups of cells were incubated for 1 h with 50 $\mu\text{mol/L}$ UTP, washed and replaced in normal medium, and then subjected to hypoxia 24, 48, or 72 h subsequent to the treatment with UTP. Data are means of at least 3 replicates in 5 separate experiments $\pm$ SD. *, Significant at $p < 0.05$, **, $p < 0.01$ compared with hypoxia.

Fig. 2.

Effect of UTP on cardiomyocyte morphology under hypoxic conditions. Six-day-old rat cardiomyocytes cultured under normoxic conditions (A) were subjected to 2 h in an hypoxic environment (B) or pretreated with 50 $\mu\text{mol/L}$ UTP and then subjected to hypoxia (C). Damaged cells were increased by hypoxia, whereas activation of UTP receptors before hypoxia reduced the percentage of cell death. Cells in the left column were stained with propidium iodide, which marks damaged cells red (medium grey in the print version, red in the Web version), and Hoechst 33342, which stains live-cell nuclei blue (lighter grey in the print version). Cells in the middle column were stained with hematoxylin and eosin, and those in the right column were stained with neutral red, which stains lysosomes. The results shown are representative of 6 experiments. Scale bars represent 10 μm .

Fig. 3.

Effect of P2 receptor agonists and antagonists on intracellular ATP levels after hypoxia in vitro. Six-day-old rat cardiomyocytes were treated for 15 min with the P2 antagonists PPADS (10 $\mu\text{mol/L}$), RB2 (50 $\mu\text{mol/L}$), or suramin (300 $\mu\text{mol/L}$) before application of UTP (50 $\mu\text{mol/L}$) for 15 min. Intracellular ATP levels were measured after 2 h of hypoxia. Data are means of at least 3 replicates in 6 separate experiments $\pm$ SD. *, Significant at $p < 0.01$ compared with hypoxia; #, $p < 0.001$ compared with normoxia.

Fig. 4.

Effect of UTP on $[Ca^{2+}]_i$ accumulation during hypoxia. Effect of hypoxia alone (A) and 30 min (B) or 24 h (C) after UTP treatment for 15 min in 6-day-old rat cardiomyocytes in vitro. Calcium was monitored during hypoxia with fluorescent dye indo-1. The fluorescence ratio of 410/490 nm, which is proportional to changes in Ca^{2+} levels, is demonstrated. Each recording was made for 10 s at times 0, 20, 40, and 60 min after the initiation of hypoxia. Each experiment shown is representative of 5 separate experiments. (D) Basal levels of $[Ca^{2+}]_i$ of the 3 groups and a normoxic control as a function of time. Values are means $\pm$ SD. *, Significant at $p < 0.05$ UTP-treated (30 min) compared with hypoxia.

Fig. 5.

Effect of pretreatment with UTP on mitochondrial Ca²⁺ overload in cardiomyocytes subjected to hypoxia. Six-day-old rat cardiomyocytes were treated with 50 μ mol/L UTP 30 min or 24 h before 2 h of hypoxia. During hypoxia, cardiomyocytes were loaded with rhod-2 AM (warm after cold incubation), and Ca²⁺ level was determined. Values are means $\pm$ SD. *, Significant at $p < 0.001$ compared with control; #, $p < 0.001$ compared with hypoxia.

Fig. 6.

Effect of P2 antagonists on $[Ca^{2+}]_i$ response to UTP. Indo-1-loaded 6-day-old rat cardiomyocytes were treated with 50 $\mu\text{mol/L}$ UTP alone (A) or pretreated with the P2 antagonists PPADS (B), RB2 (C), or suramin (D) 15 min before UTP application. Other cells were pretreated before UTP with (E) IP_3 receptor inhibitor 2APB (50 $\mu\text{mol/L}$) or (F) PLC inhibitor U73122 (2 $\mu\text{mol/L}$). The fluorescence ratio of 410/490 nm, which is proportional to changes in Ca^{2+} levels, is demonstrated. Each experiment shown is representative of 7 separate experiments. IP_3 , inositol 1,4,5-trisphosphate; 2APB, 2-aminoethoxydiphenyl borate; PLC, phospholipase C.

Fig. 7. Time-dependent effects of UTP on ERK phosphorylation in 6-day-old rat cardiomyocytes. (A) Cells were treated with 50 $\mu\text{mol/L}$ UTP for the indicated time. (B) Cells were treated with 50 $\mu\text{mol/L}$ UTP for 5 min with or without 10 $\mu\text{mol/L}$ U0126, a selective MEK1/2 inhibitor. Cell lysates were analyzed by Western blotting using a phosphospecific ERK antibody (Tyr204). The same samples were also analyzed on a separate blot using an antibody that recognizes desmin (total) to confirm equal loading on each lane. The combined ratio results (lower panel), obtained from densitometric analysis of blots, are representative of 3 identical independent experiments. Values are a percentage of the basal level of ERK phosphorylation (100%).

Fig. 8.

Effect of ERK and Akt inhibitors on UTP-induced cardioprotection. Six-day-old rat cardiomyocytes were treated for 15 min with 10 $\mu\text{mol/L}$ U0126 (a selective inhibitor of MEK1/2) and 30 $\mu\text{mol/L}$ LY294002 (a selective inhibitor of PI3-kinase) before 50 $\mu\text{mol/L}$ UTP. The cells were then washed twice and subjected to hypoxia (Hyp) for 2 h in glucose-free PBS at 37 °C. The amount of LDH released to the medium was determined. The data shown are representative of one of 3 identical independent experiments $\pm$ SD. *, Significant at $p < 0.01$ compared with control; #, $p < 0.01$ compared with hypoxia. PI3-kinase, phosphatidylinositol 3-kinase.

Fig. 9.

Effect of pretreatment with UTP on myocardial infarct size after LAD ligation in 2–3-month-old rat hearts. The area of irreversible injury (TTC negative) is presented as a percentage of the area at risk (Evans blue stained area appears dark grey in the print version). Values are means $\pm$ SD. *, Significant at $p < 0.001$ vs. sham; #, $p < 0.01$ vs. MI. LAD, left anterior descending; TTC, 2,3,5-tri-phenyltetrazolium chloride; MI, myocardial infarct.

Fig. 10. Effect of UTP treatment on ATP levels in LAD-ligated hearts of 2–3-month-old rats. The ATP level in the infarct area is shown after the animals were injected with 0.44 $\mu\text{g/kg}$ UTP 30 min before MI. Values are means $\pm$ SD. *, Significant at $p < 0.001$ vs. sham; #, $p < 0.01$ vs. MI.

Fig. 11.

Light microscopic observations of rat mitochondrial complexes after UTP treatment and exposure to hypoxia–ischemia *in vitro* and myocardial infarction *in vivo*. Mitochondrial damage is visualized as unstained areas. (A) Six-day-old cultured cardiomyocytes exposed to an hypoxic condition (Hyp) with or without pretreatment with 50 $\mu\text{mol/L}$ UTP were fixed immediately after 2 h hypoxia and stained for SDH. The colored reaction product (diformazan) indicates SDH activity. The experiment shown is representative of 6 separate experiments. Scale bars represent 10 μm . (B) Left ventricular papillary muscle area of 2–3-month-old rat hearts, sham-operated or LAD-occluded (MI), with or without UTP pretreatment 30 min before MI. Slices were cryopreserved and stained with SDH and COX. The experiment shown is representative of 6 separate experiments. Scale bar represents 200 μm . SDH, succinate dehydrogenase; COX, cytochrome *c* oxidase.

Table 1

Effect of uracil derivatives or P2 receptor antagonists on cardiomyocytes subjected to hypoxia.

Treatment	LDH release, %
Normoxia	4.2±0.6
Hypoxia	40±2.4
Hypoxia + UTP	10±1.6 [*]
Hypoxia + UTP + PPADS	32±3.6
Hypoxia + UTP + suramin	25±3.5
Hypoxia + UTP + RB2	11±0.8 [*]

Note: Six-day-old rat cardiomyocytes were pretreated for 15 min with the P2 receptor antagonists PPADS (pyridoxal-5'-phosphate-6-azophenyl-2',4'-disulfonate) (10 µmol/L), reactive blue 2 (RB2) (50 µmol/L), or suramin (300 µmol/L) before UTP treatment for 15 min. The cells were then washed twice and subjected to hypoxia for 2 h in glucose-free PBS at 37 °C. The amount of lactate dehydrogenase (LDH) released to the medium was determined and compared with the total activity of control homogenate (100%). Data are means of at least 3 replicates in 3 separate experiments ± SD.

^{*} Significant at $p < 0.01$ compared with hypoxia.