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Extracellular nucleotide derivatives protect cardiomyocytes against hypoxic stress

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ABSTRACT

Rationale: Extracellular nucleotides have widespread effects and various cell responses. Whereas the effect of a purine nucleotide (ATP) and a pyrimidine nucleotide (UTP) on myocardial infarction has been examined, the role of different purine and pyrimidine nucleotides and nucleosides in cardioprotection against hypoxic stress has not been reported.
Objective: To investigate the role of purine and pyrimidine nucleotides and nucleosides in protective effects in cardiomyocytes subjected to hypoxia.
Methods and results: Rat cultured cardiomyocytes were treated with various extracellular nucleotides and nucleosides, before or during hypoxic stress. The results revealed that GTP or CTP exhibit cardioprotective ability, as revealed by lactate dehydrogenase (LDH) release, by propidium iodide (PI) staining, by cell morphology, and by preserved mitochondrial activity. Pretreatment with various P2 antagonists (suramin, RB-2, or PPADS) did not abolish the cardioprotective effect of the nucleotides. Moreover, $P2Y_2^{-/-}$, $P2Y_4^{-/-}$, and $P2Y_2^{-/-}/P2Y_4^{-/-}$ receptor knockouts mouse cardiomyocytes were significantly protected against hypoxic stress when treated with UTP. These results indicate that the protective effect is not mediated via those receptors. We found that a wide variety of triphosphate and diphosphate nucleotides (TTP, ITP, deoxyGTP, and GDP), provided significant cardioprotective effect. GMP, guanosine, and ribose phosphate provided no cardioprotective effect. Moreover, we observed that tri/di-phosphate alone assures cardioprotection. Treatment with extracellular nucleotides, or with tri/di-phosphate, administered under normoxic conditions or during hypoxic conditions, led to a decrease in reactive oxygen species production.
Conclusions: Extracellular tri/di-phosphates are apparently the molecule responsible for cardioprotection against hypoxic damage, probably by preventing free radicals formation.

1. Introduction

Heart diseases remain the leading cause of morbidity and mortality in humans. Considerable efforts have been devoted toward improving functional recovery and reducing the extent of infarction after ischemic episodes.

The heart possesses a remarkable ability to adapt to stress conditions by changing its characteristics in a manner that makes it more resistant to further damage. Cardiac preconditioning represents a potent method to render the myocardium more resistant to irreversible damage induced by a variety of harmful conditions. In ischemic preconditioning, the heart becomes significantly protected against prolonged ischemic injury when first subjected to brief ischemia [1]. Various pharmacological agents may elicit preconditioning-like effects in experimental animals [2]. Therefore, pharmacological preconditioning may provide a safer way than short ischemia for inducing cardioprotection in humans, particularly in diseased myocardium.

Purine and pyrimidine nucleotides have a wide spectrum of biological effects and specific extracellular signaling actions in many tissues and organs [3]. Once in the extracellular fluid,

Abbreviations: DCFH-DA, 2′,7′-dichlorofluorescein-diacetate; 4-Di-1-ASP = DASPMI, 2-(4-(dimethylamino)styryl)-1-methylpyridinium iodide; E-NPP, ecto-nucleotide pyro-phosphatase; LDH, lactate dehydrogenase; NDPs, nucleotide diphosphates; NMP, nucleotide monophosphate; NTPs, nucleotide triphosphates; RB-2, Reactive blue 2; PBS, phosphate buffer saline; PI, propidium iodide; PPADS, pyridoxal-phosphate-6-azophenyl-2,4-disulfonate; PPi, inorganic pyro-phosphate; RDB, a solution of proteolytic enzymes; ROS, reactive oxygen species; SDH, Succinate dehydrogenase; WT, wild type.

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nucleotides can activate various cell responses by different purinoceptors (P2 receptors). According to molecular evidence, P2 receptors are divided into two families: P2X ionotropic ligand-gated ion channel receptors, and P2Y metabotropic G protein-coupled receptors. Currently, seven human P2X receptor subunits ($P2X_{1-7}$) and eight P2Y receptor subtypes ($P2Y_{1, 2, 4, 6, 11, 12, 13, 14}$) have been identified [4,5].

It is well known that extracellular ATP and UTP play important roles in cell survival. It was reported that UTP improves post-ischemic outcomes in a P2-receptor dependent manner in ischemic-reperfused mouse heart [6]. Our laboratory has also defined the role of UTP pretreatment in cardioprotection following chemical and hypoxic stress [7–10].

Reactive oxygen species (ROS) are essential for life, due to their involvement in cell signaling. However, excessive production of ROS processes oxidative stress implicated in many pathological conditions, including cancer, diabetes, heart diseases and more [11]. Although H_2O_2 and $^{\bullet}O_2$ (which are mitochondrial byproducts) do not cause much direct damage to biological systems, in the presence of reduced transition metals, such as Fe^{2+}, the formation of the highly reactive $^{\bullet}OH$ can occur through the Fenton reaction [12,13]. Harmful $^{\bullet}OH$ radicals will attack almost any component in living organisms, including proteins, DNA, and lipids, at a reaction rate of 10^9–10^{10} M^{-1} s^{-1} [14]. The most significant results of ROS-induced cell damage appear to be altered membrane structure due to peroxidation of membrane phospholipids containing unsaturated fatty acids and impaired enzymatic activities due to oxidation of sulfhydryl groups in proteins. Eventually, abnormal handling of Ca^{2+} in plasma membranes, mitochondria, and sarcoplasmic reticulum results in intracellular Ca^{2+} overload, the major pathogenesis of myocardial injury [15].

Recently it was reported that nucleotides have metal ion-chelation and radical scavenging ability. Nucleotides can function in a concentration-dependent manner either as pro-oxidants or as antioxidants in the Fenton reaction, this being determined by the Fe^{2+} concentration [16]. The purine nucleotide molecule contains metal binding sites both at the phosphate chain and at the purine ring (N7), and may therefore be considered as a potential biocompatible metal chelator [17,18].

Based on this information, the aims of the present study were: (1) to investigate and characterize the protective capability of various extracellular nucleotides, purines and pyrimidines and some of their derivatives, in rat cultured cardiomyocytes subjected to hypoxic stress; (2) to investigate whether the effect is mediated via purinoceptors using common antagonists; (3) to investigate the protective effect of UTP in $P2Y_2^{-/-}$ or $P2Y_4^{-/-}$ receptors knockout of cultured mouse cardiomyocytes; and (4) to search for a possible mechanism which provides cardioprotection.

Our findings indicate a novel mechanism for cardioprotective activity in cultured cardiomyocytes subjected to hypoxia, by the tri/di-phosphates, the cleaved products of extracellular nucleotides. The tri/di-phosphate residues, which possibly act mainly as metal chelators, inhibit and prevent radical formation and oxidative damage, and hence, have potentially promising therapeutic ability against hypoxic stress.

2. Materials and methods

2.1. Cell culture

Sprague–Dawley rats or C57BL/6 mice hearts (2–3 days old) were removed under sterile conditions and washed three times in phosphate buffer saline (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., Nes Ziona, Israel], prepared from a fig tree extract [19]. 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 previously described [20].

2.2. Hypoxic conditions

Cultured cardiomyocytes (5–7 days old) were washed twice from the medium with glucose-free PBS containing 5 mmol/L HEPES at pH 7.4 before exposing the myocytes to the hypoxic conditions at 37 °C. The hypoxic conditions lasted for 120 min in a hypoxic incubator in which the atmosphere was replaced by the inert gas argon (100%) in glucose-free media [20,21]. The hypoxic damage was characterized at the end of the hypoxic period by biochemical evaluation.

2.3. Assay of intracellular ATP level

After hypoxia, control and experimental cells were harvested in 1 mL of cold 5% trichloroacetic acid. The cell extract was used for the measurement of ATP content with a luciferin–luciferase bioluminescence kit (CLSII, Boehringer, Mannheim, Germany) [22].

2.4. Experiments with purinergic and pyrimidinergic ligands

Purines and pyrimidines were applied to the cardiac culture cells during hypoxia, or for 30 min following a 30 min preincubation, before hypoxia, with various antagonists (Sigma Chemicals, St. Louis, MO, USA): pyridoxal-phosphate-6-azophenyl-2,4-disulfonate (PPADS), suramin, or reactive blue 2 (RB-2).

2.5. Release of lactate dehydrogenase (LDH)

LDH activity was determined as previously described [21,23]. Briefly, 25 μL supernatant was transferred into a 96-well plate, and the LDH activity was determined with an LDH-L kit (Thermo Electron, Melbourne, Australia). The product of the enzyme was measured spectrometrically at 30 °C at a wavelength of 340 nm. The results were expressed relative to the control, the results of which were given as 100%.

2.6. Propidium iodide (PI) and Hoechst staining

The assay is based on binding of propidium iodide (5 μmol/L) to nuclei of cells whose plasma membranes have become permeable because of cell damage. The assay was performed as previously described [20]. For measurement of the amount of total cells (damaged and undamaged), the cells were treated with 300 μmol/L digitonin. After 1 h the cells were washed and stained again with PI to evaluate the total number of the cells. For counterstaining we used Hoechst 33342 (10 μmol/L) for 15 min, which stains the nuclei of all cells. Evaluation of PI and Hoechst fluorescence was done by Tecan fluorimeter and by fluorescence microscopy at 540/630 and 350/461 nm, respectively.

2.7. Hematoxylin and eosin staining

After exposure to hypoxic conditions, cardiac cells were fixed with 100% methanol for 10 min. The fixed cells were washed twice with PBS and incubated with hematoxylin for 15 min. The stained cells were rinsed in running tap water, and stained with eosin for 15 min. The cells then were rinsed again in tap water, dehydrated and mounted on a Zeiss microscope.

2.8. Mitochondrial membrane potential measurement

The method was performed as previously described [24]. Briefly, living cells, grown on round coverslips, were exposed to 2-

(4-(dimethylamino)styryl)-1-methylpyridinium iodide (4-di-1-ASP = DASPMI), dissolved in PBS (10 μmol/L) for 15 min. The coverslips were washed and mounted on chambers containing dye-free PBS + glucose. The fluorescence intensity was examined under fluorescence microscopy at 460/540 nm. Image J software was used for quantifying DASPMI stain.

2.9. Succinate dehydrogenase (SDH) activity

SDH staining was performed using a cytochemical method, based on the reduction of nitro-blue tetrazolium to diformazan. Cell culture plates were washed in PBS and incubated with PBS containing 67.5 mg/ml succinate and 1 mg/mL nitro-blue tetrazolium for 2 h at 37 °C. Cardiomyocytes were fixed with 4% formaldehyde for 10 min and cover-slipped with glycerol for light microscopy [25]. Image J software was used for quantifying SDH stained cells by measuring the color intensity of five randomly selected field from each tested group.

2.10. ROS assay

Cardiac cells were seeded in 96-well black tissue culture micro-plates. The cells received 2′,7′-dichlorofluorescein-diacetate (DCFH-DA) (2 μmol/L) for 20 min at 37 °C. After removal of the DCFH-DA, the nucleotides were added to the cells. The micro-plate was incubated at 37 °C for 1 h, during which the fluorescence was measured by Tecan fluorometer at 485/530 nm [26].

2.11. Mouse genomic DNA isolation and PCR

Isolation of genomic DNA and subsequent PCR was performed as previously described [27,28]. 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 vortexed for 30 min. For $P2Y_2$ genotyping, a triple primer strategy was performed. The two different forward primers had the following sequence: $P2Y_2$-f1: 5′-CTCACGCGCACCCTCTACTA-3′ (identifying the WT allele) and Pgk-left1: 5′-GGGGAACTTCCTGACTAGGG-3′ (identifying the inserted neo-cassette in the disrupted allele) the reverse primer for both alleles had the following sequence: $P2Y_2$-r1: 5′-GTCGGGTGCACTGCCTTTCT-3′. The amplicon resulting from the amplification of the WT segment was 551 bp long, and the amplicon for the knockout region was ~700 bp. For genotyping of $P2Y_4$ mice, two genomic PCRs were performed. The wild type allele was detected using the forward primer 5′-AGTAGAGGTTCCAGTAGAAA-3′, while the mutated allele was identified with the forward and reverse primers 5′-CGAAGTTATATTAAGGGTTC-3′ and 5′-TAATCGGTCACCCTCA-3′, respectively. The amplicon resulting from amplification of the WT segment was 337 bp long, and the amplicon corresponding to the knockout region was ~750 bp. For genotyping of $P2Y_2^{-/-}$/$P2Y_4^{-/-}$ double receptor knockout mice, the same previously described primers and methods were used. The knockouts were verified genetically and functionally (unpublished data).

2.12. Statistics

Results were expressed as mean ± S.D. Data were analyzed by analysis of variance with application of a post hoc Tukey–Kramer test. Statistical significance was determined as $^{*,\#}P < 0.01$ and $^{**}P < 0.05$ in different experiments.

2.13. Chemicals

DCFH-DA, DASPMI, propidium iodide and Hoechst 33342 were acquired from Invitrogen (Eugene, OR, USA). The primers were purchased from IDT (Integrated DNA Technologies, Coralvile, IA. USA). Other reagents were purchased from Sigma-Chemicals (St. Louis, MO, USA).

3. Results

3.1. Protective effect of extracellular nucleotides on cardiomyocytes subjected to hypoxia

UTP, as an extracellular nucleotide, has protective effects against hypoxic damage [8,10]. In order to evaluate the ability of other purine and pyrimidine nucleotides to attenuate cardiomyocyte injury following hypoxia, cultured rat cardiomyocytes were treated with extracellular GTP or CTP (0.5–50 μmol/L), and subjected to 2 h of hypoxic conditions in the presence of nucleotides. To evaluate cell damage, LDH levels in the medium were measured immediately after hypoxia. The results presented in Fig. 1A show that hypoxic untreated cells were significantly damaged, as revealed by LDH released (313.1 ± 16.1%) relative to the normoxic cells. Treatment with GTP or CTP brought about a significant attenuation of the cellular injury produced by hypoxia. Treatment with the nucleotides reduced LDH release in a dose dependent manner. LDH levels decreased from 313.1 ± 16.1% under hypoxic conditions to 107.2 ± 18.9% and 103.4 ± 16.5% following GTP treatment (15 and 50 μmol/L, respectively), and to 113.6 ± 13.5% and 101.9 ± 18.8% following CTP treatment (15 and 50 μmol/L, respec-

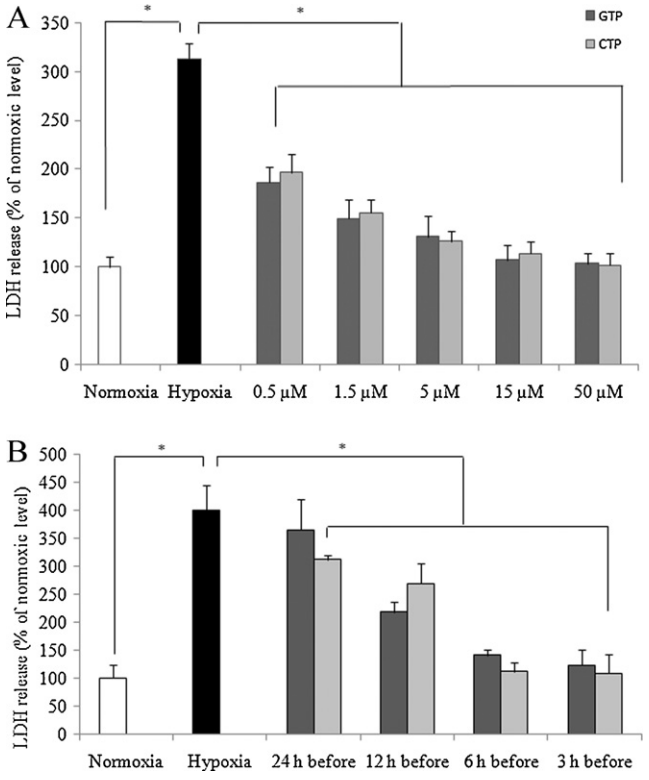

Fig. 1. Concentration and time-dependent effect of GTP and CTP on cardiomyocytes subjected to hypoxia. (A) Rat cultured cardiomyocytes were washed twice with glucose-free PBS, and incubated with various concentrations of GTP or CTP (0.5-50 μmol/L) during 2 h of hypoxic stress at 37 °C. (B) Cardiomyocytes were treated with 15 μmol/L GTP or CTP for 30 min, washed and incubated again in complete medium for 3, 6, 12, and 24 h before washing them twice with glucose-free PBS and exposing them to hypoxia. In both cases (A and B) the amount of LDH released to the extracellular fluid was determined and compared to the total activity of control. Value of 100% was given to the level of LDH released from untreated cells washed in glucose-free PBS but not subjected to hypoxia. Data are means of at least three replicates in five separate experiments ± S.D. $^{*}P < 0.01$.

tively). At lower nucleotide concentrations (below 15 μmol/L), LDH release was only partially decreased, indicating lower cardioprotective ability. Extracellular nucleotides (50 μmol/L) did not cause cell damage when given under normoxic conditions (data not shown). When the cells were treated with the nucleotides GTP or CTP (15 μmol/L) for 30 min under normoxic conditions, and removed before being subjected to hypoxia, a decrease in LDH release was also obtained (data not shown). These results demonstrate that treatment of cardiomyocytes with extracellular nucleotides (GTP or CTP), protected the cells from hypoxic damage.

3.2. Prolonged protection by nucleotides of cardiomyocytes subjected to hypoxia.

In order to evaluate the time dependence of cardioprotection against hypoxic stress by the nucleotides, cardiomyocytes were pretreated for 30 min with GTP or CTP (15 μmol/L) and then washed out – 24, 12, 6, and 3 h before initiation of hypoxic conditions. As revealed by LDH release in Fig. 1B, when the cells were pretreated shortly before the hypoxic stress, enhanced cardioprotective effect was obtained: LDH released following hypoxia 6 and 3 h after pretreatment with the indicated nucleotides: GTP (140.8 ± 17.6% and 123.5 ± 10.9%, respectively) or CTP (112.2 ± 14.9% and 108.9 ± 33.1%, respectively), was similar to the normoxic LDH level (100%), in contrast to the hypoxic untreated cells (400.2 ± 45.7%). On the other hand, when pretreatment was farther in time (24 and 12 h) from the hypoxic stress, lower cardioprotection was obtained for GTP (366.1 ± 53.7% and 218.5 ± 17.6% respectively) and for CTP (312.2 ± 7.1% and 269.9 ± 35%, respectively), similar to the hypoxic untreated cells. These results establish that extracellular nucleotides probably activate mechanism that lasts up to about 6 h.

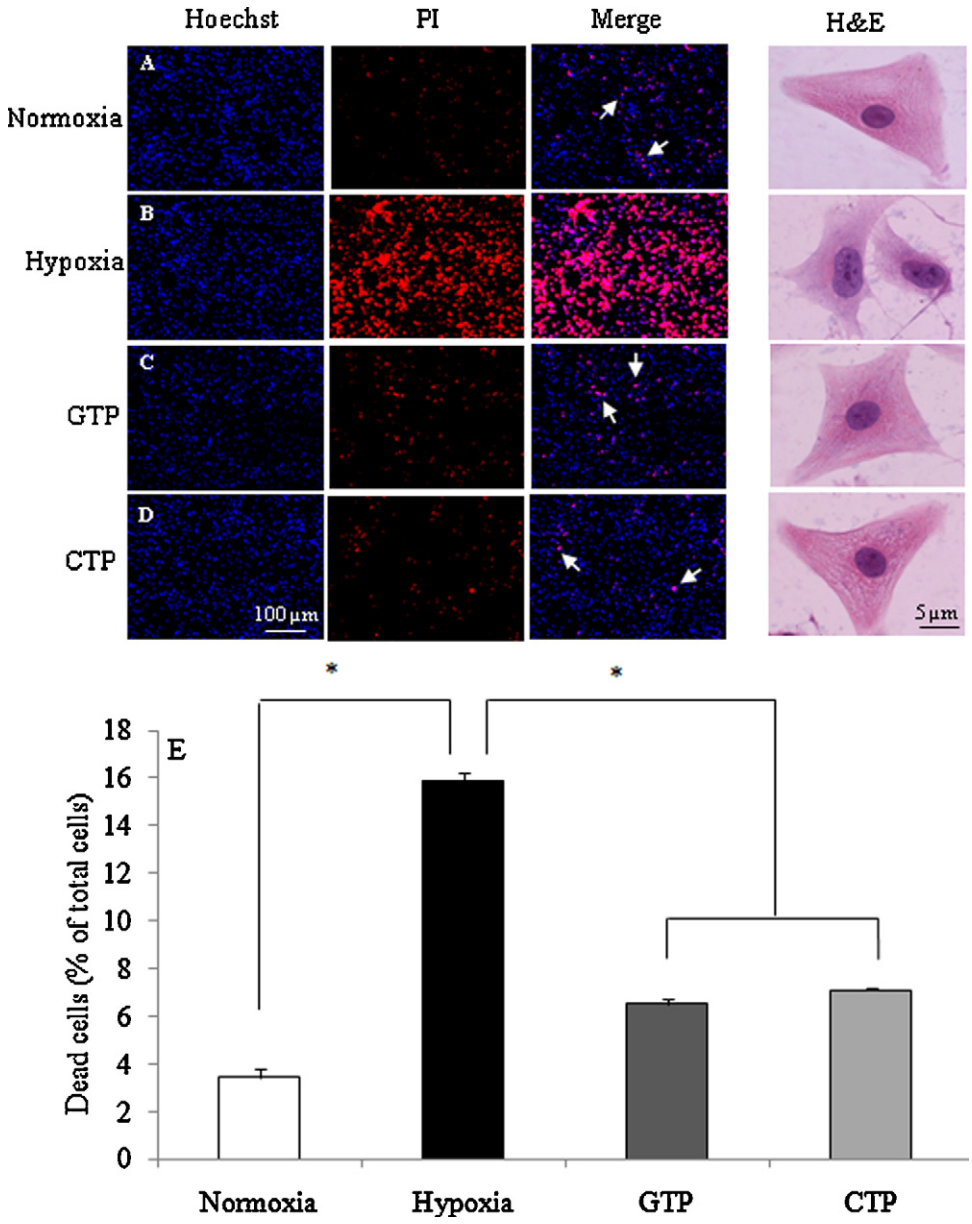

Fig. 2. Viability and morphology of GTP- or CTP-treated cardiomyocytes subjected to hypoxia. Cultured cardiomyocytes were subjected to 2 h hypoxia with or without 15 μmol/L GTP or CTP treatment. One group of cells was stained with Hoechst 33342 (blue), which stains live-cell nuclei (left panel, first column) and with propidium iodide (red), which marks damaged cells (left panel, second column). Arrow demonstrates some dead cells observed. A second group of cells was stained with hematoxylin and eosin (A–D, right panel). Quantitative processing of cell viability was determined by spectrophotometer (E). The qualitative results shown (A–D) are representative of three experiments. The quantitative results (E) are means of five separate experiments ± S.D. $^{*}P < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.3. Cardiomyocyte viability and morphology following nucleotide treatment and hypoxia

In order to characterize the protective effect of extracellular nucleotides against hypoxic damage, cell viability and morphology were assayed. For this purpose, rat cultured cardiomyocytes were washed twice with glucose-free PBS and exposed to hypoxia with or without 15 μmol/L GTP or CTP. Two hours later, cells were stained with propidium iodide (PI) and with Hoechst 33342. Cell loss was presented as the number of dead cells (propidium iodide stained) relative to the total number of cells (Hoechst 33342 stained). Two hours of hypoxia of untreated cardiomyocytes caused cell loss of 15.8 ± 0.31%, whereas cell loss under normoxic conditions was 3.42 ± 0.33%. On the other hand, GTP or CTP treatment during hypoxia attenuated cell loss caused by hypoxia to 6.5 ± 0.23% and 7 ± 0.13% (Fig. 2E).

The protective effect of the nucleotides was also demonstrated by morphology. Following 2 h of hypoxia, the cells were stained with hematoxylin and eosin. The results demonstrate typical irreversible oncotic damage of untreated cardiomyocytes subjected to hypoxia: vacuoles and asymmetry of the myofilaments, and edematous areas in the cytoplasm and around the nucleus (Fig. 2B, right panel). GTP or CTP treatment during the hypoxic conditions maintained symmetry of the myofilaments, a globular nucleus, and a clear border of the cell membrane, similar to the normoxic cells (Fig. 2C and D, right panel). The results in Fig. 2 show that GTP and CTP have significant ability to maintain cell viability under hypoxic conditions.

3.4. Extracellular nucleotides preserve mitochondrial activity

In order to evaluate mitochondrial function following hypoxic stress with or without GTP or CTP treatment, rat cultured cardiomyocytes were stained with DASPMI to estimate mitochondrial membrane potential. Fig. 3 demonstrates that following hypoxic conditions, untreated hypoxic cardiomyocytes exhibited low mitochondrial membrane potential (20 ± 2.2%), showing dim yellow fluorescence mainly around the picnotic nucleus, (Fig. 3B and A, respectively, left panel). Hypoxic cells treated with GTP or CTP (15 μmol/L) exhibited normal mitochondrial membrane potential (92.2 ± 4.9% and 90.8 ± 6.3%, respectively) with bright yellow fluorescence, which was distributed in the cytosol (Fig. 3C and D, left panel), similar to the normoxic cells (100%) (Fig. 3A, left panel).

To further characterize mitochondrial function, we stained and evaluated SDH activity. Untreated cardiomyocytes exposed to hypoxia demonstrated low SDH activity (38.3 ± 3.2%) (Fig. 3B and A, respectively, right panel). GTP- or CTP-treated cells exhibited normal SDH activity (86.9 ± 5.5% and 87.6 ± 8.1%, respectively), similar to normoxic cells (100%) (Fig. 3C and D, respectively, right panel) as was quantified by Image J software that was used for measuring the color intensity of the cells. We also examined ATP levels after hypoxic stress. Fig. 3E demonstrates that GTP or CTP in hypoxic cells maintained normal ATP levels (15.5 ± 0.8 and 11.8 ± 1.8 nmol/L mg protein^{-1}, respectively), in contrast with the untreated hypoxic cells (3.9 ± 0.8 nmol/L mg protein^{-1}). These results demonstrate that cardiomyocytes, which were treated with nucleotides (GTP or CTP) during hypoxic conditions, maintained mitochondrial function and preserved ATP levels, which is crucial for cell viability.

3.5. Effect of purinergic receptor antagonists on cardioprotection mediated by nucleotides

In order to verify whether the protective effect is mediated via P2 receptors, cardiomyocytes were treated with the P2 receptor antagonists; suramin, PPADS, or RB-2. After 30 min with the antagonists, 15 μmol/L GTP or CTP were added. 30 min later, the cells were washed twice in glucose-free PBS and subjected to hypoxic conditions. The results summarized in Table 1 demonstrate high LDH release to the medium after hypoxic conditions (457.5 ± 47.6%) vs. the LDH released in normoxic conditions (100%). Cells which were treated with P2 antagonists – 300 μmol/L suramin, 10 μmol/L PPADS, or 15 μmol/L RB-2 – showed similarly elevated LDH release (463.3 ± 65.9%, 502.2 ± 42%, and 438 ± 45.5%, respectively), relative to untreated hypoxic cells (457.5 ± 47.6%). On the other hand, GTP- or CTP-pretreated hypoxic cells showed dramatically lower LDH release (213.7 ± 30.2% and 219.9 ± 50.5%, respectively). LDH levels in cells which were pretreated with the antagonist suramin and GTP or CTP (212.2 ± 47.9% and 203.7 ± 45.9%, respectively), PPADS and GTP or CTP (195.6 ± 19.5% and 175.2 ± 24.8%, respectively), and with RB-2 and GTP or CTP (188.4 ± 16.5% and 232.6 ± 37.1%, respectively), were close to the LDH levels in the GTP-

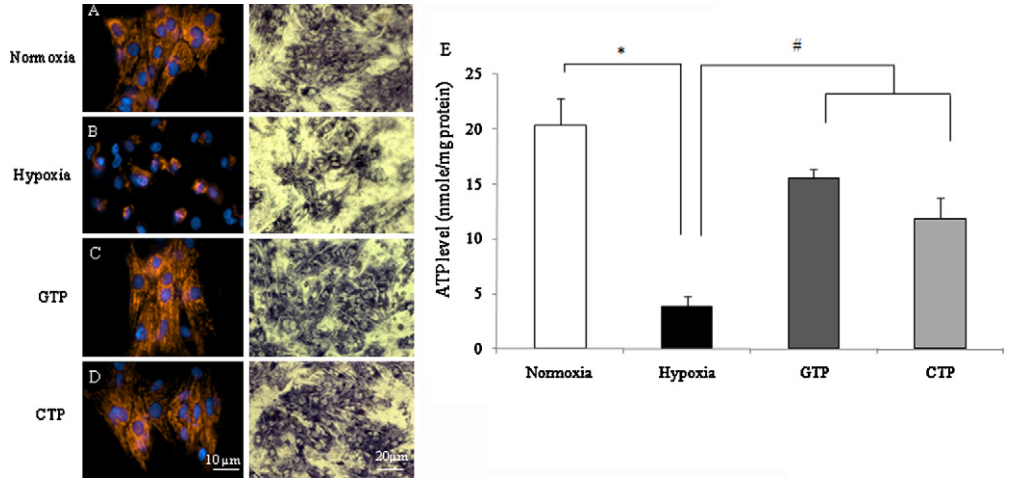

Fig. 3. Mitochondrial function of nucleotide-treated cardiomyocytes subjected to hypoxia. (A) Rat cultured cardiomyocytes were subjected to 2 h hypoxic conditions with or without 15 μmol/L GTP or CTP. The cells were stained with DASPMI, and with Hoechst 33342 (blue) (A–D left column). (B) Cells were stained for SDH activity (A–D right column). (E) Cells were homogenized and used for the measurement of ATP content. The results shown are representative of five experiments. $^{*}P < 0.01$ relative to normoxia, $^{\#}P < 0.01$ relative to hypoxia. DASPMI bar = 10 μm, SDH bar = 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 1
Effect of P2 antagonists on protection by extracellular nucleotides of cardiomyocytes subjected to hypoxia.

Treatment	LDH (% of normoxic level)
Normoxia	100
Hypoxia	457.5 ± 47.6*
Hypoxia + suramin	463.3 ± 65.9*
Hypoxia + PPADS	502.2 ± 42*
Hypoxia + RB-2	438 ± 45.5*
Hypoxia + GTP	213.7 ± 30.2#
Hypoxia + CTP	219.9 ± 50.5#
Hypoxia + suramin + GTP	212.2 ± 47.9#
Hypoxia + suramin + CTP	203.7 ± 45.9#
Hypoxia + PPADS + GTP	195.6 ± 19.5#
Hypoxia + PPADS + CTP	175.2 ± 24.8#
Hypoxia + RB-2 + GTP	188.4 ± 16.5#
Hypoxia + RB-2 + CTP	232.6 ± 37.1#

Rat cultured cardiomyocytes were treated with suramin (300 μmol/L), PPADS (10 μmol/L), or RB-2 (15 μmol/L) 30 min before adding GTP or CTP. The cells were then subjected to hypoxia for 2 h. Data are means of at least three replicates in five separate experiments ± S.D.
* $P < 0.01$ compared with normoxia.
# $P < 0.01$ compared with hypoxia.

or CTP-treated hypoxic cells. Under hypoxic conditions, the presence of the antagonists caused extra damage relative to hypoxia alone, therefore we tested them only in a pretreatment manner. Although the antagonists we used are not receptor-specific, these results suggest that the protective effect is probably not mediated via P2 receptors.

3.6. Cardioprotection by UTP in $P2Y_2^{-/-}$, $P2Y_4^{-/-}$, and $P2Y_2^{-/-}/P2Y_4^{-/-}$ receptor knockout cultured mouse cells subjected to hypoxia

It is well establish that UTP has a potent affinity to $P2Y_2$ and $P2Y_4$ receptors [29]. Our laboratory has previously shown that rat cardiomyocytes express $P2Y_2$ and $P2Y_4$ receptors [10]. In order to verify the involvement of these receptors in mediating cardioprotection against hypoxia, cultured mouse cardiomyocytes which were $P2Y_2^{-/-}$, $P2Y_4^{-/-}$, and $P2Y_2^{-/-}/P2Y_4^{-/-}$ receptor knockouts were used, in comparison to cardiomyocytes from WT mouse. The cells were washed twice from the medium with glucose-free PBS, treated with UTP (15 μmol/L), and subjected to 2 h of hypoxia, so that UTP was present during hypoxia. We also compared the cardioprotection of 30 min UTP pretreatment prior to the hypoxic conditions, wherein UTP was not present during the stress. In both cases we obtained similar result, described in Fig. 4A: UTP brought about cardioprotection, as measured by LDH release, in WT cells (283 ± 36.4% in hypoxic UTP-treated cells vs. 723.6 ± 12.1% in hypoxic untreated cells). A similar effect was obtained in $P2Y_2^{-/-}$ receptor knockout cells (114.4 ± 17.1% in hypoxic UTP-treated cells vs. 371.3 ± 12% in hypoxic untreated cells), in $P2Y_4^{-/-}$ receptor knockout cells (377.7 ± 41.6% in hypoxic UTP-treated cells vs. 987.8 ± 36.2% in hypoxic untreated cells), and in $P2Y_2^{-/-}/P2Y_4^{-/-}$

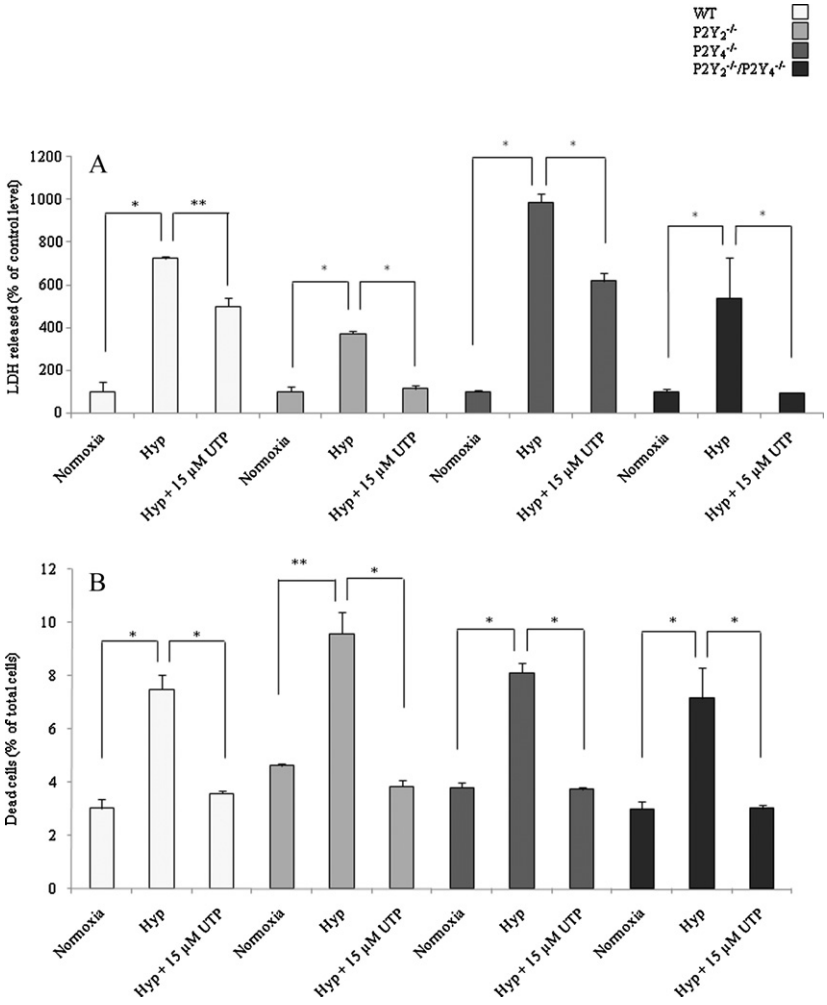

Fig. 4. Effect of extracellular UTP on cardioprotection in $P2Y_2^{-/-}$, $P2Y_4^{-/-}$, and $P2Y2_2^{-/-}/P2Y_4^{-/-}$ receptor knockouts subjected to hypoxia. (A) Cultured cardiomyocytes of WT, $P2Y_2^{-/-}$, $P2Y_4^{-/-}$, and $P2Y2_2^{-/-}/P2Y_4^{-/-}$ receptor knockouts mice treated with UTP followed by hypoxia. The amount of LDH released to the extracellular fluid was determined and compared to the total activity of control. (B) The same mice groups were stained with PI and cell viability was determined. Data are means of at least three replicates in three separate experiments ± S.D. $^{*}P < 0.01$, $^{**}P < 0.05$.

double receptor knockout cells (96 ± 2.4% in hypoxic UTP-treated cells vs. 537.2 ± 93.3% in hypoxic untreated cells). The amount of dead cells was also determined by PI staining. As presented in Fig. 4B, all groups had a significant cardioprotective effect: UTP led to reduction of cell death in WT cells (3.0 ± 0.34% in hypoxic UTP-treated cells vs. 7.5 ± 0.52% in hypoxic untreated cells), in $P2Y_2^{-/-}$ receptor knockout cells (3.86 ± 0.23% in hypoxic UTP-treated cells vs. 9.59 ± 0.81% in hypoxic untreated cells), in $P2Y_4^{-/-}$ receptor knockout cells (3.75 ± 0.07% in hypoxic UTP-treated cells vs. 8.11 ± 0.36% in hypoxic untreated cells), and in $P2Y_2^{-/-}/P2Y_4^{-/-}$ double receptor knockout cells (3.04 ± 0.12% in hypoxic UTP-treated cells vs. 7.16 ± 1.14% in hypoxic untreated cells).

These results provide evidence that in mouse cultured cardiomyocytes, the protective effect mediated by UTP is probably not taking place via $P2Y_2$ and/or $P2Y_4$ receptors. Our hypothesis is that all the nucleotides mentioned above, in providing cardioprotection, are acting via a shared mechanism, which probably does not involve P2 receptors.

3.7. Extracellular nucleotide derivatives protect cardiomyocytes subjected to hypoxia

The effect of various purine and pyrimidine nucleotide analogs and derivatives on cardioprotection against hypoxic stress was investigated. We used various derivatives, which included: triphosphate nucleotides (TTP and ITP), a triphosphate deoxy-nucleotide (dGTP), a diphosphate nucleotide (GDP), a monophosphate nucleotide (GMP), and a nucleoside (guanosine). In order to examine the protective effect, cardiomyocytes were incubated under hypoxic conditions with or without the derivatives mentioned above (15–150 μmol/L). After hypoxia, the LDH level was determined to evaluate cell damage. Table 2 demonstrates protective effect by lowered LDH release, relative to hypoxic untreated cells (352.1 ± 3.7%), if the cells were treated with 15 μmol/L TTP or ITP (142 ± 36.6% and 141.5 ± 34.4%, respectively). A similar protective effect was obtained even if the cells were pretreated with 15 μmol/L dGTP (137.7 ± 22.4%). Moreover, the cells were protected, although to quite a lesser extent, if they were treated with 50 μmol/L GDP (179.9 ± 28.9%). A protective effect was not observed with either GMP (365.4 ± 21.1%) or guanosine (351.1 ± 23.4%).

The role of the ribose and the phosphate moieties of the nucleotides in cardioprotection was investigated. As demonstrated in Table 2, ribose phosphate did not provide cardioprotection (373.5 ± 15%). Interestingly, 15 μmol/L triphosphate or diphosphate (pyrophosphate), given before or during the hypoxia, led to remarkably significant cardioprotection (102.3 ± 12.8% and 109.1 ± 26.4%, respectively), even better than the triphosphate nucleotides mentioned above. A dose response of tri/di-phosphate exposure prior to hypoxia appears in Fig. 5. The most effective doses for providing cardioprotection were 5–50 μmol/L for both triphosphate and diphosphate.

These results demonstrate that the protection against hypoxic stress can be achieved if the cells are treated with any triphosphate nucleotide, and in a somewhat less preferred manner—with the diphosphate nucleotide forms. Moreover, superior cardioprotection can also be obtained with tri/di-phosphate *per se*.

3.8. Effect of extracellular nucleotides on ROS concentration

In order to investigate the cardioprotective mechanism, ROS levels after hypoxic stress were determined. Rat cultured cardiomyocytes were washed twice with glucose-free PBS and treated with 15 μmol/L GTP, 50 μmol/L GDP, 15 μmol/L pyrophosphate, and 15 μmol/L triphosphate, followed by hypoxia. Then, the cells were loaded with DCFH-DA in order to measure ROS concentrations. Fig. 6A demonstrates high ROS levels in hypoxic untreated cells relative to 2 h normoxic incubated cells (162.7 ± 19.3% and 100%, respectively). Treatment with the tri/di-phosphates mentioned above provided a significant reduction in ROS levels as follows: for GTP – 108.2 ± 15%, for GDP – 121.6 ± 20.2%, for pyrophosphate – 101.8 ± 15.1%, for triphosphate – 103.5 ± 8.3%.

In order to investigate the antioxidative ability of extracellular nucleotides and tri/di-phosphates, rat cultured cardiomyocytes were loaded with DCFH-DA. After 20 min, the cells were washed and treated with 15 μmol/L GTP, 50 μmol/L GDP, 15 μmol/L pyrophosphate, and 15 μmol/L triphosphate. Every 20 min under normoxic conditions, ROS levels were evaluated by fluorescence measurements. As demonstrated in Fig. 6B, 20 min after the above-mentioned treatments, we noticed a significant attenuation in fluorescence intensity, which indicated an attenuation of ROS production. This effect lasted, and even strengthened, as time progressed. After 100 min, the fluorescence values were 202.5 ± 19.9% for control untreated cells, 166.4 ± 6.1% for 15 μmol/L GTP, 178.2 ± 9.2% for 50 μmol/L GDP, 163.5 ± 12.1% for 15 μmol/L pyrophosphate, and 171.2 ± 23.1% for 15 μmol/L triphosphate, as compared to basal levels at time = 0 min (100%).

We hypothesize that the decrease in ROS concentration after the nucleotides or the tri/di-phosphates were applied probably protected the cells against hypoxic stress. Nucleotides, or to be more specific—their tri/di-phosphate moieties, apparently prevent free radical formation during hypoxia, thereby protecting cells from hypoxic damage.

Table 2
Protective effect of nucleotide derivatives and moieties on cardiomyocytes subjected to hypoxia.

Treatment	LDH release (% of normoxic level)
Normoxia	100
Hypoxia	352.1 ± 3.73%*
Hypoxia + TTP	142.9 ± 36.6%#
Hypoxia + ITP	141 ± 34.4%#
Hypoxia + dGTP	137.7 ± 22.4%#
Hypoxia + GDP	179.9 ± 28.9%**
Hypoxia + GMP	365.4 ± 21.1%
Hypoxia + guanosine	351.1 ± 23.4%
Hypoxia + ribose phosphate	373.5 ± 15%
Hypoxia + pyrophosphate	109.1 ± 26.4%#
Hypoxia + triphosphate	102.3 ± 12.8%#

Rat cultured cardiomyocytes were subjected to 2 h hypoxia, with or without 15–100 μmol/L of various nucleotides, nucleotide derivatives, and nucleotide moieties. Data are means of at least three replicates in five separate experiments ± S.D.
* $P < 0.01$ compared with normoxia.
# $P < 0.01$ compared with hypoxia.
** $P < 0.05$ compared with hypoxia.

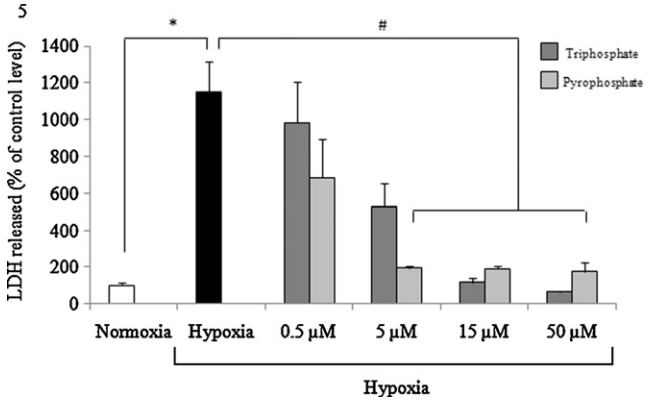

Fig. 5. The effect of various concentrations (0.5–50 μmol/L) of tri/di-phosphate, added during hypoxia, on cardioprotection. Data are means of at least three replicates in four separate experiments ± S.D. *$P < 0.01$ compared with normoxia, #$P < 0.01$ compared with hypoxia.

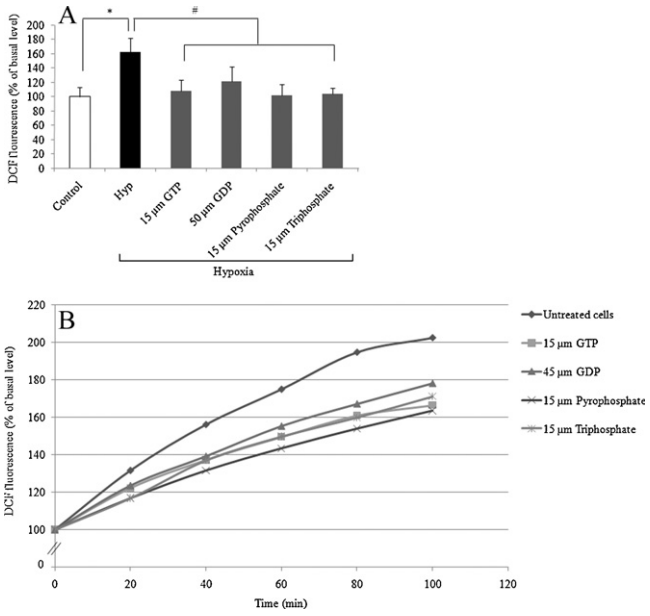

Fig. 6. Effect of nucleotides and their derivatives on ROS concentration. (A) Rat cultured cardiomyocytes were incubated in normoxic conditions or treated with GTP, GDP, pyrophosphate, and triphosphate, followed by hypoxia. The cells then were stained with DCFH-DA (20 μmol/L) for 20 min at 37 °C and washed twice, and the fluorescence at 485/530 nm was analyzed. (B) Rat cultured cardiomyocytes on microplates were loaded with DCFH-DA for 20 min at 37 °C. The cells then were washed twice and treated with GTP, GDP, pyrophosphate, and triphosphate, in normoxic conditions at 37 °C and the development of the fluorescence was measured at 485/530 nm. Value of 100% was given to fluorescence value at time = 0 for each treated cell. Data represent means of at least 8 replicates in five separate experiments ± S.D. $^{*}P < 0.01$ compared with normoxia, $^{\#}P < 0.01$ compared with hypoxia.

4. Discussion

Our work presents a novel strategy for cardiomyocyte protection against hypoxic stress by various extracellular nucleotides and nucleotide derivatives. GTP or CTP nucleotides exhibited a dose-dependent and time-dependent manner of cardioprotection. GTP- or CTP-treated hypoxic cells significantly reduced cell death, maintained cell morphology, and preserved mitochondrial membrane potential, SDH activity, and ATP levels.

Many studies have shown the protective effect of extracellular nucleotides (ATP/UTP, and their derivatives), which are mediated via P2 receptors [6,30–33]. The primary goal during our exploration was to investigate the receptors which mediate the cardioprotective response. Surprisingly, none of the antagonists (suramin, PPADS, or RB-2), abolished the protective effect induced by the nucleotides. We further used $P2Y_2^{-/-}$, $P2Y_4^{-/-}$, and $P2Y_2^{-/-}/P2Y_4^{-/-}$ receptor knockout mice, and examined the protective effect mediated by UTP, which is known for its powerful affinity to these receptors. Interestingly, LDH levels and PI staining after hypoxic stress indicate cardioprotection in all 3 knockout groups. It could be that UTP is degraded to UDP and this nucleotide is known to activate $P2Y_6$ receptor to give the protection obtained. However, this possibility is unlikely because we needed 50 μM of UDP (data not shown) compared to 15 μM of UTP required to get the protection. Furthermore, because pyrophosphate is sufficient to protect the cells, we think $P2Y_6$ is not involved in this protection.

Based on these findings, we provide evidence that the cardioprotective effect mediated by UTP, in mouse cultured cardiomyocytes, appears not to be mediated via $P2Y_2$, and/or $P2Y_4$ receptors. Our hypothesis is that all the nucleotides (including ATP) and their derivatives, are acting via a similar mechanism, which probably is not mediated via P2 receptors.

The cardioprotective capability of other nucleotides (TTP, ITP, dGTP, GDP, GMP) and some structurally related molecules (guanosine, ribose phosphate, pyrophosphate, and triphosphate), were evaluated. We report here on significant cardioprotection against hypoxic stress by all the tested triphosphate nucleotides. Furthermore, GDP provided significant cardioprotection, although with quite a lower efficacy than triphosphate nucleotides. On the other hand, GMP and ribose phosphate did not provide cardioprotection. Surprisingly, significant protection was obtained when the cell were treated with tri/di-phosphate, without a nucleobase or ribose moiety.

Although the protective capability of other nucleotide analogs (e.g. dCTP, CDP, CMP, cytidine, etc.) was not examined, we hypothesize that in order to obtain cardioprotection against hypoxic stress, the identity of the nucleobase is relatively unimportant, whereas the presence of a tri/di-phosphate moiety appears to be essential.

Previous studies have found that: (1) the yield of $^{\bullet}OH$ in the presence of the nucleotide derivatives is determined by chelation of ferrous ion by polyphosphates and by preferential $^{\bullet}OH$ scavenging by the nucleoside portion of the molecule [33], (2) extracellular nucleotides and nucleosides have strong antioxidant capability as radical scavengers and as metal ion chelators [16], and (3) the primary (rapid) mechanism of inhibition of the Fenton or Haber-Weiss-like reaction involves metal ion-chelation, and radical scavenging is the secondary (slow) mechanism of inhibition [17,18]. Based on these findings, we searched for a possible mechanism for providing cardioprotection. We examined the effect of the nucleotides and its derivatives to decrease ROS concentration following exposure to hypoxic conditions or under normoxic conditions. Indeed, significant reductions in cytosolic ROS concentration were observed after exposure of cells to GTP or tri/di-phosphate prior to hypoxia. A lesser effect was observed if the cells were treated with GDP. We also observed a significant decrease (15–20%) in intracellular ROS levels in GTP-, GDP-, and tri/di-phosphate-treated cardiomyocytes under normoxic conditions, even without exposing the cells to stress, an effect which was long lasting and which even became more pronounced over time. Because ROS and their by-products are capable of causing oxidative damage and may be cytotoxic to the cells, our assumption is that low basal cytosolic ROS concentrations, caused by tri/di-phosphate nucleotides or by tri/di-phosphate *per se*, can prevent future stress damage by preventing ROS formation. Furthermore, the nucleotides treatment attendance during the hypoxic conditions may provide intensive protection against ROS produced during the hypoxia.

GMP and guanosine did not provide cardioprotection, but as reported that the nucleosides can act as free radical scavengers based on their N1 and N7 nitrogen atoms [17,34], it is possible that they would provide cardioprotection against hypoxia if their entry into cells was unhindered.

Based on our findings, we assume that the NTPs need to be cleaved outside the cells by nucleases in order to allow the pyrophosphate to enter the cells and serve as chelator. It is well known that ecto-nucleotide pyro-phosphatase(s) (E-NPP) cleave nucleotide triphosphates (NTPs) to nucleotide monophosphate (NMP) + inorganic pyro-phosphate (PPi) [35], and that ecto-alkaline phosphatase(s) hydrolyze many different phosphoric acid esters, including NTPs, nucleotide diphosphates (NDPs) and NMPs, releasing inorganic phosphate (Pi) [36]. Because GDP provides cardioprotection, and due to an unknown ecto-nucleotide pyrophosphatase function of cleaving NDPs to nucleoside and PPi (which can act as chelator), further research is carried out nowadays in order to explore such possibility. Another study is taking place in order to determine the receptor responsible for the cardioprotection due to tri/di-phosphates, because the extracellu-

lar tri/di-phosphates cannot enter the cells and need to activate receptor, transporter or ionic channels of the cell membrane.

Based on the results presented in this article, our summary hypothesis is as follows: inorganic triphosphate or pyrophosphate, whether administered directly, or obtained by cleavage of triphosphate nucleotides (e.g., by E-NPP to PPi), enters into the cells via transporters, and may act as a chelator of divalent cations, particularly Fe^{2+}, and thereby prevent free radical formation from H_2O_2 and $^{\bullet}O_2$ produced in the mitochondria, as a result of the Fenton reaction. Therefore, using tri/di-phosphates as pharmacological preconditioning agents may provide a safer and more promising alternative to ischemic preconditioning, for inducing cardioprotection in humans against various kinds of stress.

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