Inosine and equilibrative nucleoside transporter 2 contribute to hypoxic preconditioning in the murine cardiomyocyte HL-1 cell line

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Naydenova Z, Rose JB, Coe IR. Inosine and equilibrative nucleoside transporter 2 contribute to hypoxic preconditioning in the murine cardiomyocyte HL-1 cell line. Am J Physiol Heart Circ Physiol 294: H2687–H2692, 2008. First published April 18, 2008; doi:10.1152/ajpheart.00251.2007.—The purine nucleoside adenosine is a physiologically important molecule in the heart. Brief exposure of cardiomyocytes to hypoxic challenge results in the production of extracellular adenosine, which then interacts with adenosine receptors to activate compensatory signaling pathways that lead to cellular resistance to subsequent hypoxic challenge. This phenomenon is known as preconditioning (PC), and, while adenosine is clearly involved, other components of the response are less well understood. Flux of nucleosides, such as adenosine and inosine, across cardiomyocyte membranes is dependent on equilibrative nucleoside transporters 1 and 2 (ENT1 and ENT2). We have previously shown in the murine cardiomyocyte HL-1 cell line that hypoxic challenge leads to an increase in intracellular adenosine, which exits the cell via ENT1 and ENT2. Exogenously added inosine (5 μM) preconditions cardiomyocytes in an A1 adenosine receptor-dependent manner since preconditioning can be blocked by the A1 adenosine receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (1 μM) but not the A3 adenosine receptor antagonist MRS-1220 (200 nM). These data suggest that cardiomyocyte responses to hypoxic PC are more complex than previously thought, involving both adenosine and inosine and differing, but overlapping, contributions of the two ENT isoforms.

PURINE NUCLEOTIDES (such as ATP) and their metabolites (such as adenosine) are physiologically essential molecules in the heart. ATP is the universal currency of chemical energy in the cell, and more ATP is produced and consumed (per unit time) in cardiovascular tissue than anywhere else in the human body (26, 32). Ischemia and hypoxia lead to an imbalance in ATP consumption/production, and the consequences can include arrhythmia, cellular damage, and ultimately cell death (18). However, cardiomyocytes can resist the effects of ischemia and hypoxia if they are briefly exposed to a challenge either pharmacologically or hypoxically (14, 15, 20, 21, 27). This effect, known as preconditioning (PC), has been extensively studied, and various factors, including adenosine and receptor-coupled signaling pathways, have been proposed to be involved (1, 19, 27). Adenosine, a key element in PC, is not lipophilic and crosses cell membranes via nucleoside transporters (2, 12). In cardiomyocytes, equilibrative nucleoside transporters (ENTs), which facilitate the movement of purine nucleosides down their endogenous concentration gradient, are responsible for the flux of adenosine across the plasma membrane (4–6). Extracellular adenosine interacts with adenosine receptors (A1, A2A, A2B, and/or A3), and receptor activation results in the activation of signaling pathways that “resist” the stress caused by ischemia/hypoxia. ENTs are thus critical elements in modulating the local effects of adenosine. Moreover, the potentiation of adenosine receptor activation by drugs that inhibit ENTs, such as dipyridamole (DIPY), is a clinically relevant pharmacological intervention (9, 24, 34).

We previously used the murine cardiomyocyte HL-1 cell line to investigate the role of ENTs in hypoxic or pharmacological PC and shown that PC involves adenosine, mouse (m)ENT1, and adenosine receptor activation (5, 6). However, HL-1 cells also possess ENT2, which has been reported to transport purine nucleosides and to have a higher affinity for inosine, a major and metabolically stable adenosine metabolite (8, 31). A growing body of data suggests that other purine nucleosides or nucleobases (e.g., inosine and adenosine) have physiologically relevant actions in other tissues as a result of cell surface receptor signaling (7, 29, 30, 33). Moreover, inosine has been implicated in cellular responses to ischemia in the brain and skeletal muscle and has been reported to activate A3 receptors (7, 29, 30, 33). Inosine is readily produced by the deamination of adenosine and/or by the deamination of IMP to inosine by 5'-nucleotidase. Intriguingly, recent data have suggested that IMP hydrolysis contributes significantly to adenosine and inosine pools (33). Despite these observations, very little is known about the contribution of inosine to PC in the heart. Given our observation of high concentrations of inosine in HL-1 cells in response to hypoxia, we speculated that inosine might contribute to PC in cardiomyocytes and used the murine HL-1 cell line to identify the relative contributions of ENT1/ENT2 to adenosine /inosine release and to determine if inosine is involved in PC.

MATERIALS AND METHODS

Materials. Claycomb medium and L-glutamine solution were purchased from JRH Biosciences (Lenexa, KS). FBS, antibiotic/antimycotic solution, norepinephrine, inosine, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), nitrobenzylthioinosine (NBTI), DIPY, and 8-(3-chlorostyryl)caffeine (CSC) were purchased from Sigma-Aldrich Canada. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Hypoxic atmosphere of 2% O₂-5% CO₂-93% N₂. Control cells were maintained in a Plexiglas GasPak container and maintained at 37°C with a humidified atmosphere. Control cells were exposed to normoxia for equivalent periods of hypoxia. Nonpreconditioned cells were exposed to 20 h of hypoxia. Cells were treated with 5 μM inosine for 15 min prior to 20 h of hypoxia. Control cells were exposed to normoxia for equivalent time periods.

For hypoxia challenge, HL-1 cells were transferred to a vented Plexiglas GasPak container and maintained at 37°C with a humidified atmosphere of 2% O₂-5% CO₂-93% N₂. Control cells were maintained at 5% CO₂-95% air at 37°C. For the hypoxic challenge, degassed medium was used. Oxygen concentrations in the chamber were measured using a Fyrite Oxygen Analyzer.

Adenosine receptors. To determine whether adenosine receptor activation was involved in pharmacological PC with inosine, HL-1 cells were treated with adenosine receptor agonists and antagonists that we have previously confirmed to be effective in this cell line (5). Thus, HL-1 cells were treated with the general adenosine receptor antagonist CGS-15943 (4 nM), the specific A₁ adenosine receptor antagonist DPCPX (1 μM), the specific A₂ adenosine receptor antagonist CSC (500 nM), and the specific A₃ adenosine receptor antagonist MRS-1220 (200 nM) for 20 min before and during the treatment with 5 μM inosine for 15 min followed by 20 h of hypoxia. We used pharmacological agents at concentrations that ensure maximal blockade of the respective receptors without interfering with ENT-dependent transport by pretesting them in adenosine transport assays (5).

Cell viability. Following PC and/or hypoxia, cell viability was determined by a standard trypan blue exclusion assay. Eight fields of view were counted for each sample, and values shown as percentages of dead cells (means ± SE).

HPLC analysis. Extracellular adenosine and inosine were measured as previously described for PC-12 cells (17). HL-1 cells were plated on 60-mm dishes and grown to 75–80% cell confluency. Culture plates were precoated with antibiotic/antimycotic solution. Culture plates were precoated with antibiotic/antimycotic solution. Culture plates were precoated with antibiotic/antimycotic solution. Culture plates were precoated with antibiotic/antimycotic solution.

Fig. 1. Hypoxic preconditioning (PC) enhances HL-1 cell viability after chronic hypoxia. Cell viability was determined after the following treatments of HL-1 cells: cells maintained in normoxia (5% CO₂ and 95% air, 20 h); cells exposed to hypoxia (2% O₂, degassed media, 20 h); and cells hypoxically preconditioned (90-min hypoxia and 60-min normoxia, 20 h of hypoxia). The role of adenosine transporters was investigated by treating HL-1 cells with 500 nM nitrobenzylthionine (NBPTI) during PC followed by 20 h of hypoxia. Pooled data are means ± SE; n ≥ 6. *P < 0.05 compared with hypoxia; ΔP < 0.01 compared with hypoxia.

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RESULTS

We (5, 6) have previously shown that adenosine is released from HL-1 cardiomyocytes via ENT1 following hypoxic challenge, suggesting that ENT1 is a gatekeeper in PC. Therefore, inhibition of ENT1 by NBPTI could effectively abolish PC unless ENT2 and inosine are involved. To determine whether inosine is an effective PC agent, we used our previous approach (5), and, since both ENT1 and ENT2 exist in HL-1 cells (4), we investigated the relative contributions of ENT1 and ENT2 to PC.

Using our previously determined PC protocol, we found that inhibition of mENT1 alone using NBPTI did not prevent PC (Fig. 1). We speculated that purine nucleoside-dependent PC could still be occurring due to purine nucleoside flux via ENT2 or retention of intracellular pools of adenosine metab-
olites leading to equivalent cell viability compared with PC. To determine if there were changes in purinergic nucleotides and their metabolites following hypoxic challenge, we compared extracellular concentrations of adenosine and inosine following hypoxic challenge and in the presence of the ENT1 inhibitor NBTI (Fig. 2). These data showed that levels of inosine in the extracellular medium of cells exposed to hypoxic challenge were significantly higher (~20-fold) than those of adenosine. Moreover, inhibition of mENT1 (by NBTI) did not significantly affect extracellular concentrations of inosine (suggesting efflux via mENT2), whereas inhibition of both mENT1 and mENT2 (using NBTI + DIPY) significantly reduced extracellular inosine concentration, although not to basal levels (suggesting some extracellular production). These data suggest that mENT2 could be responsible for the release of inosine, which could in turn contribute to PC.

Since it is clear that hypoxic challenge leads to significant extracellular levels of inosine, we then asked if inosine could act as a pharmacological PC agent, analogous to adenosine. Therefore, we tested whether inosine could precondition HL-1 cells under identical conditions to those previously described (Fig. 3). Based on our observation that extracellular concentrations of inosine reached 7 μM after 30 min of hypoxia, we used 5 μM inosine to pharmacologically precondition the cells in these experiments. We found that inosine is an effective PC agent and that it appears to act via A1 receptors, similarly to adenosine, but has no or very little effect through A3 receptors, in contrast to adenosine, which we have previously found to act through both receptors (5). Finally, to ensure that inosine was not triggering protection by elevating extracellular adenosine (and thus A1 adenosine receptor agonism) through competition for uptake, we measured 2-chloradenosine uptake in the presence and absence of 5 μM inosine and found no inhibitory effect (data not shown).

It is evident that there are substantial levels of extracellular inosine following hypoxic challenge, and we hypothesized that some of this inosine is being released by ENT2; thus, we needed to confirm that the inosine was being produced intracellularly and released rather than adenosine being released and rapidly deaminated to inosine extracellularly. Therefore, we measured intracellular levels of inosine following hypoxic challenge in the presence of ENT1 and ENT2 inhibitors (Fig. 4) and compared these with previously described extracellular levels. We found that there was a significant increase in intracellular inosine following hypoxia and that it was primarily released via ENT2 with a smaller component released via ENT1.

While our data are suggestive of flux of inosine via ENT2, transport of this purine by ENT2 has not been demonstrated in HL-1 cells. Therefore, we confirmed that ENT2 could transport inosine under typical conditions used for transport characterizations (4). Our data (Fig. 5) showed that exogenously added inosine was transported by both ENT1 and ENT2 at approximately equal levels, whereas adenosine was primarily transported by ENT1.

Finally, we were interested in the source of inosine being released from cells. In a previous study (5) aimed at measuring...
In conclusion, we propose that inosine is an effective and physiologically relevant PC agent that can be produced intracellularly and released by cardiomyocytes via ENTs following hypoxic challenge and that contributes to PC via A1 adenosine receptors (Fig. 7).

**DISCUSSION**

Adenosine is well established to play a role in PC in a number of physiological settings, including the cardiovascular system (19). However, there is now evidence that adenosine metabolites may play also role in cardiovascular physiology. More than 20 years ago, the purine nucleoside inosine was reported to be a protective agent in myocardial ischemia (11). More recently, there have been reports that inosine reduces ischemic injury in the brain (13, 28) and that it has immunomodulatory functions (13). Inosine is a hydrophilic purine nucleoside and is transported across cell membranes by members of the ENT family, with ENT2 appearing to be its preferred route (8). Interestingly, ENT2 is highly expressed in muscle (8, 23), suggesting a possible physiological role for inosine and ENT2 in the cardiovascular system. Thus, the presence of ENT2, along with ENT1, in cardiomyocytes raised the possibility of differing or overlapping physiological roles for these proteins. Moreover, the reported differential transport preferences of ENT1 and ENT2 for adenosine and inosine, in addition to the ability of ENT2 to transport nucleobases, raised the possibility that purine nucleosides other than adenosine were relevant in cardiovascular physiology. Since adenosine and ENT1 have already been implicated in hypoxic PC in HL-1 cells (5, 6), we were interested in determining if there was also a contribution by ENT2 and inosine to this process. Our data demonstrate that ENT2 and inosine contribute to PC in HL-1 cells, supporting the notion that inosine is a physiologically relevant neuro- and cardioprotective molecule (13, 11, 28).

In our study, the majority of inosine produced in response to hypoxic challenge, and responsible for PC, appears to be intracellular (probably from the metabolism of adenosine via adenosine deaminase, which is found both intracellularly and extracellularly in cardiomyocytes). However, we also noted the presence of some extracellular inosine even with complete ENT inhibition, supporting previous observations of high levels (and a possible extracellular source) of inosine. These data correlate with a recent study (33) using adenosine deaminase knockout mice in which inosine levels were not as low in the cardiovascular system as would be expected if all inosine were being produced by the deamination of adenosine. Thus,

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**Fig. 4.** Hypoxic challenge leads to an increase in the level of intracellular inosine and its efflux via ENTs in HL-1 cells. 

**A:** intracellular concentrations of inosine in the presence or absence of ENT inhibitors 500 nM NBTI or 500 nM NBTI + 30 μM DIPY after 30 min hypoxia treatment. Pooled data are of 2 experiments and are means ± SE; n = 3. *P < 0.001 compared with hypoxia; ΔP < 0.001 compared with normoxia.

**B:** extracellular concentrations of inosine under the same conditions as in A. Pooled data are means ± SE (n = 3); with each condition conducted at least 6 times. *P < 0.001 compared with hypoxia; ΔP < 0.001 compared with normoxia.

**Fig. 5.** In HL-1 cells, mouse ENT2 is responsible for about half of the inosine transport. Transport of [3H]inosine was measured at 5 s in the presence of the ENT1 inhibitor NBTI (100 nM). Pooled data are of 3 experiments, with each condition conducted 6 times; data are means ± SE. *P < 0.001 compared with adenosine + NBTI.
IMP may be a significant, and underappreciated, source of inosine in cardiomyocytes (33).

While inosine has been reported to interact with A3 receptors, for which it has been reported to have a high affinity in mast cells (16), we found no evidence of an interaction with A3 receptors. Rather, we noted that the PC effects of inosine are A1 adenosine receptor dependent, which correlates with previous findings demonstrating that loss of the A1 adenosine receptor compromises ischemic tolerance (25, 33). These observations suggest that cell type-specific differences in adenosine physiology, relating to receptor specificity and coupling, may exist (22).

Taken together, our data suggest that inosine (and possibly other adenosine metabolites) needs to be more thoroughly investigated in a myocardial setting to ensure that the complexity of factors involved in PC and cardiomyocyte physiology are fully understood. The ratio of local extracellular concentrations of inosine to adenosine may play a role in determining the cellular response given the differing, but perhaps overlapping, affinities for adenosine receptors and transporters (13). While adenosine is generally considered to be a beneficial purine nucleoside, excess extracellular adenosine can also be detrimental to a number of cell types, leading to apoptosis and inflammation (3). Thus, cellular control of the flux and receptor-dependent signaling of purine nucleosides is likely to be important for appropriate cellular responses in metabolically active cells and tissues such as those in the cardiovascular. Taken together, our observations suggest that cardiomyocyte responses to hypoxic PC may involve a more complex set of players than

Fig. 6. Hypoxic challenge leads to a decrease in the level of intracellular ATP and an increase in the levels of inosine, AMP, and ADP in HL-1 cells. A–D: intracellular levels of inosine (A), AMP (B), ADP (C), and ATP (D) in the presence or absence of ENT inhibitors 500 nM NBPT or 500 nM NBPT + 30 μM DIPY were determined by HPLC after 30 min of hypoxia treatment. In A, pooled data are of 2 experiments and are means ± SD, with each condition conducted 5 times. *P < 0.001 compared with hypoxia; ΔP < 0.01 compared with normoxia. In B–D, pooled data are of 3 experiments and are means ± SE, with each condition conducted 6 times. In B, *P < 0.01 compared with hypoxia; ΔP < 0.001 compared with normoxia. In C, *P < 0.001 compared with normoxia; ΔP < 0.001 compared with hypoxia.

Fig. 7. Pathways of purine nucleoside metabolism in HL-1 cardiomyocytes. 5′-NT, 5′-nucleotidase.
REFERENCES


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previously thought, and future studies should take into account the contribution of inosine and differing roles of the various ENT isoforms.