Compensatory upregulation of the adenosine system following phenylephrine-induced hypertrophy in cultured rat ventricular myocytes

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Compensatory upregulation of the adenosine system following phenylephrine-induced hypertrophy in cultured rat ventricular myocytes. Am J Physiol Heart Circ Physiol 298: H545–H553, 2010. First published December 4, 2009; doi:10.1152/ajpheart.00417.2009.—Adenosine has been shown to exert direct antihypertrophic effects on the heart, and plasma adenosine levels have been shown to be elevated in patients with heart failure. It has therefore been proposed that endogenously synthesized adenosine may function as a cardiac antihypertrophic factor. The present study was aimed to determine whether the adenosine system is altered in a potential adaptive manner following phenylephrine-induced hypertrophy in cultured neonatal rat ventricular myocytes. Phenylephrine produced significant hypertrophy as determined by cell size and atrial natriuretic peptide gene expression, which was accompanied by significantly increased gene and protein expression of adenosine A1, A2a, and A3 receptors. These effects and the hypertrophic response were prevented by the α1-adrenoceptor antagonist prazosin as well as pharmacological agonists of all adenosine receptor subtypes. The upregulation of adenosine receptors by phenylephrine was also abrogated by adenosine 5′-nucleoside transporter 2 although expression levels of equilibrative nucleoside transporter 1 were unaffected. Taken together, our results suggest an adaptive upregulation of the adenosine system in the presence of a nucleoside transporter and adenosine deaminase inhibitor, the combination of which abrogated the hypertrophic effect of phenylephrine. The latter effect was reversed by adenosine receptor antagonists. Phenylephrine also produced a significant upregulation in expression levels of equilibrative nucleoside transporter 1 although expression levels of equilibrative nucleoside transporter 2 were unaffected. Taken together, our results suggest an adaptive upregulation of the adenosine system to phenylephrine-induced cardiomyocyte hypertrophy that serves to limit the hypertrophic effect of α1-adrenoceptor activation.

ventricular hypertrophy; adenosine; nucleoside transport; adenosine deaminase; adenosine receptors

Ventricular hypertrophy is a major determinant of mortality and morbidity in patients with heart failure, a factor that has resulted in the emergence of hypertrophy as an important therapeutic target for treating heart failure (7). The mechanisms underlying the hypertrophic program are exceedingly complex, involving a multiplicity of factors (6). Although there are numerous endogenous candidates that serve as potent stimuli for initiating hypertrophy, a paucity of endogenous antihypertrophic factors have been identified. Adenosine, a purine nucleoside produced by numerous tissues, including the heart, is an important signaling molecule in the cardiovascular system, and it has been shown to play a regulatory role in cellular metabolism and exert cardioprotective effects (11, 20, 24, 28). We have also previously reported that adenosine selectively diminishes acute phenylephrine-induced effects, including positive inotropic responses and elevation in intracellular Ca2+ concentrations (12). In recent years, adenosine has emerged as an important endogenous antihypertrophic factor that functions independently of its cardioprotective influence (9, 16, 17). Indeed, elevated plasma adenosine concentrations have been demonstrated in patients with heart failure, and a correlation between plasma adenosine concentrations and the severity of heart failure has been reported (8). The latter observation suggests that elevations in adenosine levels in heart failure reflect a compensatory process. It is also interesting that increasing plasma adenosine concentrations with nucleoside transport inhibitors was shown to reduce the severity of heart failure (16). Taking these findings into consideration, the adenosine system may represent an important endogenous regulator of the myocardial remodeling process in response to hypertrophic stimuli.

Among the key determinants of adenosine production in cardiomyocytes are the cytosolic and ectosolic 5′-nucleotidases that are responsible for the enzymatic dephosphorylation of 5′-AMP to adenosine (4). Adenosine kinase and adenosine deaminase also play important roles by initiating the metabolism of adenosine to AMP and inosine, respectively (5). Furthermore, the cardiac cell is equipped with nucleoside transporters that mediate the release of adenosine to act in an autocrine/paracrine manner, or its cellular uptake to terminate activation of cell surface receptors and replenish the nucleotide pool (3).

In view of the complexities of adenosine regulation, it is clear that the net effect of adenosine-induced cardiomyocyte responses is dependent not only on adenosine production but also the presence of adenosine receptors and adenosine transporters. To date, little is known about the role of the adenosine system in cardiac hypertrophy and whether the various components of the adenosine system are altered in response to hypertrophic stimuli. We hypothesized that adenosine represents an endogenous adaptive response to hypertrophic stimulation that then acts to limit the magnitude of hypertrophy. Accordingly, the present study was undertaken to examine whether cardiomyocyte hypertrophy would result in any changes in the adenosine system in a cultured neonatal rat ventricular myocytes model and, if so, to determine the possible mechanisms involved.

MATERIALS AND METHODS

Experimental protocols. For the first set of studies, we assessed the effect of the α1-adrenoceptor agonist phenylephrine and the attendant hypertrophy on cardiomyocyte expression of adenosine receptor sub-
types. Cells were serum-starved for 24 h before all experiments after which cells were treated with phenylephrine (10 μM). We recorded the temporal changes in adenosine receptor subtype A1, A2a, and A3 mRNA and protein expression after exposure to phenylephrine for 6 and 24 h. To further assess the regulatory role of phenylephrine-induced hypertrophy on the expression of adenosine receptor subtypes, the effect of phenylephrine was subsequently studied in the presence of the following subtype-selective adenosine receptor agonists: the A1 receptor agonist N6-cyclopentyladenosine (CPA, 1 μM), the A2a receptor agonist CGS-21680 (100 nM), and the A3 receptor agonist N6-(3-iodobenzyl)-adenosine-5'-N-methylcarboxamide (IB-MECA, 100 nM). In addition, the effects of their corresponding antagonists 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 10 μM), 8-(3-chlorostyryl)-caffeine (CSC, 10 μM), and MRS-1523 (1 μM) respectively, were determined. Preliminary studies confirmed that neither adenosine receptor agonists nor antagonists alone affected expression of adenosine receptor subtypes.

We next determined whether phenylephrine affects cardiomyocyte adenosine production. Accordingly, cells were plated in a six-well plate at a concentration of 6 × 105 cells/well. After 24 h serum starvation, cells were incubated in 1 ml of serum-free cell culture medium for 24 h after which samples of the medium were obtained for HPLC analysis of adenosine release from cardiomyocytes. To examine whether adenosine release would be augmented under hypertrophic conditions, cells were treated with 10 μM phenylephrine for 24 h. Additional experiments were done in the presence of adenosine metabolite and/or transport inhibitors. For these experiments, the adenosine deaminase inhibitor erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA, 5 μM) and/or the nucleoside transport inhibitor nitrobenzylthioinosine (NBMPR, 1 μM) was added 1 h before phenylephrine addition.

We determined whether endogenously produced adenosine could serve as a negative feedback modulator of phenylephrine-induced cardiomyocyte hypertrophy. For these experiments, expression levels of atrial natriuretic peptide (ANP) were assessed by real-time PCR in cells treated with phenylephrine in the presence or absence of EHNA and/or NBMPR for 6 or 24 h. Additional studies were done to examine the nature of adenosine receptor involvement in mediating the anti-hypertrophic effect by determining the effects of subtype-specific receptor antagonists. Cells were pretreated (1 h before phenylephrine administration) with EHNA (5 μM) and/or NBMPR (1 μM) in the absence or presence of DPCPX (10 μM), CSC (10 μM), MRS-1523 (1 μM), or 8-(p-sulfophenyl)theophylline (SPT, 10 μM). When studied, these adenosine receptor antagonists were administered 30 min before the addition of EHNA or NBMPR.

For the last set of studies, we determined changes in cardiomyocyte expression of adenosine receptor subtypes in response to ectosolic 5'-nucleotidase inhibition by determining the effects adenosine 5'- (α,β-methylene)diphosphate (AOPCP, 50 μM), a potent and specific inhibitor of ectosolic 5'-nucleotidase. AOPCP was added 1 h before phenylephrine administration, and sequential changes in adenosine receptor expression were recorded. Additional experiments were done in the presence of EHNA (5 μM) and/or NBMPR (1 μM), which were administered 30 min before AOPCP.

Primary culture of neonatal cardiomyocytes. All protocols for the use of animals were approved by the University of Western Ontario Animal Care and Use Subcommittee and conformed to the guidelines of the Canadian Council on Animal Care (Ottawa, Ontario, Canada). Primary culture from 1- to 4-day-old Sprague-Dawley rat pups (Charles River Canada, St. Constant, Quebec, Canada) was performed essentially as previously described (9). Briefly, rats were decapitated, and the ventricles were washed, cut, and subsequently digested with collagenase II. The reaction was terminated with the addition of 20% FBS. The cellular extracts were strained and centrifuged at 514 g for 5 min. The supernatant was then aspirated, and the resulting pellet was resuspended in cell culture media. The suspended cell mixture underwent two rounds of preplating to eliminate fibroblasts and other nonmyocyte components. The final myocyte preparation was plated in cell culture media containing Dulbecco’s modified Eagle medium/ Ham’s F-12 supplemented with 10% FBS, 10 μg/ml transferrin, 10 μg/ml insulin, 10 ng/ml selenium, 50 U/ml penicillin, 2 mg/ml BSA, 5 μg/ml linoleic acid, 3 mM pyruvic acid, 0.1 mM minimum essential medium nonessential vitamins, and 10% minimum essential medium vitamin solution, 0.1 mM bromodeoxyuridine, 100 μM l-ascorbic acid, and 30 mM HEPES, pH 7.2.

Cardiomyocyte surface area analysis. Cells were plated at a concentration of 1 × 106 cells/plate and viewed using a Leica (Wetzlar, Germany) inverted microscope equipped with a Polaroid digital camera at 20× magnification. Cell surface area was measured using SigmaScan Software (Systat, Richmond, CA) from 30 randomly selected cells per experiment and averaged to give an n value of 1.

RNA isolation, reverse transcription, and real-time PCR analysis. Total RNA was isolated from cells plated at a density of 6 × 106 cells/plate by the Trizol method according to the manufacturer’s instructions. Briefly, cells were harvested in 700 μl Trizol reagent, after which 200 μl of chloroform were added, vortexed, and centrifuged at 12,000 g for 20 min at 4°C. After separating the aqueous layer, RNA was precipitated with the addition of 500 μl isopropanol and centrifuged at 10,000 g for 20 min at 4°C. The supernatant was then aspirated, and the resulting pellet was resuspended in 30 μl diethylpyrocarbonate water. Total RNA was quantified spectrophotometrically at a wavelength of 260 nm. A total of 2 μg RNA was used to synthesize single-stranded cDNA by Superscript II H-Reverse-transcriptase according to the manufacturer’s instructions. The cDNA was diluted 10-fold, and 1 μl of the diluted cDNA was used in a 20-μl PCR reaction. Gene expression was performed with SYBR green jumpstart Taq ready mix DNA polymerase, and fluorescence was measured and quantified using a DNA engine Opticon II (MJ Research/Bio-Rad, Waltham, MA). Table 1 displays genes examined as well as primers, annealing temperatures, and cycle number used for each gene amplified by real-time PCR. PCR conditions and cycle numbers were optimized for each gene. PCR conditions for amplification of all genes were 30 s at 94°C followed by annealing at the gene-specific temperature (see Table 1) for 20 s and elongation at 72°C for 30 s. Melting curve analysis showed a single product formation for each gene amplified. Gene expression was normalized using the housekeeping gene 18S rRNA as an internal control.

SDS-PAGE and Western blotting for adenosine receptor subtypes. Cells plated at a density of 6 × 106 cells/plate were harvested in 100 μl of lysis buffer (50 mM Tris·Cl, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 2 mM EDTA, 2 mM EGTA, 50 mM NaF, 200 μM Na3VO4, 10 mM Na2P2O7, 40 mM β-glycerophosphate, and protease inhibitors) and subsequently homogenized and centrifuged at 10,000 g for 10 min at 4°C. The supernatant was collected and quantified by the Bradford Protein Assay Kit. A total of 50 μg of protein were loaded on 12% SDS-PAGE at 100 volts for 2 h and transferred at 100 volts for 2 h on a 0.45-μm nitrocellulose membrane. Membranes underwent Ponceau S staining to verify transfer efficiency and washed before blocking for 1 h with 5% dry skim milk. Membranes were probed with rabbit anti-A1, anti-A2a, and anti-A3 receptor antibodies at a dilution of 1:1,500, 1:500, and 1:500, respectively, overnight followed by a secondary antibody for 1 h, and blots were detected using enhanced chemiluminescence reagent. Actin (1:2,000 antibody dilution) was used as a loading control, and adenosine receptor abundance was normalized to actin levels before statistical analysis.

HPLC analysis for extracellular adenosine concentrations. Adenosine release from cardiomyocytes was determined by reverse-phase HPLC analysis of their cell culture media. Cell culture media obtained from treated cells first underwent sample preparation consisting of a solid-phase extraction with Bakerbond C18 extraction tubes and elution with 1 ml of methanol. Samples were then concentrated when the eluent was allowed to dry in 40°C conditions for 10 min. The final mixture was reconstituted in 100 μl of the mobile phase. A 20-μl sample was transferred to a glass insert and injected on a 3.0 ×...
Table 1. Primers for real-time PCR analysis

<table>
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<th>Name</th>
<th>Sequence (5'→3')</th>
<th>Cycle No.</th>
<th>Annealing temperature, °C</th>
<th>Accession No.</th>
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<td>rENT1(+)</td>
<td>GGCTTCTTGACCTGATG</td>
<td>40</td>
<td>58</td>
<td>AF015304</td>
</tr>
<tr>
<td>rENT1(-)</td>
<td>CTTCTTGACCTGATG</td>
<td>40</td>
<td>58</td>
<td>AF015305</td>
</tr>
<tr>
<td>rENT2(+)</td>
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<td>45</td>
<td>58</td>
<td>AB001089</td>
</tr>
<tr>
<td>A1R(+)</td>
<td>GTGATTTGGCTGCTGAGGT</td>
<td>45</td>
<td>58</td>
<td>NM_053294</td>
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<td>45</td>
<td>58</td>
<td>NM_017161</td>
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<tr>
<td>A2AR(+)</td>
<td>TCTGGCTATGCCATGACGA</td>
<td>45</td>
<td>58</td>
<td>DQ05463</td>
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<tr>
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<td>58</td>
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<tr>
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<td>55</td>
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</tr>
<tr>
<td>A3R(-)</td>
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<tr>
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</tr>
</tbody>
</table>

rENT, rat equilibrative nucleoside transporter; A1R, A2AR, and A3R, adenosine receptor subtypes 1, 2a, and 3, respectively; ANP, atrial natriuretic peptide.

150-mm 5-μm Agilent Zorbax SB-C18 column maintained at 40°C in a Hewlett Packard 1090 HPLC system. The mobile phase (4% acetonitrile-96% ammonium formate, pH 3.0) was run at 0.5 ml/min, and the eluent was detected by a Hewlett Packard 1050 variable-wavelength ultraviolet detector at a wavelength of 254 nm.

Drugs and chemicals. TRIzol, Superscript II RNase H-Reverse Transcriptase, and SYBR green jumpstart Taq ready mix DNA polymerase were purchased from Invitrogen (Carlsbad, CA). Antibodies against adenosine receptor subtypes A2a and A3 were purchased from Millipore (Billerica, MA), whereas antibodies against adenosine receptor subtype A1 were purchased from Alpha Diagnostics Internatonal (San Antonio, TX). Bradford Protein Assay kits were from Bio-Rad Laboratories (Mississauga, Ontario, Canada). All other chemicals and drugs were purchased from Sigma-Aldrich Canada (Oakville, Ontario, Canada). The concentrations of drugs used in the present study reflect those found to exert maximal effects in initial experiments aimed at identifying optimal concentrations of each agent. We further assessed selectivity of the effects of antagonist against their respective receptors by performing experiments in which the effect of the specific receptor antagonist was also tested against agonists acting on either of the other two receptors. At the concentrations tested, adenosine receptor antagonists were effective only in preventing the antihypertrophic effect of their respective agonists.

Statistical analysis. Results are given as means ± SE. Statistical significance was determined by using a one-way ANOVA, and post hoc comparisons were performed using the Student-Newman-Keuls test. Differences were considered significant when P < 0.05.

RESULTS

Upregulation of adenosine receptors in response to α1-adrenergic receptor stimulation. We first determined whether phenylephrine-induced hypertrophy is associated with alterations in the cardiomyocyte adenosine receptor system by treating ventricular myocyte cultures with phenylephrine for 6 or 24 h and determining the expression of adenosine receptor subtypes A1, A2a, and A3 using real-time PCR and Western blotting. As shown in Fig. 1A, phenylephrine produced a significant upregulation in the three adenosine receptor subtypes at both time points, which was also observed with respect to protein levels as determined by Western blotting (Fig. 1B). Addition of prazosin (10 μM), an α1-adrenergic receptor antagonist, abolished phenylephrine-induced effects on adenosine receptor expression (Fig. 2). Prazosin also completely prevented the hypertrophic response to phenylephrine as determined by both cell surface area and ANP expression (data not shown).

Phenylephrine-induced adenosine receptor upregulation is prevented by respective adenosine receptor agonists. We have previously shown the inhibition of phenylephrine-induced cardiomyocyte hypertrophy by activation of multiple adenosine receptor subtypes (9). We considered it of interest to assess whether adenosine receptor activation also modifies the upregulation of these receptors following phenylephrine admin-

![Fig. 1. Effect of phenylephrine (PE, 10 μM) on expression of adenosine receptor (AR) subtypes. A: changes in adenosine receptor mRNA expression as assessed by real-time PCR in rat ventricular cardiomyocytes following exposure to PE for either 6 or 24 h. B: corresponding changes in AR protein expression as assessed by Western blotting with representative blots above bars. Values are shown as means ± SE from 6 separate experiments. *P < 0.05 vs. control.](http://ajpheart.physiology.org/)
studies have suggested the involvement of adenosine receptor upregulation.

Previous only as a key enzyme involved in the regulation of adenosine production but also as a signaling molecule during the hypertrophic response following $\alpha_1$-adrenoceptor stimulation.

Phenylephrine elevates extracellular adenosine concentrations in the presence of metabolic and transport inhibitors. To further assess the possible role of endogenous adenosine as a compensatory mechanism in phenylephrine-induced hypertrophy, we assessed first the ability of phenylephrine to modulate the release of adenosine from cultured myocytes and, secondly, whether any changes in adenosine production are associated with altered hypertrophic responses. For the former, adenosine content was measured in culture media after 24 h treatment of myocytes with phenylephrine. Under control conditions, adenosine concentrations averaged $0.65 \pm 0.03$ ng/ml, a value that was not increased by phenylephrine alone nor in the presence of either EHNA, an adenosine deaminase inhibitor, or NBMPR, an adenosine transport inhibitor (data not shown). Treatment with EHNA + NBMPR (24 h) produced a moderate although not statistically significant increase in adenosine concentration ($0.84 \pm 0.07$ ng/ml). However, treatment with phenylephrine in the presence of both EHNA and NBMPR significantly increased adenosine levels by more than fourfold to $3.94 \pm 1$ ng/ml ($P < 0.05$ vs. control, $n = 6$, Fig. 6A). Moreover, no hypertrophic responses were observed following phenylephrine administration under these conditions (Fig. 6, B and C). To assess whether the lack of hypertrophic responses in the presence of EHNA and NBMPR could reflect an adenosine-dependent inhibition of the hypertrophic response, we carried out identical experiments in the presence of adenosine receptor blockers. Indeed, as summarized in Fig. 7, the ability of EHNA + NBMPR to abrogate phenylephrine-induced hypertrophy was prevented by the selective adenosine receptor antagonists DPCPX, CSC, and MRS-1683 as well as SPT, a nonselective antagonist. Taken together, the results suggest that the ability of the EHNA + NBMPR combination to inhibit phenylephrine-induced hypertrophy was because of enhanced adenosine production, resulting in activation of multiple adenosine receptor subtypes.

Upregulation of equilibrative nucleoside transporter 1 mRNA in phenylephrine-treated cardiomyocytes. Intracellularly formed adenosine has one of two fates, metabolism by adenosine kinase and adenosine deaminase or export out of the cell by equilibrative nucleoside transporters (ENTs) to initiate receptor activation. We therefore determined the effect of phenylephrine on cardiomyocyte ENT1 and ENT2 mRNA expression. As shown in Fig. 8, treatment with phenylephrine for either 6 or 24 h resulted in a significant upregulation in ENT1 mRNA, although expression levels of ENT2 were unaffected.

Lack of effect of either endothelin-1 or aldosterone on adenosine receptor expression. We also determined whether the effect of phenylephrine on the adenosine system is shared by other prohypertrophic factors by determining the effect of endothelin (ET)-1 (10 nM) or aldosterone (100 nM) on adenosine receptor expression 6 and 24 h after agonist addition. However, neither ET-1 nor aldosterone had any effect on adenosine receptor expression despite hypertrophic responses that were quantitatively identical to those seen with phenylephrine. Moreover, adenosine receptor activation with either EPA, CGS-21680, or IB-MECA had no effect on the hypertrophy observed 6 or 24 h after addition of either ET-1 or aldosterone (data not shown).
DISCUSSION

The major finding of this study is the demonstration of an upregulation of the adenosine system in response to α₁-adrenoceptor-stimulated hypertrophy in cultured neonatal rat cardiomyocytes. This was manifested by significant increases in the relative expression of adenosine A₁, A₂a, and A₃ receptors and the equilibrative nucleoside transporter ENT1 as well as significantly elevated extracellular adenosine concentrations in hypertrophied cells. However, the latter was observed only under conditions of nucleoside transporter and adenosine deaminase inhibition, which would suggest a rapid metabolism and reuptake of the nucleoside following stimulation of its production by phrenylephrine. Taken together, we postulate from our findings that the upregulation of multiple components of the adenosine system following phrenylephrine administration represents a compensatory mechanism of the cardiomyocyte to inhibit the hypertrophic effect of α₁ adrenoceptor activation. Indeed, it appears that this compensatory response of upregulation of the adenosine system is specific for α₁-receptor activation, since we were unable to observe any effect in response to hypertrophy produced by either ET-1 or aldosterone, although the influence of either hypertrophic stimuli still needs to be determined. It should be noted that, although there is robust evidence from animal studies implicating α₁-adrenoceptor activation in cardiac pathology, its role in clinical pathology and particularly in the development of heart failure under conditions of excess adrenergic activation is not completely understood (23). Based on the present results, one can speculate that the state of the adenosine system may dictate the nature of the contribution of α₁-adrenoceptors to the heart failure process.

The ability of phenylephrine to upregulate expression levels of all three adenosine receptor subtypes studied was somewhat surprising, since these receptors couple to diverse signaling processes. However, we have previously demonstrated that activation of all three receptor subtypes exerts antihypertrophic effects (9), an observation further shown in the present report. However, the issue of specificity of adenosine receptor involvement in mediating antihypertrophic effects of the nucleoside is far from resolved. For example, another report impli-
selective $A_1$ receptor activation as the mediator for the antihypertrophic effect of adenosine with no antihypertrophic effect evident either with IB-MECA or CGS-21680 (17). To further add complexity to the issue of receptor specificity mediating the antihypertrophic effect of adenosine, Lu and colleagues (18) recently reported an unexpected reduction in left ventricular hypertrophy in mice lacking the $A_3$ receptor that were subjected to aortic banding, an observation suggestive of a pro-hypertrophic role for this adenosine receptor subtype.

The ability of adenosine receptor agonists to prevent the upregulation of adenosine receptor expression was an interesting observation and one that we propose reflects the ability of these agents to prevent the hypertrophic response. This would suggest that the upregulation of adenosine receptors following phenylephrine administration does not reflect only a pharmacological response to $\alpha_1$-adrenoceptor activation but one that also requires the presence of the hypertrophic response. Related to the preceding discussion, this finding also highlights the ability of multiple adenosine receptor activation to inhibit the hypertrophic response to phenylephrine. This phenomenon was reinforced by the finding that the antihypertrophic influence of combined EHNA and NBMPR treatment (thus blocking endogenous adenosine reuptake or catabolism) was reversed by all adenosine receptor antagonists.

The mechanisms by which phenylephrine upregulates the adenosine system in uncertain; however, it is unlikely to occur simply as a result of hypertrophy per se since both ET-1 and aldosterone were without effect despite a similar hypertrophic influence as that seen with phenylephrine. The consequences of $\alpha_1$-adrenoceptor activation are multifaceted and include superoxide and nitric oxide generation as well as the activation of various cell signaling processes that could contribute to transcriptional modification, resulting in altered receptor expression (19, 26). Phenylephrine also stimulates ectosolic 5'-nucleotidase activity; therefore, we determined whether this could mediate the upregulation of adenosine receptors following administration of phenylephrine. Indeed, deficiency in ectosolic 5'-nucleotidase has been shown to exacerbate heart failure and left ventricular hypertrophy in mice subjected to aortic constriction-induced pressure overload (27). In the present study, pharmacological inhibition of ectosolic 5'-nu-

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Fig. 4. Effect of PE (10 $\mu$M) on cardiomyocyte hypertrophy as assessed by cell surface area (A) and atrial natriuretic peptide (ANP) expression (B) either alone or in the presence of their respective agonist +/− antagonist. Values are shown as means ± SE from 6 separate experiments. *$P < 0.05$ vs. control.
cleotidase with AOPCP completely abrogated the adenosine receptor upregulation seen with phenylephrine administration, suggesting that extracellularly generated adenosine may contribute to adenosine receptor upregulation. However, this was observed in the absence of any effect on phenylephrine-induced hypertrophy, which likely reflects insufficient levels of endogenous adenosine because of its rapid inactivation (see below).

Various factors can affect myocardial adenosine receptor expression, including myocardial ischemia, diabetes, and aging (1, 2, 10, 14); however, the present report is the first to demonstrate changes in adenosine receptor abundance in response to α1-adrenoceptor-dependent cardiomyocyte hypertrophy. In this regard, the adenosine receptor responses to other forms of cardiac insult have been relatively complex in contrast to the present report showing a uniform upregulation of all adenosine receptor subtypes.

**Fig. 5.** Effect of PE (10 μM) on expression of AR subtypes either alone or in the presence of adenosine 5′-(α,β-methylene)diphosphate (AOPCP, 50 μM). A: changes in AR mRNA expression as assessed by real-time PCR in rat ventricular cardiomyocytes following exposure to PE for either 6 or 24 h. B: corresponding changes in AR protein expression as assessed by Western blotting with representative blots above bars. Values are shown as means ± SE from 6 separate experiments. *P < 0.05 vs. control.

**Fig. 6.** Effect of 24 h PE (10 μM) treatment on adenosine levels in the culture medium (A), cardiomyocyte ANP expression (B), and cell surface area (C) either alone or in the presence of combined treatment with erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA, 5 μM) and nitrobenzylthioinosine (NBMPR, 1 μM). Values are shown as means ± SE from 6 separate experiments. *P < 0.05 vs. the other two groups. Although not shown, neither EHNA nor NBMPR alone had effects on any parameter.
receptor subtypes. For example, Ashton et al. (2) demonstrated a reduction in the A3 receptor, an increase in the A2b receptor, and no changes in the A1 or A2a receptor in isolated mouse hearts subjected to ischemia and reperfusion. However, the responses to pathological changes may be substantially modified by aging, since these authors found that, in aged hearts, a selective downregulation of A1 receptors was observed without any effect on other receptor subtypes. Moreover, aging per se reduced A3 receptor expression levels, whereas Jenner et al. (14) reported a reduction in adenosine A1 and A2a receptor expression in the aged rat heart. Taken together, these studies suggest that aging may be an important influence on the response of the adenosine system to pathological changes, including the stimulation of the hypertrophic program. Thus the reduction in adenosine receptor abundance in the aged myocardium may limit the potential antihypertrophic effect of adenosine, at least with respect to \( \alpha_1 \)-adrenoceptor-dependent processes.

In conclusion, we have demonstrated in this study that the adenosine system is upregulated by phenylephrine and the accompanying hypertrophy and that changes in receptor profile, adenosine production, and transport are orchestrated such

Fig. 7. Effects of AR antagonists on the anti-hypertrophic effect of combined EHNA (5 \( \mu \)M) and NBMPR (1 \( \mu \)M) treatment in cardiomyocytes treated for 24 h with PE (10 \( \mu \)M). Values are shown as means ± SE from 6 separate experiments. *\( P < 0.05 \) vs. control.

![Graph showing effects of AR antagonists on anti-hypertrophic effect](...)

Fig. 8. Effect of 24 h PE (10 \( \mu \)M) treatment on equilibrative nucleoside transporter 1 (ENT1) and equilibrative nucleoside transporter 2 (ENT2) mRNA expression in cultured cardiomyocytes. SPT, 8-(p-sulfophenyl)theophylline. Values are shown as means ± SE from 6 separate experiments. *\( P < 0.05 \) vs. control.

![Graph showing ENT1 and ENT2 mRNA expression](...)
that the cardiomyocyte hypertrophic response is minimized.
When taken together, our results point to cardiomyocyte-depended adenosine as an endogenous compensatory anti hypertrophic factor, at least with respect to α₁-adrenoceptor dependent hypertrophy. Although adenosine receptor expression was unaffected by either ET-1 or aldosterone, whether this system responds to other hypertrophic stimuli remains to be determined. Moreover, the precise mechanism(s) by which endogenous adenosine mitigates the hypertrophic response to α₁-adrenoceptor activation remains to be determined with further studies. Based on the present study, manipulation of the endogenous adenosine system in the heart may provide an effective therapeutic approach to mitigating ventricular hypertrophy.

GRANTS
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DISCLOSURES
No conflicts of interest are declared by the authors.

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