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Inhibition of Phenylephrine-induced Cardiomyocyte Hypertrophy by Activation of Multiple Adenosine Receptor Subtypes

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ABBREVIATIONS: ANP, atrial natriuretic peptide; CGS21680, 2-p-(2-carboxyethyl)phenethylamino-5'-N-ethylcarboxamidoadenosine; CPA, N^6 cyclopentyl adenosine; CSC, 8-(3-chlorostyryl) caffeine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; ERK, extracellular signal-regulated protein kinase; IB-MECA, N^6-(3-iodobenzyl)adenosine-5'—methyluronamide; MAPK, mitogen-activated protein kinase; MRS1523, 3-propyl-6-ethyl-5[ethyl(thio)carbonyl]-2-phenyl-4-propyl-3-pyridinecarboxylate; MEM, minimal essential medium; NHE1, sodium-hydrogen exchanger isoform 1; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; RGS, regulator of G-protein signaling; RT-PCR, reverse transcription-polymerase chain reaction; PCR, polymerase chain reaction.

Plasma adenosine levels are elevated in cardiovascular disease including hypertension and heart failure and the nucleoside has been proposed to serve as an endogenous anti-myocardial remodelling factor. We studied the modulation of phenylephrine-induced hypertrophy by adenosine receptor activation in isolated neonatal cultured ventricular myocytes. Phenylephrine (10 μM) increased cell size by 35% and significantly increased expression of atrial natriuretic peptide. These effects were reduced by the stable adenosine analogue 2-chloro adenosine and completely blocked by the adenosine A_1 receptor agonist CPA (1 μM) , the A_{2A} receptor agonist CGS21680 (100 nM) and the A_3 receptor agonist IB-MECA (100 nM). The antihypertrophic effects of all three agonists were completely reversed by their respective antagonists. Phenylephrine significantly upregulated expression of the immediate early gene *c-fos* especially within the first 30 min of phenylephrine treatment. These effects were almost completely inhibited by all adenosine receptor agonists. Although phenylephrine also induced early stimulation of both p38MAPK and ERK, these responses were unaffected by adenosine agonists. The expression of the G-protein regulatory factors RGS2 and RGS4 were increased by nearly three-fold fold by phenylephrine treatment although this was completely prevented by adenosine receptor agonists. These agents also blocked the ability of phenylephrine to upregulate Na-H exchange isoform 1 (NHE1) expression in hypertrophied myocytes. Thus, our results demonstrate an antihypertrophic effect of adenosine acting via multiple receptor subtypes through a mechanism involving downregulation of NHE1 expression. The ability to prevent RGS upregulation further suggests that adenosine receptor activation minimizes signalling which leads to hypertrophic responses.

Key Words: adenosine, adenosine receptors, myocyte hypertrophy, MAP kinase, RGS proteins, Na-H exchange

Introduction

Adenosine, a product of adenine nucleotide catabolism, has been demonstrated to exert numerous effects on the cardiovascular system. For example, the vasodilating property of adenosine is well-established and it has been proposed that adenosine is an important regulator of coronary vascular resistance particularly under hypoxic situations; a phenomenon termed the "Berne hypothesis." Extensive evidence has been presented demonstrating a cardioprotective effect of adenosine, as well as adenosine analogues, against ischemic and reperfusion injury using a variety of both *in vitro* and *in vivo* approaches (reviewed in Headrick et al., 2003) which may be due, at least in part, to inhibition of the deleterious effects of oxidative stress on the heart (Karmazyn and Cook, 1992).

There is emerging evidence that adenosine could represent an important regulator of the myocardial remodelling process in response to various stimuli. Plasma adenosine levels are elevated in experimental hypertension (Ohnishi et al., 1988; Yamada et al., 1992) and elevated plasma concentrations of the nucleoside have been demonstrated in patients with congestive heart failure irrespective of causative factor (Funaya et al., 1997). With respect to the latter, the degree of elevation in plasma adenosine concentrations was dependent on the severity of heart failure according to New York Heart Association [NYHA] classification with the greatest increases [more than 5 fold] observed in NYHA class IV patients (Funaya et al., 1997). A study from the same group also demonstrated that administration of nucleoside transport inhibitors dipyridamole and dilazep (which increase adenosine levels) for 6 months reduced the severity of heart failure with an improvement in both ejection fraction and maximal oxygen consumption

with the beneficial effects reversing after drug discontinuation (Kitakaze et al., 1998). Elevated levels of adenosine in cardiovascular disease may therefore represent a protective mechanism, for example elevated adenosine could counter the deleterious effects of sympathetic nervous stimulation by its well-known direct vasodilating effect or by inhibition of neurotransmitter release. Adenosine could further exert antihypertensive effects by blocking the renin angiotensin system (Ohnishi et al., 1988). In rats with pressure overloaded hypertrophy the adenosine uptake blocker dipyridamole reduced abnormal left ventricular chamber filling and preserved adrenergic responsiveness in pressure overload-induced hypertrophy (Chung et al., 1998).

Extensive studies on the potential direct effects of adenosine on the remodelling process have not been carried out particularly in terms of understanding mechanisms. In the present report, we used neonatal cultured rat ventricular myocytes to assess the effect of adenosine, and more specifically, selective adenosine receptor agonists on hypertrophy induced by the α_1 adrenoceptor agonist phenylephrine.

Methods

Cell Cultures

Primary myocyte cultures were prepared from neonatal Harlan Sprague-Dawley rat heart ventricles as described previously (Karmazyn et al., 2003). Isolated primary myocytes were plated onto glass coverslips for physiologic studies, or onto Primaria^{TM} (Falcon) culture dishes or flasks for collection of cell extracts. Myocytes were maintained for 48 h in medium containing Dulbecco's modified Eagle medium/Ham's F-12 supplemented with 10% fetal bovine serum, 10 μg/ml transferrin, 10 μg/ml insulin, 10 ng/ml selenium, 50 units/ml penicillin, 50 μg/ml streptomycin, 2 mg/ml bovine serum albumin, 5 μg/ml linoleic acid, 3 mM pyruvic acid, 0.1 mM minimum essential medium non-essential amino acids, 10% minimal essential medium (MEM) vitamin solution, 0.1 mM bromodeoxyuridine, 100 μM L-ascorbic acid, and 30 mM HEPES, pH 7.2.

Experimental Protocol

Cells were serum-starved 24 h prior to all experiments after which cells were treated for 24 hours with 10 μM phenylephrine. To assess the effect of adenosine in modulating hypertrophic responses we first determined the effect of the stable adenosine analogue, 2Cl-adenosine (10 μM). The effect of phenylephrine was subsequently studied in the presence of the following adenosine receptor selective agonists: the A_1 receptor agonist N^6 cyclopentyl adenosine (CPA 1 μM), the A_{2A} receptor agonist 2-p-(2-carboxyethyl)phenethylamino-5'-N-ethylcarboxamidoadenosine (CGS 21680, 100 nM) and the A_3 receptor agonist N^6-(3-iodobenzyl)adenosine-5'-–methyluronamide (IB-MECA, 100 nM). Additionally, the effect of

their corresponding antagonists, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 10 μM), 8-(3-chlorostyryl) caffeine CSC (10 μM)) and 3-propyl-6-ethyl-5[ethyl(thio)carbonyl]-2-phenyl-4-propyl-3-pyridinecarboxylate (MRS 1523, 1 μM), respectively was determined. The concentrations of drugs used in the present study reflect those which were found to exert maximal effects in initial experiments aimed at identifying optimal concentrations of each agent. To confirm selectivity of the effects of antagonist against their respective receptors, experiments were performed in which the effect of the specific receptor antagonist was also tested against agonists acting on either of the other two receptors. For all experiments, 2Cl-adenosine, adenosine receptor agonists or the agonist-antagonist combination was added 10 minutes before administration of phenylephrine.

Measurement of Cell Surface Area

Cardiomyocyte surface area was determined for 50 randomly selected cells per experiment and averaged to provide an N value of one. Cells were visualized with a Leica inverted microscope equipped with a Polaroid digital camera using 20x magnification. Cell area was determined using Mocha software.

Determination of Gene Expression

RT-PCR was used to analyze NHE-1 and ANP mRNA expression. The NHE-1 primers used were primer 1 5'-TCTGTGGACCTGGTGAATGA-3' and primer 2 5'-GTCACTGAGGCAGGGTTGTA-3' with a predicted product size of 210 bp and a competitor size of 292 bp. The forward and reverse primers for rat ANP were 5'-CTGCTAGACCACCTGGAGGA-3' and 5'-AAGCTGTTGCAGCCTAGTCC-3', respectively

with a PCR product of 320 bp. Samples were then electrophoresed in 3 % agarose gels containing ethidium bromide and quantified via computer densitometry. GAPDH was used as the reference gene for ANP expression whereas for NHE1 expression the gene product was standardized using competitive PCR where NHE1 mRNA expression was compared to amplification of competitor fragments. For GAPDH the forward and reverse primers were 5'-AAAGGGCATCCTGGGCTACA-3' and 5'-CAGTGTTGGGGGCTGAGTTG-3', respectively.

Real time PCR was used to assess mRNA expression of *c-fos* and RGS using a DNA Engine Opticon 2 system (MJ Research, MA) according to the manufacturer's instruction. The ribosomal 18S rRNA gene was used as an internal control. The following primer sequences were used: for RGS2, forward; 5'-AGCAAATATGGGCTTGCTGCAT-3', reverse; 5'-GCCTCTTGGATATTTTGGGCAATC-3'; for RGS4, forward; 5'GACTTCTGGATCAGCTGTGA-3', reverse; 5'-CAGTCTGCAGAACTCTTGGC-3', for *c-fos*, forward; 5'-GTCTGCGTTGCAGACCGAGATTGC-3', reverse; 5'-CTCCAGCTCTGTGACCATGGG-3' and for 18SrRNA forward: 5'GTATCCCGTTGAACCCCATT-3', reverse 5'-CCATCCAATCGGTAGTAGCG-3'.

Western Blotting for p38MAPK, ERK and NHE-1

The cells were plated at a concentration of 6 x 10^6 cells/ 6 cm dish. After washing with PBS two times, the cells were scraped into 100 µl lysis buffer (20 mM Tris, 150 mM NaCl, 1% Triton-X 100, 10% glycerol, 2 mM EDTA, 2mM EGTA, 50 mM NaF, 200 µM Na_3VO_4, 10 mM $Na_4P_2O_7$, 40 mM β-glycerophosphate, 10 µg/ml leupeptin, 1 µM pepstatin A, 1 mM PMSF, and 1 µM colyculin A). The lysate was transferred to a 1.5 ml Ependorf tube, homogenized and

centrifuged at 10000 x g for 5 min at 4 ^{0}C. The supernatant was transferred to a fresh tube. The protein concentration was assayed by Bradford protein assay kit (Bio-Rad). 30 μg protein were loaded in 10% SDS-PAGE, and transferred to nylon membrane (Amersham). The membranes were blocked in 5% dry milk for 3 hours, primary antibody for 2h, secondary antibody for 1h, and then detected by ECL reagent (Amersham). Antibodies were purchased from Cell Signaling and used at 1:500, 1:1000 and 1:2000 dilutions for NHE-1, p38MAPK and ERK, respectively .

Statistical Analysis

Results are given as means ± SEM. Statistical analyses were performed by using one-way analysis of variance (ANOVA). The post-test comparison was performed by the method of Bonferroni. Differences were considered significant when $P<0.05$.

Results

Figure 1 demonstrates representative example of cells exposed to phenylephrine in the presence or absence of adenosine agonists. The hypertrophic phenotype was evident in phenylephrine-treated cells but not in those treated with any of the adenosine agonists. Quantitative data are shown in Figures 2 - 5. As shown in Figure 2, 2Cl-adenosine, the non hydrolysable form of the nucleoside, blocked the phenylephrine-induced hypertrophy as determined by cell size as well as ANP expression. We next examined the nature of adenosine receptor involvement in mediating the antihypertrophic effect of adenosine by determining the effects of specific receptor agonists. These agents included the A_1 receptor agonist CPA, the A_{2A} receptor agonist CGS21680 and the A_3 receptor agonist IB-MECA. These results are summarized in Figures 3-5. Surprisingly, all three agonists equally inhibited the hypertrophic effect of phenylephrine. To further demonstrate receptor specificity in mediating the antihypertrophic effect of adenosine receptor agonists we determined whether these effects could be reversed by their respective antagonists. As shown in Figures 3-5, all adenosine receptor antagonists reversed the effect of their respective agonists against phenylephrine-induced hypertrophy. Moreover, none of the agonists on their own exerted any effects on basal cell area or ANP expression (Figures 2-5). To confirm receptor antagonist selectivity against their respective agonists we tested the relative ability of each antagonist to alter the response of either of the other two agonists against phenylephrine-induced hypertrophy (n=6 for all groups). Phenylephrine on its own increased cells size by 39 ± 7% which was inhibited by the A1 receptor agonist CPA alone (5 ± 2% increase), or in the presence of the A_{2A} antagonist CSC (13 ± 2%) or the A3 antagonist MRS (9

± 2%). Similarly, the effect of the A_{2A} agonist CGS 21680 was also unaffected by the A1 receptor antagonist DPCPX (9 ± 3%) or the A3 antagonist MRS (4 ±1%). Finally, in the last set of studies the effect of the A3 agonist was unaffected by either DPCPX (7 ± 2%) or CSC (8 ± 3%). Thus, although a slight attenuation of the antihypertophic effect of CPA was observed with CSC, the results confirm the relative selectivity of adenosine receptor antagonists against their specific receptor subtypes.

The ability of adenosine receptor agonists to activate MAP kinase was next determined by studying the early phosphorylation of p38MAPK and p44/42 (ERK). Phenylephrine increased ERK phosphorylation with peak values attained by 5 and 15 minutes after drug addition (Figure 6) However, there was generally no effect of any of the adenosine receptor agonists. Virtually identical results were observed with respect to p38MAPK (Figure 7). MAP kinase activities in cells treated for longer than 60 minutes were identical to basal values and unaffected by any treatment (not shown). Increased proto-oncogene expression has also been implicated in the development of cardiomyocyte hypertrophy and we therefore determined mRNA expression of *c-fos* after phenylephrine addition in the absence or presence adenosine receptor agonists. As shown in Figure 8 phenylephrine produced a rapid *c-fos* induction during the initial 30 min of phenylephrine treatment, however this was significantly attenuated by all adenosine receptor agonists.

Recent evidence suggests the involvement of NHE1 in cardiomyocyte hypertrophy as well as an ability of adenosine to inhibit phenylephrine stimulated NHE1 activation in cardiomyocytes, through an A_1 receptor mechanism. Accordingly, we studied the relationship between NHE1

expression and the antihypertrophic effects of adenosine receptor antagonists. As Figure 9 illustrates, 24 hr exposure to phenylephrine increased cardiomycyte NHE1 expression, however, this was completely prevented by adenosine receptor activation, the latter effect being reversed in the presence of the receptor antagonist. Moreover, using Western blotting we observed that NHE-1 protein abundance was increased by 37 ± 3.8 % by phenylephrine alone ($P<0.05$ from control) whereas in the presence of either CPA, CGS21680 or IB-MECA the effects of phenylephrine were reduced to 3.8 ± 0.53%, 6.2 ± .86% and 13 ± 3.8% ($P>0.05$, n=5 for all groups).

Phenylephrine also increased the expression of both RGS2 and RGS4, which are important in regulating Gq-dependent processes, by nearly three-fold although these effects were completely abrogated by all three adenosine receptor agonists as well as 2Cl-adenosine (Figure 10).

Discussion

We studied the effect of adenosine receptor modulators on the hypertrophic effect of the α_1 adrenoceptor agonist phenylephrine, an agent which has been used extensively to initiate hypertrophy in cultured myocytes. 2Cl-adenosine, the non selective adenosine receptor agonist which is not subjected to extensive hydrolysis by adenosine deaminase as is the parent nucleoside, effectively inhibited the hypertrophy produced by phenylephrine although the effect was generally less than that produced by individual receptor agonists alone. Although speculative, perhaps this reflects a non-specific effect of the nucleoside or some degree of either hydrolysis or cellular uptake of 2Cl-adenosine by the nucleoside transporter thereby limiting its efficacy.

We then studied the effect of agonists acting on the A_1, A_{2A} and A_3 receptors on their ability to modulate the phenylephrine induced hypertrophy. These receptor agonists were selected based on our ability to identify the abundant presence of these receptor subtypes using Western blotting whereas only faint A_{2B} receptor blots were seen (not shown). Moreover, the present lack of availability of highly selective A_{2B} receptor ligands led us to concentrate our studies on the other three receptor subtypes. It was surprising that the antihypertrophic effects of adenosine receptor agonists were similarly observed with agents acting on the A_1, A_{2A} and A_3 receptors in relatively equal fashion. Nonetheless, despite the fact that these receptors are coupled to diverse cell signalling processes activators of multiple adenosine receptor subtypes have also been shown to protect the ischemic and reperfused myocardium (Yao and Gross 1993; Auchampach et al., 2004; Boucher et al., 2004). In the present study, the antihypertrophic effect of adenosine

agonists was associated with prevention of early upregulation of the immediate early gene *c-fos* which is likely an important event during the development of early hypertrophy (Babu et al., 2000). The early activation of both ERK and p38MAPK, both of which are also likely important in the hypertrophic response (Zechner et al., 1997; Bueno and Molkentin, 2002) was unaffected by any of the adenosine receptor agonists.

In general, our study concurs with the recent report of Liao and coworkers who similarly demonstrated an antihypertrophic effect of 2Cl-adenosine in neonatal rat ventricular myocytes although in that study the effect was mimicked only by an A_1 receptor agonist whereas both an A_{2A} and an A_3 agonist were ineffective (Liao et al., 2003). The reasons for the differences in receptor-mediated effects are uncertain although the effects which we observed were unlikely to involve nonspecific effects of either the A_{2A} or A_3 agonists since their antihypertrophic effects, as well as those of the A_1 agonist CPA, were prevented by their respective antagonists which on their own were devoid of direct effects. Moreover, at the concentrations used the effects of these antagonists were restricted to their respective agonists suggesting a high degree of selectivity. We also used identical pharmacological tools to probe the adenosine system such that differences in agents used could not account for the differences in responses seen in the two studies although the concentration of phenylephrine in our study was 10 fold lower than that used by Liao et al (2003) since in our hands this concentration produced maximum effect on hypertrophy. However, whether this accounts for the different responses seen in the two studies remains to be determined. It is interesting however to add that a recent study reported an antimitogenic effect of adenosine in cardiac fibroblasts which could further contribute to an

the nucleoside (Dubey et al., 2001). However, in that study these effects were found to be exclusively dependent on adenosine A_{2B} receptor activation with no effect of either an A1 or an A_{2A} receptor agonists (Dubey et al., 2001). Thus, the exact nature of the receptors mediating the antihypertrophic or antiremodelling effect of adenosine requires further studies.

Our study was also designed to explore the potential mechanisms underlying the antihypertrophic effect of adenosine. As previously suggested (Kitakaze and Hori, 2000) there are a number of potential mechanisms by which adenosine could exert salutary effects in heart failure such as attenuation of norepinephrine release or decreased endothelin, angiotensin II or cytokine production. Moreover, collagen and protein synthesis by isolated cardiac fibroblasts is inhibited by adenosine suggesting that adenosine has an antifibrosis effect (Dubey et al., 2001). Lastly, it should be emphasized that in our study the antihypertrophic effect of adenosine or adenosine receptor agonists was restricted to determining their effects against phenylephrine and it remains to be determined whether the effect we report with multiple adenosine receptor agonists can also be applied to other hypertrophic stimuli or to the in vivo situation.

We also examined NHE1 expression for a number of reasons. For example, there is now strong evidence which suggests an important role for this transporter in mediating cardiac hypertrophy (Cingolani, 1999; Karmazyn, 2001). Secondly, there is also evidence that adenosine inhibits α_1 adrenergic-mediated activation of NHE1 in cardiac cells through a mechanism mediated by A_1 receptors (Avkiran and Yokoyama, 2000). Although the effect of A_{2A} or A_3 receptor activation on NHE1 activity in cardiac cells has not been reported, preliminary studies

in our laboratory indicate that CGS21680 and IB-MECA at concentrations effective in reducing hypertrophy are also effective in inhibiting NHE1 activity in cardiomyocytes subjected to intracellular acidosis by ammonium chloride pulsing. Thus it appears that activation of multiple adenosine receptor subtypes could inhibit NHE1 activity. In the spontaneously hypertensive rat, treatment with an NHE1 inhibitor significantly attenuates myocardial hypertrophy and fibrosis (Camilion de Hurtado et al., 2002) whereas standard antihypertensive treatment with an ACE inhibitor or a calcium channel or AT_1 receptor blocker normalizes upregulated myocardial NHE1 activity (Alvarez et al., 2002). Taken together the possibility that NHE1 is related to the antihypertrophic effect of adenosine receptor activation was considered. Our results did indeed demonstrate that the antihypertrophic effect of adenosine receptor activation was associated with completely blocked NHE1 upregulation which occurred after phenylephrine administration in the absence of adenosine receptor agonists. Although this does not prove a cause and effect relationship, the results suggest that inhibition of NHE1 upregulation may account at least in part for the antihypertrophic effect of adenosine receptor agonists.

Adenosine receptors have been initially classified primarily with respect to their ability to affect adenylate cyclase activity. For example adenosine A_1 and A_3 receptor stimulation results in adenylate cyclase inhibition whereas A_{2A} receptor mediates adenylate cyclase activation (reviewed in Schulte and Fredholm, 2003). Thus, it is unlikely that the ability of adenosine receptor activation to modulate adenylate cyclase activity was a common mechanism for the antihypertrophic effect of adenosine receptor activation. It is interesting that phenylephrine significantly increased both RGS2 and RGS4 expression which was completely prevented by

adenosine receptor agonists. RGS proteins which act as GTPase activating proteins thus limiting

G-protein dependent responses are upregulated in a variety of cells and tissues by different stimuli, including the activation of Gq- and Gs-coupled receptors. Gq-mediated increases in RGS2 have been attributed to increased intracellular calcium, increased protein kinase C activity, or both (reviewed in Chidiac and Roy, 2003). Thus the increase in RGS2 observed herein in response to the α_1 adrenoceptor activation may be related to these effects although this needs to be determined with further experiments. It is interesting that although RGS proteins appear to be antihypertrophic, overexpression of RGS4 in transgenic mice has been shown to enhance mortality following left ventricular pressure overload produced by aortic coarctation possibly due to reduced cardiac compensation (Rogers et al., 1999). The mechanism(s) via which adenosine receptor agonists attenuate RGS protein upregulation is at present unclear. We have previously demonstrated that adenosine A_1 receptor activation inhibits phenylephrine-induced cardiomyocyte changes including the increase in the calcium transient, however no effect was seen with either an A_{2A} or A_3 agonist (Hoque et al., 2000). Cardiomyocyte adenosine receptors are not known to decrease phospholipase C activity and indeed some adenosine receptor subtypes are linked to PKC activation (Henry et al., 1996). Intriguingly, one study has shown RGS2 to be increased in human heart failure and that symptomatic relief resulting from using a left ventricular assist device (LVAD) was accompanied by a decrease in this protein back to levels observed in nonfailing hearts (Takeishi et al., 2000). It is interesting that the use of LVAD has been shown to reverse left ventricular hypertrophy (Zafeiridis et al., 1998). While

extrapolation to the present study should be done cautiously the finding may be analogous to the antihypertrophic effect of adenosine. Taken together with the present results, a logical scenario may be that RGS proteins are upregulated to minimize deleterious signaling under hypertrophic conditions, returning to normal levels when such stresses are relieved, ie with adenosine receptor activation. However, the precise role of either RGS2 or RGS4 in mediating hypertrophic or antihypertrophic responses requires further studies particularly in view of, as already alluded to above, a potential deleterious effect of RGS protein upregulation under certain conditions (Rogers et al., 1999). Indeed, an in depth study to determine the role of RGS proteins in general in mediating the antihypertrophic influence of adenosine would be important.

Although neonatal cardiomyocytes have been used extensively to study the cellular and molecular basis for hypertrophic responses, nonetheless studies with these cells should be interpreted cautiously because of potential differences when compared to cardiomyocytes from adult animals. Our results add strength to the concept that endogenous adenosine serves as antihypertrophic factors especially under conditions where plasma levels of the nucleoside are elevated such as hypertension or heart failure.

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organization, and cardiac-specific gene expression. *J Cell Biol* **139**:115-127.

Figure Legends.

Figure 1. Representative micrographs illustrating cardiomyocytes exposed for 24 hours under control conditions or with phenylephrine alone (PE) or in the presence of 2 chloroadenosine (2Cl-Ad), the A_1 receptor agonist CPA, the A_{2A} receptor CGS21680 (CGS) or the A_3 receptor agonist IB-MECA (IB). Original magnification= 20x.

Figure 2. The effect of 2Cl-adenosine (2Cl-Ade, 10 μM) on phenylephrine (PHE, 10 μM) induced hypertrophy as determined by cell surface area and ANP expression. Bars indicate mean ± SE from 10 separate experiments. *P<0.05 from control; $^{+}$P<0.05 from PHE alone. Bottom panel shows representative agarose gel for PCR products. All values were determined 24 hours after phenylephrine treatment.

Figure 3. The effect of the adenosine A_1 agonist CPA (1 μM) in the absence or presence the A_1 antagonist DPCPX (10 μM) on phenylephrine (PHE, 10 μM) induced hypertrophy as determined by cell surface area and ANP gene expression. Bars indicate mean ± SE from 10 separate experiments. *P<0.05 from control; $^{+}$P<0.05 from PHE alone. Bottom panel shows representative agarose gel for PCR products. All values were determined 24 hours after

phenylephrine treatment.

Figure 4. The effect of the adenosine A_{2A} agonist CGS21680 (CGS, 100 nM) in the absence or presence the A_{2A} antagonist CSC (10 μM) on phenylephrine (PHE, 10 μM) induced hypertrophy as determined by cell surface area and ANP expression. Bars indicate mean ± SE from 10 separate experiments. *P<0.05 from control; $^{+}$P<0.05 from PHE alone. Bottom panel shows representative agarose gel for PCR products. All values were determined 24 hours after phenylephrine treatment.

Figure 5. The effect of the adenosine A_3 agonist IB-MECA (IB, 100 nM) in the absence or presence the A_3 antagonist MRS (1 μM) on phenylephrine (PHE, 10 μM) induced hypertrophy as determined by cell surface area and ANP expression. Bars indicate mean ± SE from 10 separate experiments. *P<0.05 from control; $^{+}$P<0.05 from PHE alone. Bottom panel shows representative agarose gel for PCR products. All values were determined 24 hours after phenylephrine treatment.

Figure 6. Profiles of p44/42 and phospho p44/42 (P-p44/42) levels in cardiomyocytes after addition of phenylephrine (PHE, 10 μM) alone or in the presence of the A_1 receptor agonist CPA (1 μM), the A_{2A} receptor agonist CGS 21680 (CGS, 100 nM) and the A_3 receptor agonist IB-MECA (100 nM). Bars indicate mean ± SE from 10 separate experiments with

corresponding examples of Western blots for both p44/42 and P-p44/42. *P<0.05 from control.

Figure 7. Profiles of p38 and phospho p38 (P-p38) levels in cardiomyocytes after addition of phenylephrine (PHE, 10 μM) alone or in the presence of the A_1 receptor agonist CPA (1 μM), the A_{2A} receptor agonist CGS 21680 (CGS, 100 nM) and the A_3 receptor agonist IB-MECA (100 nM). Bars indicate mean ± SE from 10 separate experiments with corresponding examples of Western blots for both p44/42 and P-p44/42. *P<0.05 from control.

Figure 8. Profiles of *c-fos* mRNA levels in cardiomyocytes after addition of phenylephrine (PHE, 10 μM) alone or in the presence of the A_1 receptor agonist CPA (1 μM), the A_{2A} receptor agonist CGS 21680 (CGS, 100 nM) and the A_3 receptor agonist IB-MECA (100 nM). Data were obtained using real time PCR. Bars indicate mean ± SE from 8 separate experiments. *P<0.05 from respective values whereas $^+$ denotes significantly higher values from 5 min controls. All values were determined 24 hours after phenylephrine treatment.

Figure 9. Effect of phenylephrine (PHE, 10 μM) on NHE1 gene expression in cardiac myocytes. Cells were treated with phenylephrine alone or in the presence adenosine agonists in the presence or absence of their respective antagonists. Bars indicate mean ± SE from 10 separate experiments. *P<0.05 from control. All values were determined 24 hours after phenylephrine treatment.

Figure 10. Profiles of RGS2 and RGS4 mRNA levels in cardiomyocytes after addition of phenylephrine (PHE, 10 μM) alone or in the presence of the A_1 receptor agonist CPA (1 μM), the A_{2A} receptor agonist CGS 21680 (CGS, 100 nM), the A_3 receptor agonist IB-MECA (100 nM) or the adenosine analogue 2Cl-Ade (10 μM). Data were obtained using real time PCR. Bars indicate mean ± SE from 8 separate experiments. *P<0.05 from control. All values were determined 24 hours after phenylephrine treatment.

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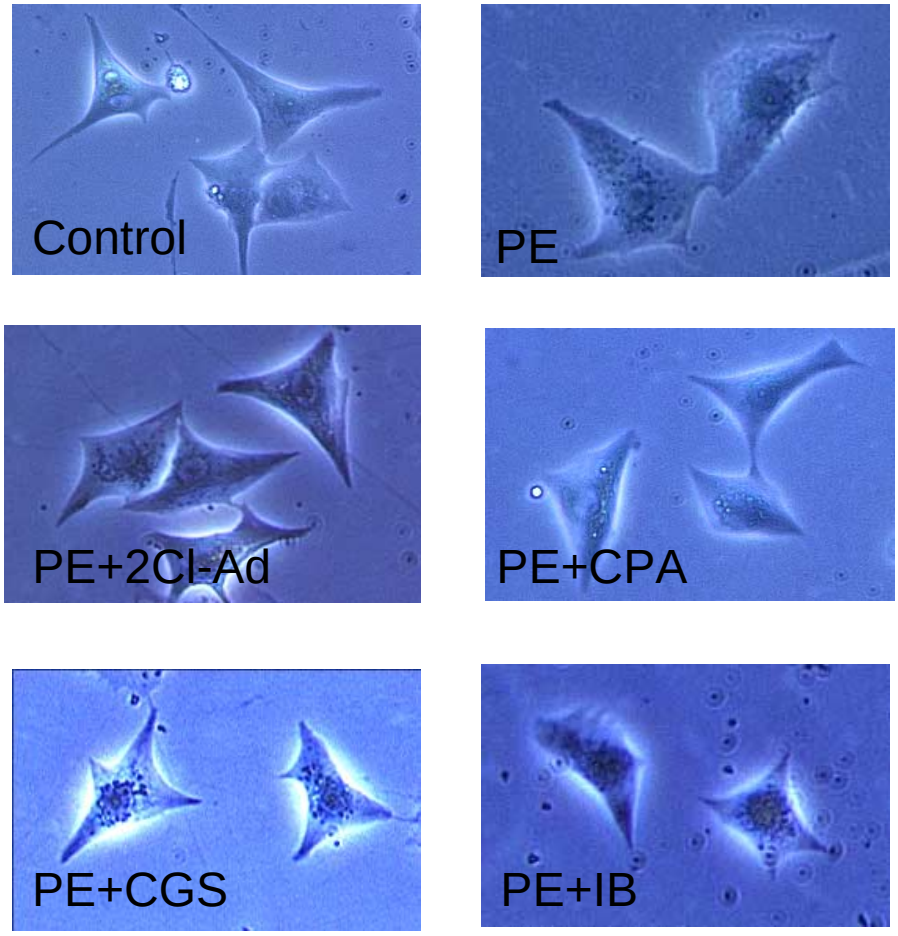

Figure 1

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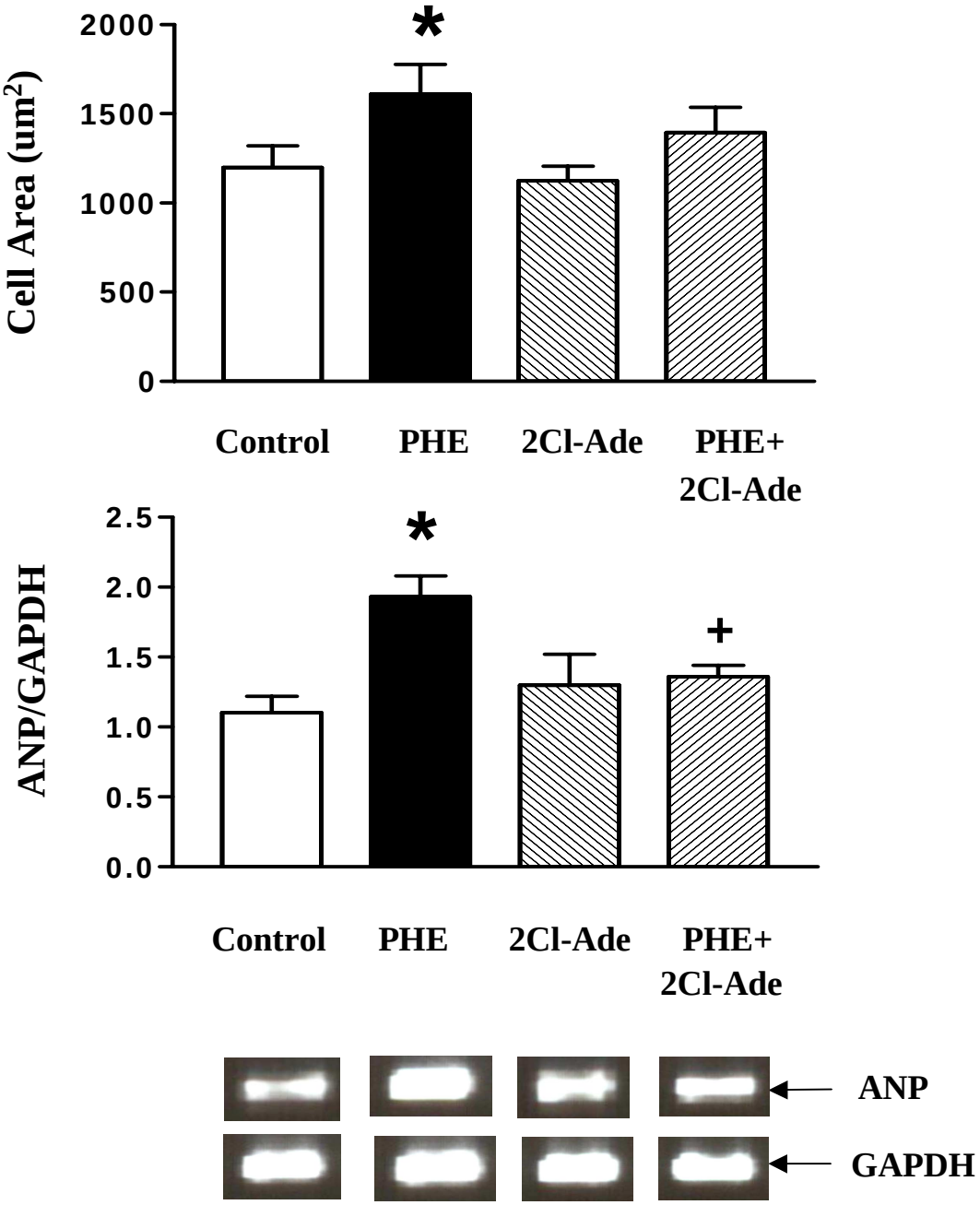

Figure 2

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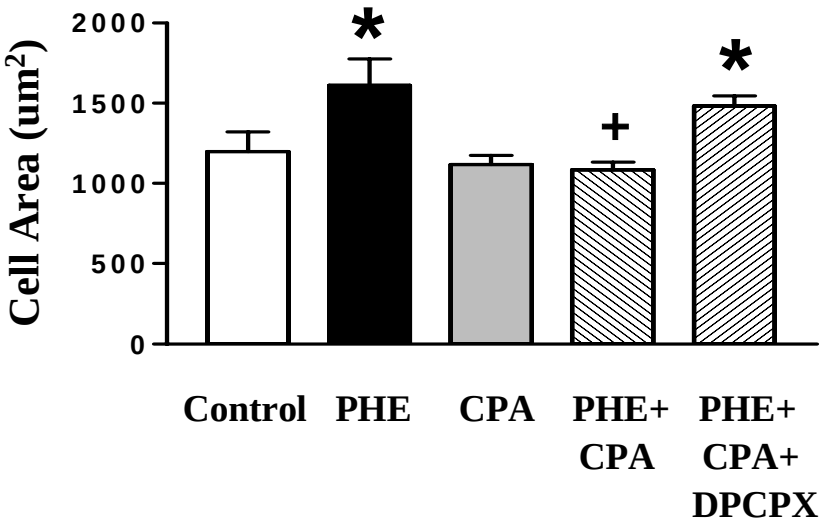

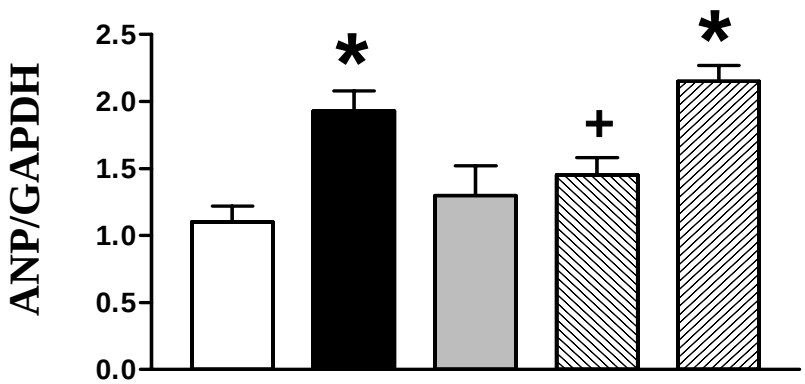

Figure 3

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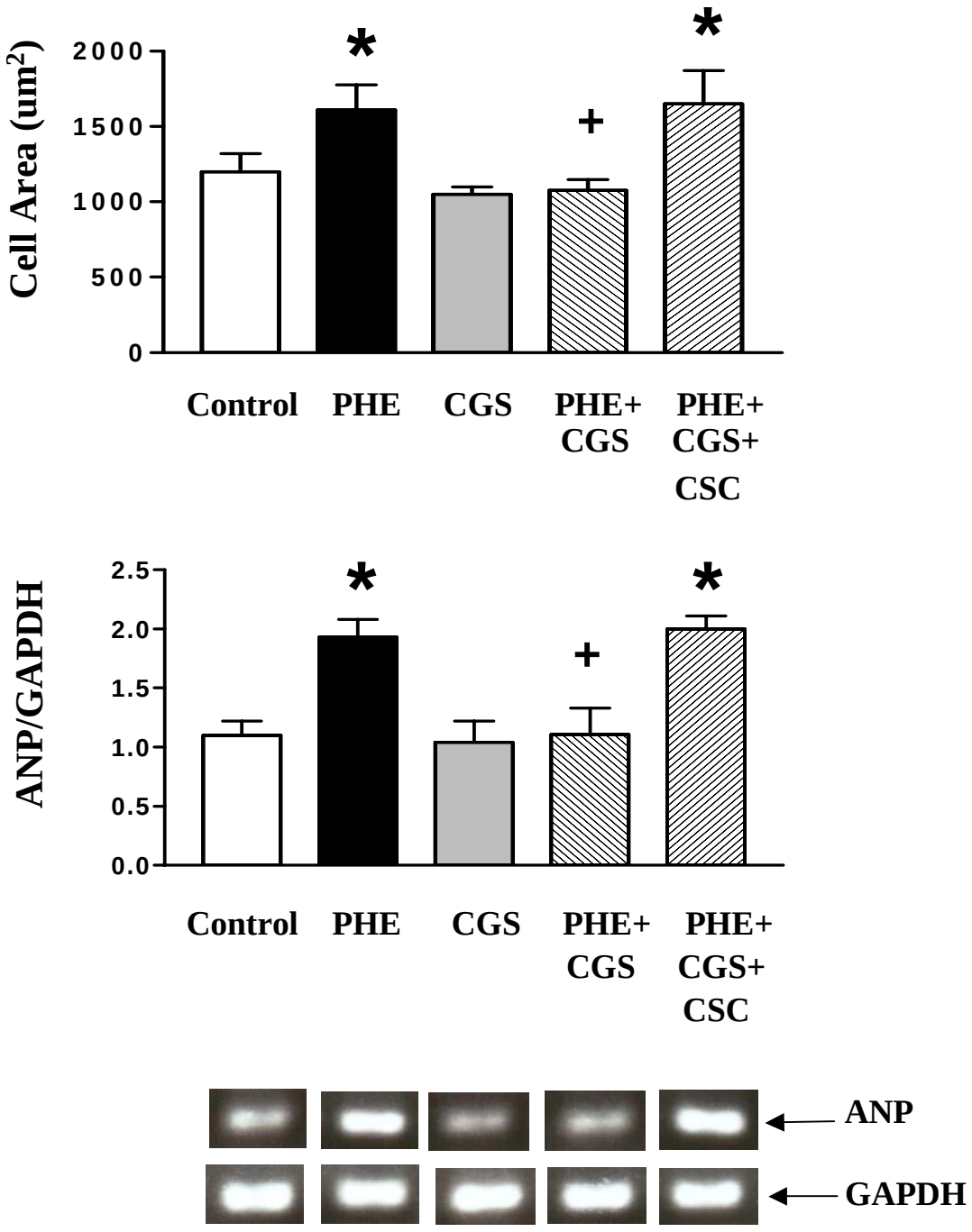

Figure 4

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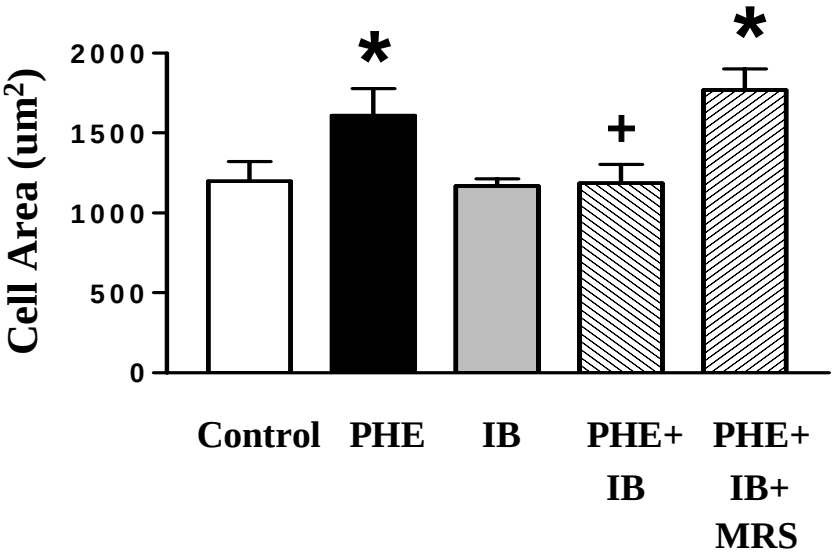

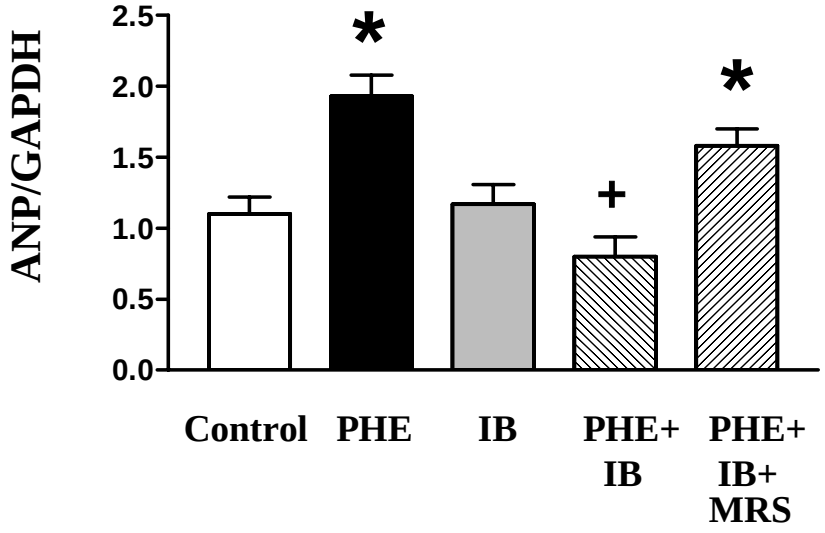

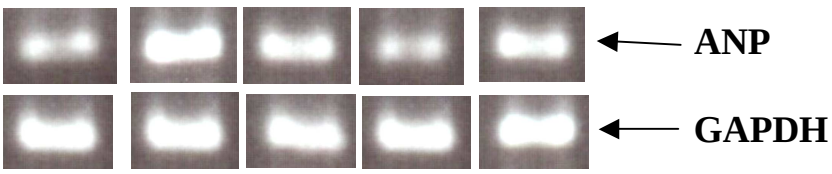

Figure 5

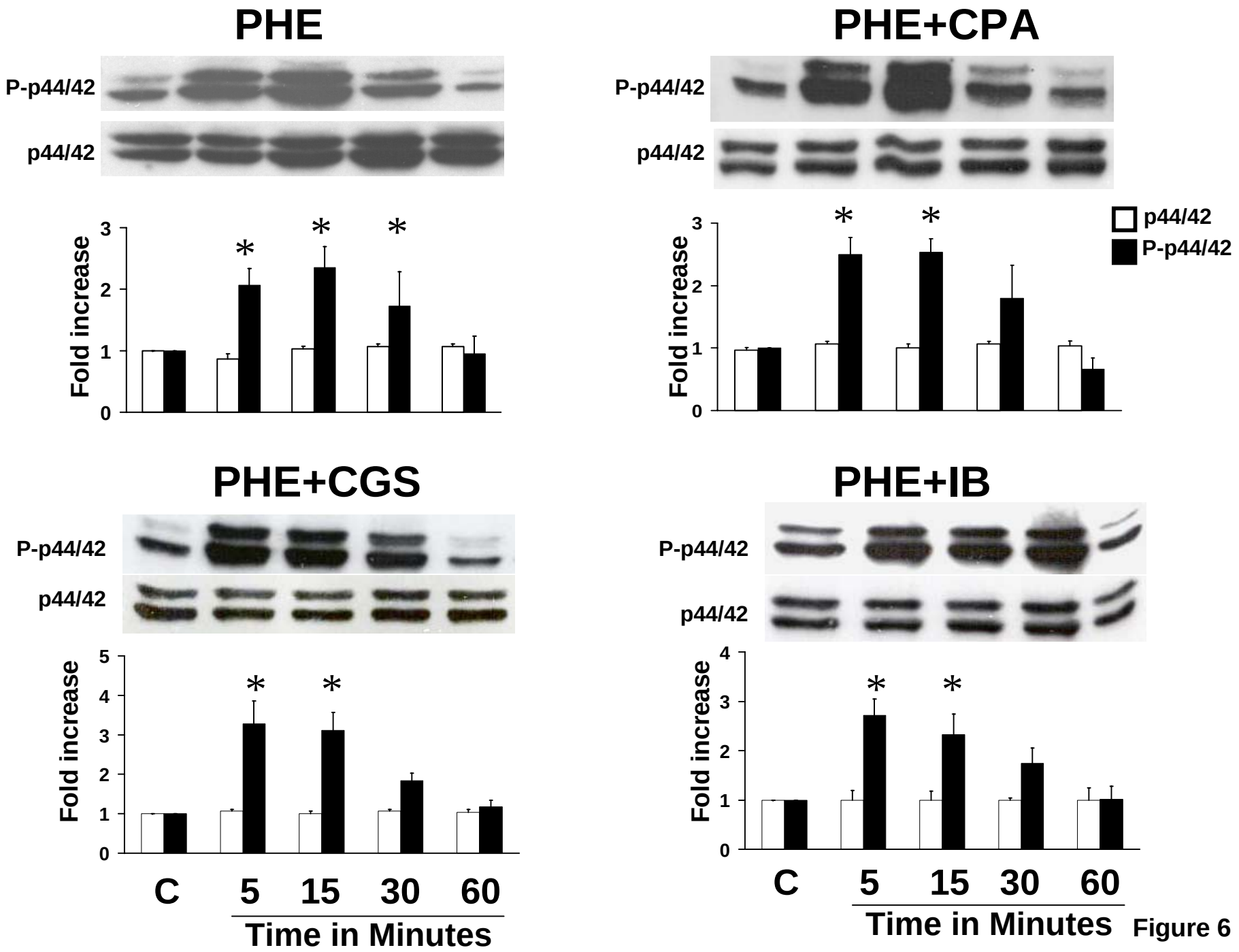

Figure 6

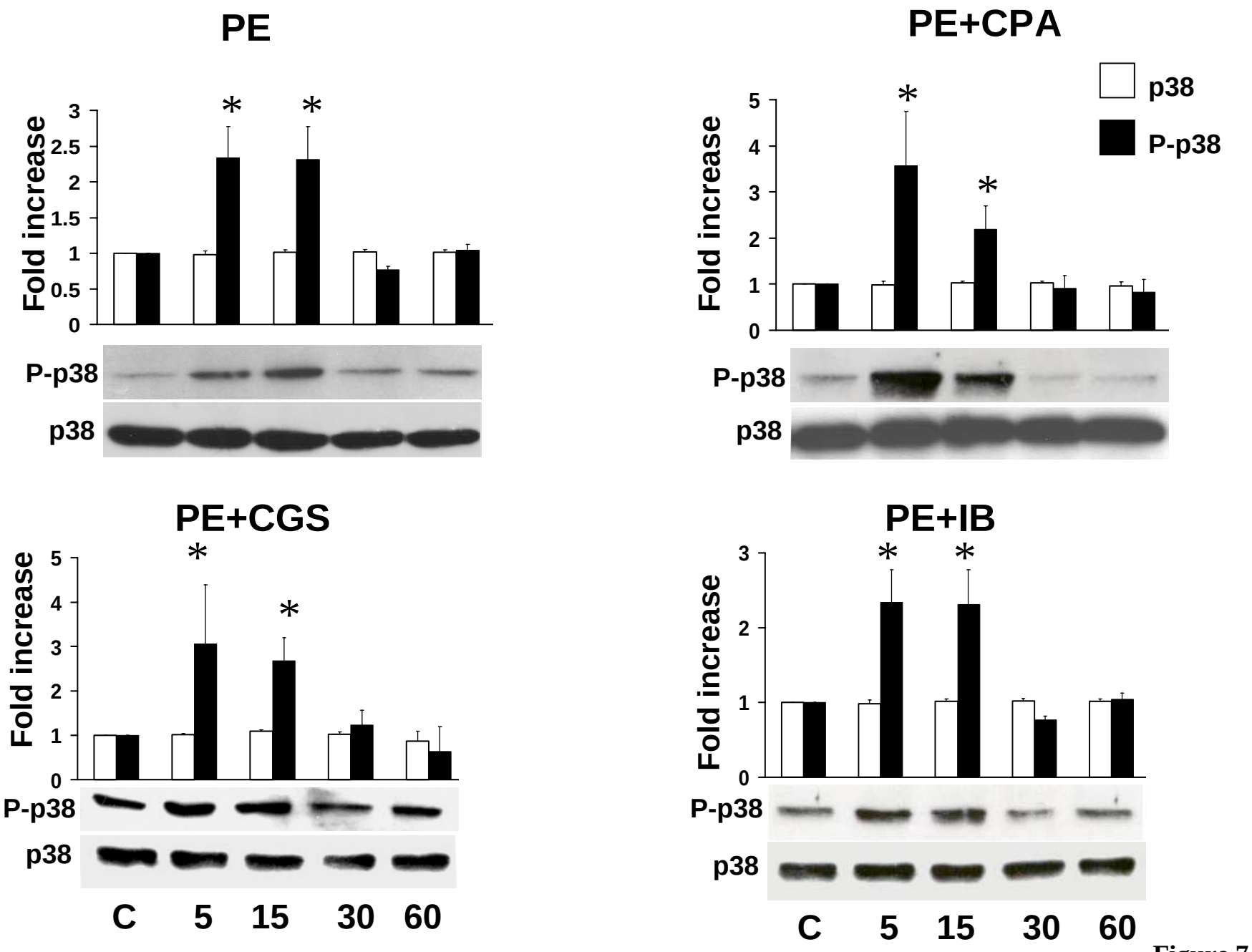

Figure 7

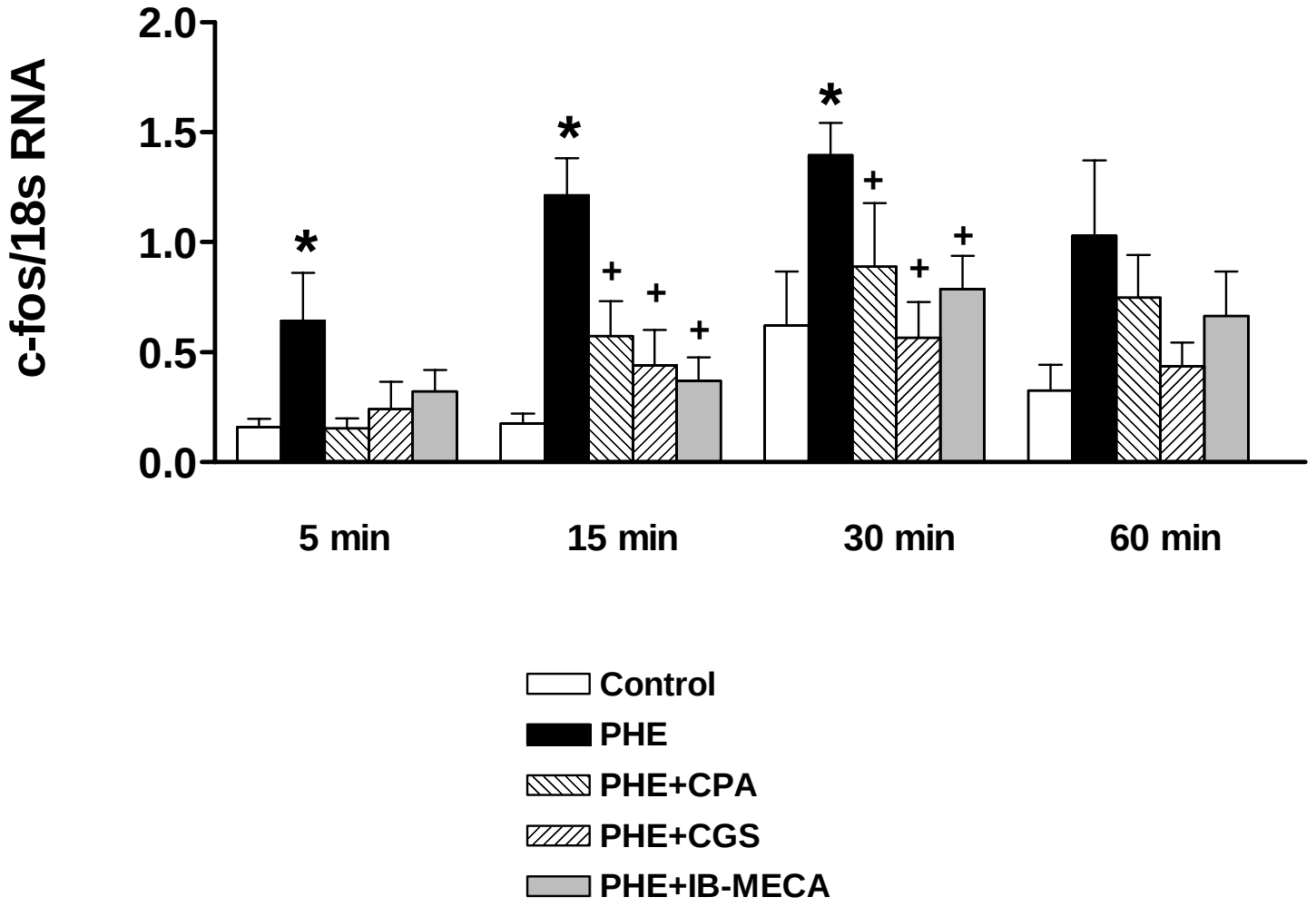

Figure 8

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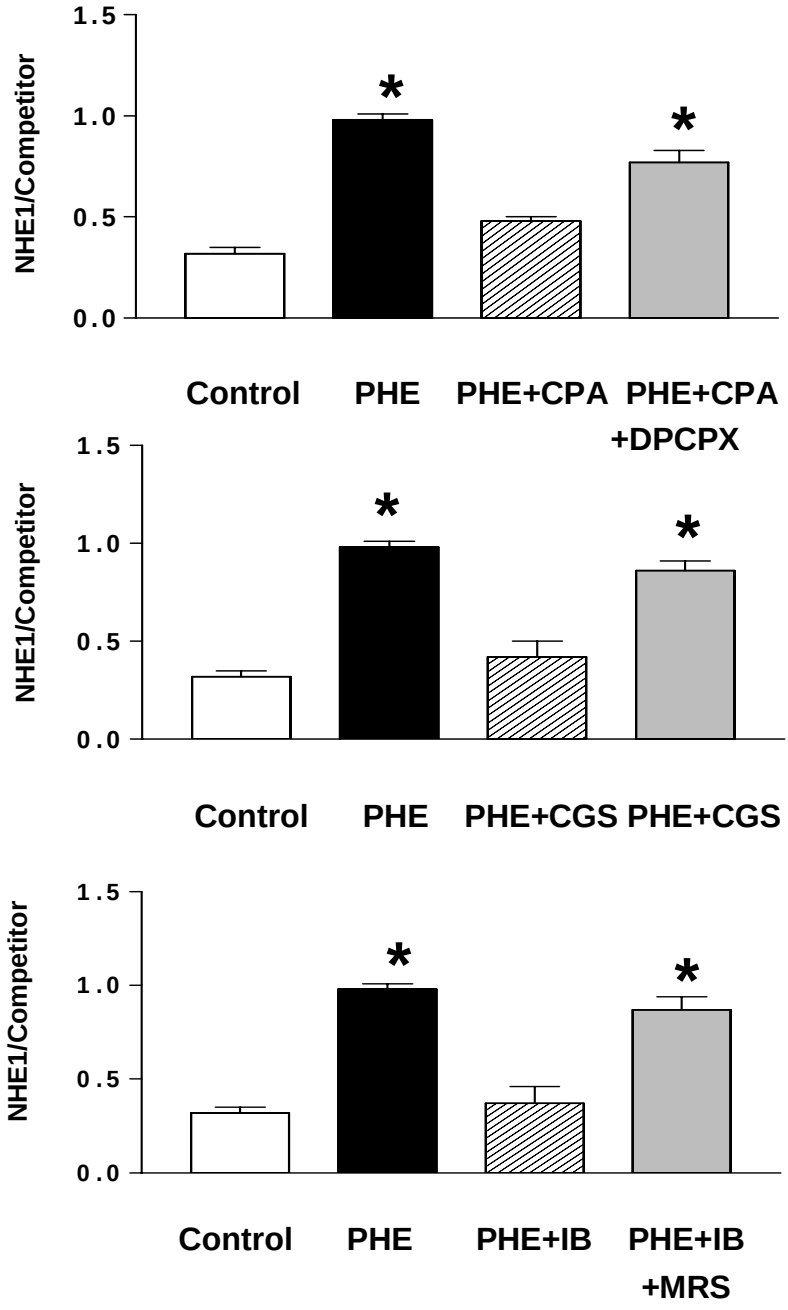

Figure 9

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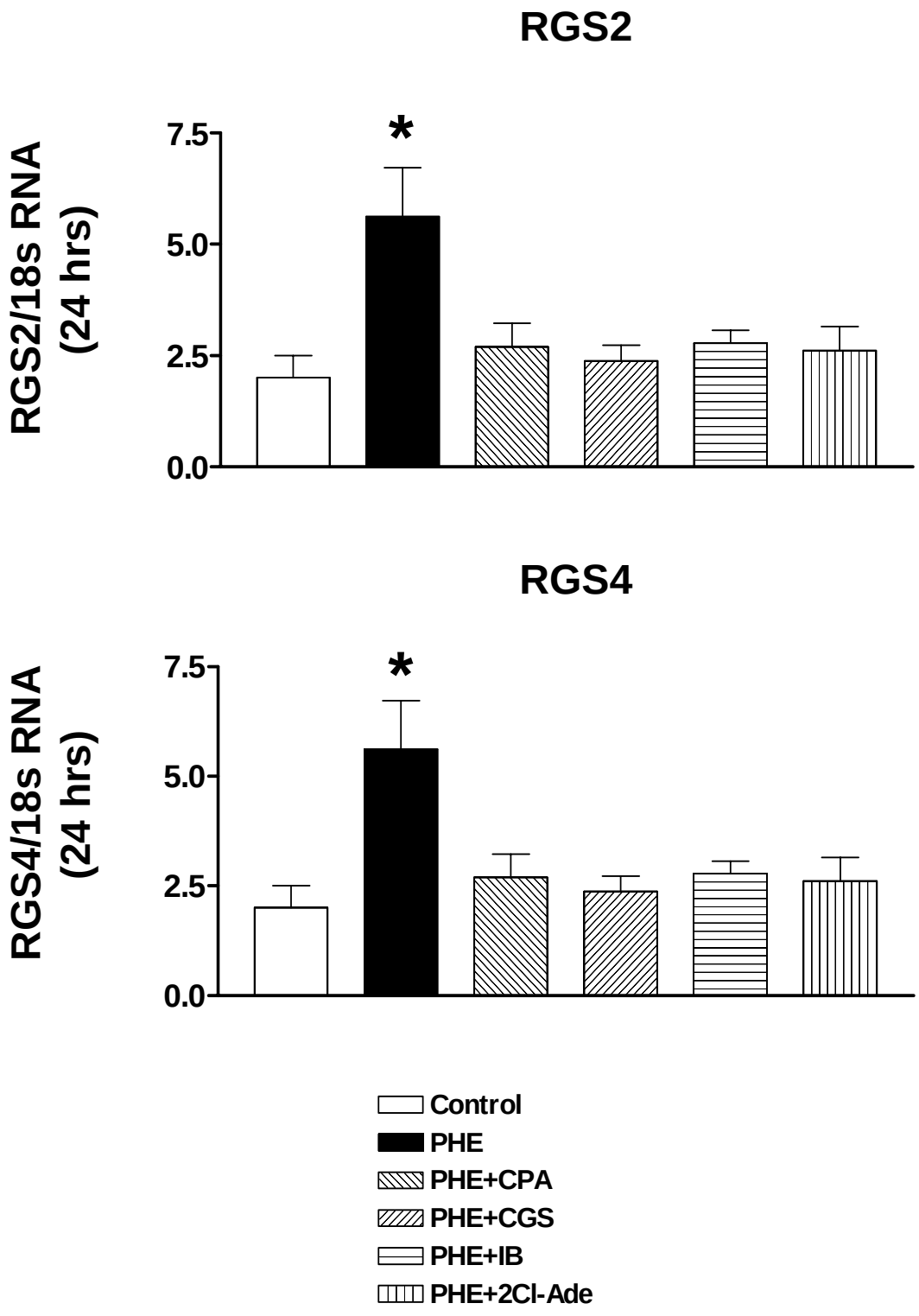

Figure 10