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# Obligatory role for endogenous endothelin in mediating the hypertrophic effects of phenylephrine and angiotensin II in neonatal rat ventricular myocytes: evidence for two distinct mechanisms for endothelin regulation

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A list of abbreviations used in the paper:

AngII: angiotensin II

ECE-1: endothelin converting enzyme1

ERK: extracellular signal-regulated protein kinase

ET-1: endothelin 1

JNK: c-Jun –terminal kinase

PE: phenylephrine

PPRD: phosphoramidon

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**Abstract**

Various Gq protein coupled receptor agonists such as the $\alpha 1$ adrenoceptor agonist phenylephrine, angiotensin II and endothelin-1 are potent hypertrophic factors. There is evidence of potential crosstalk between these agents particularly in terms of endothelin-1 as playing a central role in mediating the actions of other hypertrophic factors. Using cultured rat neonatal ventricular myocytes we assessed the potential crosstalk between these factors and sought to examine the potential underlying mechanisms. Twenty four hours exposure to either agent produced significant hypertrophy as determined by cell size and molecular markers. Although the hypertrophic effects of phenylephrine and angiotensin II were expectedly prevented by $\alpha 1$ and $AT_1$ receptor antagonists, respectively, these effects were also blocked by the $ET_A$ receptor antagonist BQ123 but not by the $ET_B$ antagonist BQ788. Both phenylephrine and angiotensin II significantly increased protein expression of both endothelin receptor subtypes. Both phenylephrine and angiotensin II produced significant activation of p38 as well as extracellular signal-regulated protein kinase and c-Jun –terminal kinase although this was unaffected by endothelin receptor blockade. Further studies revealed that the effects of phenylephrine and angiotensin II were mediated by stimulated endothelin -1 production occurring via two separate mechanisms: angiotensin II by increasing the levels of the endothelin-1 precursor prepro endothelin-1 and phenylephrine by upregulating endothelin converting enzyme 1. Our results indicate that the endothelin-1 system plays an obligatory role

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in the hypertrophic response to both phenylephrine and angiotensin II in cultured myocytes through a mechanism independent of mitogen-activated protein kinase activation.

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## Introduction

Regulation of cardiomyocyte hypertrophy occurs via complex mechanisms but to a large degree involves receptor-mediated intracellular processes initiated by paracrine, autocrine and hormonal factors (Oshima et al., 2002). For example, one such group of hypertrophic agonists including the $\alpha 1$ adrenergic agonist phenylephrine (PE), angiotensin II (AngII) and endothelin-1 (ET-1) bind to specific Gq-protein-coupled receptor to initiate the intracellular response (Dorn and Brown, 1999). In cultured cardiac myocytes, activation of G protein-coupled receptors by PE, AngII and ET-1 not only induces cardiac hypertrophy (Simpson et al., 1982; Dzau, 1988; Shubeita et al., 1990) but also upregulates the activity of mitogen-activated protein kinases (MAPKs) (Clerk and Sugden, 2000; Molkentin and Dorn, 2001) a superfamily composed of three overall branches of the serine/threonine kinases extracellular signal-regulated protein kinase (ERK), c-Jun –terminal kinase (JNK), and p38.

Recent evidence suggests crosstalk between AngII and ET-1 (Mulder et al., 1997). For example, AngII has been shown to stimulate the production of ET-1 in neonatal rat ventricular myocytes through a mechanism involving prepro ET-1 stimulation (Ito et al., 1993). The potential role of the ET-1 system in regulating hypertrophic responses to AngII is of importance in terms of understanding the mechanistic basis for hypertrophic responses and also in terms of designing therapeutic strategies aimed at limiting the hypertrophic phenotype. However, it is not known whether ET-1 plays an obligatory role in the hypertrophic responses to other factors and

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whether there is reciprocity in crosstalk between these agents. Accordingly, the present study was designed to address these questions as well as the potential underlying mechanisms. ET-1 has also been shown to activate $ET_A$ or $ET_B$ receptors on cardiomyocytes to induce cellular hypertrophy (Ito et al., 1993; Cullen et al., 2001) but there is no evidence to date if the expression of $ET_A$ and $ET_B$ can be regulated by PE or AngII. Accordingly, we also sought to determine if PE or AngII can directly modulate ET receptors in cultured cardiomyocytes.

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## Materials and Methods

### Primary Neonatal Cardiac Myocytes Culture

Myocytes were prepared from the ventricles of 4-day-old Sprague-Dawley rats as described in detail previously (Gan et al., 2003). In brief the ventricles were excised, washed and cut into small pieces in 15 ml Hanks' Balanced Salt Solution (HBSS) (Invitrogen, Burlington, ON, Canada), then digested in 60 ml of HBSS containing 800 U collagenase (Worthington Biochemical Corporation, Lakewood, NJ, USA)/ventricle. The digestion was performed in a circulating water bath to keep the reaction temperature at $37^0$C. The digestion was terminated by adding the same volume of 20% Fetal Bovine Serum (FBS). The cells were sorted by a cell strainer to remove undigested particles and then centrifuged at 600 x g for 5 min at $4^0$C. The cell pellet was resuspended in a plating medium containing 10% FBS, 0.1 mM Bromodeoxyuridine, and was preplated in tissue culture flasks for two times of 20 min to reduce contaminating non-myocytes after which the cells were transferred into Primaria$^{TM}$ cell culture dishes (Becton Dickinson Labware, Mississauga, ON, Canada) and cultured for 48 hours. The medium was replaced with a serum-free maintenance medium and incubated for another 24 hours before being used for study. Approximately 95% of cells prepared by this method demonstrated sarcomeric myosin heavy chain staining, indicating relatively low nonmyocyte contamination (Rajapurohitam et al., 2003).

### Experimental Design

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Myocytes were treated with either PE (10 μM), Ang II (100 nM) or ET-1 (10 nM) for 24 hours. For some experiments, the cells were first treated with the appropriate antagonist for 15 min prior to agonist addition. These included the $\alpha_1$ adrenoceptor antagonist prazosin (1μM), the $AT_1$ antagonist [$Sar^1$-$Ile^8$] - Angiotensin II (1 μM), the $AT_2$ antagonist PD 123319 (1 μM), the $ET_A$ antagonist BQ123 (100 nM) and the $ET_B$ antagonist BQ788 (1 μM). In addition, the endothelin converting enzyme-1 (ECE-1) inhibitor phosphoramidon (PPRD) (10 μM) was used in some studies and added using a protocol identical to the receptor antagonists. All agents used were from Sigma (Oakville, ON, Canada).

**Cell Area Measurement**

The cells were plated at a density of 1 x $10^6$ cells/ 6 cm dish to obtain individually plated cells. At the end of the treatment period the cells were washed twice with PBS after which they were viewed using a Leica DMIL inverted microscope equipped with a Polaroid digital camera. Eight random photographs were taken from each sample and surface area from at least 5 cells from each photograph was determined in a blinded fashion using Mocha software (Jandel Scientific, USA). Thus, surface area from at least 40 cells was averaged to obtain one "n" value.

**RT-PCR and Real Time PCR**

Myocytes were plated at 6 x $10^6$ cells/ 6 cm dish. After washing twice with PBS, RNA was isolated by adding 1 ml Trizol reagent (Invitrogen, Burlington, ON, Canada) to each dish. 5 μg of

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total RNA were applied for reverse transcription by Superscript II reverse transcriptase (Invitrogen, Burlington, ON, Canada). 1 μl from the 20 μl cDNA product was used for each PCR reaction. RT-PCR was performed using a Genius DNA Engine thermocycler (Mandel Scientific Inc. Guelph, ON, Canada) with Platinum Taq DNA polymerase (Invitrogen, Burlington, ON, Canada). Real time PCR was performed with a DNA Engine Opticon Real-Time System (MJ Research, Waltham, MA, USA) with SYBR Green JumpStart Taq ReadyMix kit (Sigma, Oakville, ON, Canada) according to the manufacturer's instructions. The primers and the PCR programs used are listed in Table 1A and Table 1B respectively.

**Western Blot Analysis**

Cells were plated at a concentration of 6 x $10^6$ cells/ 6 cm dish. After washing twice with PBS 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 1.5 ml Eppendorf tubes, homogenized and then centrifuged at 10000 x g for 5 min at $4^0$C. The supernatant was transferred to a fresh tube and the protein concentration was assayed by Bradford Protein Assay Kit (Bio-Rad, Mississauga, ON, Canada). 30 μg protein were loaded in 10% SDS-PAGE, and transferred to nylon membrane (Amersham, Little Chalfont Buckinghamshire, UK). The membranes were blocked in 5% dry milk for 3 hours, primary antibody for 2h, secondary antibody for 1h, and then detected by ECL

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reagent (Amersham, Little Chalfont Buckinghamshire, UK). $ET_A$, $ET_B$ and actin antibodies were from Chemicon (Temecula, CA, USA) and used at a 1:500 dilution except for actin which was used at a dilution of 1:2000 dilution. Antibodies against pp44/42, ERK, pp38, p38, pJNK and JNK were purchased from Cell Signaling (Beverly, MA, USA) and used at a 1:1000 dilution.

**Enzyme Immunometric Assay**

Myocytes were plated at 3 x $10^6$ cells/ 3.5 cm dish. Big ET-1 and ET-1 protein levels in the culture media were measured using Enzyme Immunometric Assay kits (Assay Design, Chicago, IL, USA) according to the manufacturer's instructions. The cross-reactivity of rat big ET-1 antibody with either ET-1, ET-2 or ET-3 was <0.1% whereas cross-reactivity of the ET-1 antibody with either ET-2 or ET-3 was 3.32% and < 0.1%, respectively.

**Statistical Analysis**

All values in the figures and text are presented as mean ± SEM. Sample size per experiment is indicated in the results. Data were analyzed by one-way ANOVA with $P<0.05$ considered to represent significant differences between groups.

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## Results

### Effect of ET receptor blockade on the hypertrophic effects of PE and AngII

As an initial assessment of the potential role of the ET system to the hypertrophic effects of other factors we first determined whether blocking ET receptors can alter the response to either PE or AngII. As shown in Figure 1, both PE (Panel A) and AngII (Panel B) significantly increased cell surface area by approximately 40% and 50%, respectively. The $ET_A$ receptor antagonist BQ123 completely abrogated the hypertrophic response to both agents whereas the $ET_B$ receptor antagonist BQ788 was without effect.

To further assess the nature of the hypertrophic phenotype in response to agonists we determined the mRNA expression of two molecular hypertrophic markers, atrial natriuretic peptide (ANP) and myosin light chain-2 (MLC-2) by RT-PCR and real time PCR (Figure 2). Both PE and AngII significantly increased expression of these two markers which was prevented by the $ET_A$ receptor antagonist BQ123 and the $ET_B$ receptor antagonist BQ788.

### Effect of $\alpha 1$ adrenoceptor and AngII receptor blockade on ET-1 induced cardiac hypertrophy

Studies were carried out, as summarized in Table 2, to assess whether the hypertrophic effect of ET-1 can be modulated by blocking $\alpha 1$ or AngII receptors. However, neither the $\alpha 1$ adrenoceptor antagonist prazosin, the $AT_1$ receptor antagonist [$Sar^1$-$Ile^8$] - Angiotensin II or the $AT_2$ receptor antagonist PD 123319 had any effect on ET-1 induced cardiac hypertrophy.

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Furthermore, prazosin has no effect on AngII induced hypertrophy whereas neither of the AngII antagonists influenced PE induced hypertrophy (Table2).

**Effect of PE and AngII on $ET_A$ and $ET_B$ receptor protein expression in cardiac myocytes**

Figure 3 summarizes our results aimed at assessing whether ET receptor expression is affected by either PE or AngII. Both the $ET_A$ and the $ET_B$ receptor were significantly increased after 24 hour treatment with either agent. We next wished to determine whether the upregulation of ET receptors occurred via direct effects or secondary mechanisms, possibly via ET-1 upregulation. Initial studies determined the effect of PPRD which inhibits the ECE-1 that converts big ET-1 to ET-1. As shown in Figure 3, PPRD which had no effect of its own completely prevented the upregulation of the $ET_A$ receptor by both PE (Panel A) as well as AngII (Panel B). In contrast, PPRD directly increased $ET_B$ expression on its own and has no effect on $ET_B$ upregulation produced by either PE (Panel C) or AngII (Panel D).

**Effect of PPRD on PE and AngII induced hypertrophy**

As shown in Figure 4, the hypertrophic effect of both PE and AngII was completely prevented by PPRD which on its own was without effect, thus suggesting that endogenous ET-1 mediates the hypertrophic effect of these two agents.

**Comparative effects of PE and AngII on ECE-1 and ET-1 expression**

The ability of PPRD to prevent the upregulation of the $ET_A$ receptor by both PE and AngII

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prompted us to examine whether ECE-1 could be directly modified by either agent as a potential explanation for $ET_A$ upregulation. As shown in Figure 5, PE significantly increased ECE-1 expression threefold whereas AngII was completely without effect. In contrast, however, AngII increased expression of prepro ET-1 directly although prepro ET-1 expression was unaffected by PE. Moreover, the magnitude of prepro ET-1 upregulation was virtually identical to the effect of PE on ECE-1.

Taken together, the results suggest an ability of PE and AngII to stimulate ET-1 production via two distinct mechanisms. To demonstrate this directly, we next studied the relative ability of each agent to augment synthesis of big ET-1, the immediate ET-1 precursor, as well as ET-1 itself by determining the levels of these peptides in culture medium following agonist addition. As shown in Figure 6 both PE and AngII significantly increased ET-1 production whereas, in contrast, only AngII significantly increased the levels of big ET-1. The increased ET-1 production induced by PE was abolished by the $\alpha_1$ antagonist prazosin whereas the increased ET-1 and big ET-1 production induced by AngII was inhibited by the $AT_1$ blocker [$Sar^1$-$Ile^8$]-AngII and to a lesser degree by the $AT_2$ antagonist PD 123319. The ability of both AngII receptor antagonists to exert inhibitory effects was surprising although this may infer a role for multiple receptor subtypes in mediating the effect of AngII on the ET-1 system.

**Effect of treatments on MAPK activity**

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All three hypertrophic factors used in this study activate MAPK pathways in cardiac myocytes and we therefore explored if the prevention of cardiac hypertrophy by BQ123 is related to MAPK activity. As shown in Figure 7, PE significantly increased MAPK activity as evidenced by increased phosphorylation of p44/42 (ERK), p38 and JNK whereas AngII has little effect. Moreover, PE and AngII induced MAPK activation was unaffected by either the $ET_A$ receptor antagonist BQ123 or the $ET_B$ receptor antagonist BQ788.

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## Discussion

ET-1 has initially been identified as a potent vasoconstricting factor produced by vascular endothelial cells although it has also been demonstrated to be synthesized by cardiac cells (Sakai et al., 1996). ET-1 acts via two G-protein-coupled receptors ($ET_A$, $ET_B$) although the $ET_A$ receptor represents 90% of endothelin receptors in cardiomyocytes (Fareh et al., 1996; Sakai et al., 1996). ET-1 has been implicated in the pathophysiology of myocardial infarction and congestive heart failure (Ezra et al., 1989; Stewart et al., 1991; Grover et al., 1993), the latter role likely attributable to its potent hypertrophic actions thus potentially contributing to the myocardial remodelling process (Yorikane et al., 1993). ET-1 and ET receptor expression can be increased by numerous factors such as cytokines, hormones, autocoids, fluid sheer as well as various other factors (Rubanyi and Polokoff, 1994). AngII has been shown to upregulate prepro ET-1 mRNA level in cultured cardiac myocytes (Ito et al., 1993)potentially suggesting that stimulation of ET-1 synthesis mediates at least some of the effects of this hormone. To assess and expand this hypothesis, we studied the effect of two hypertrophic factors, AngII and the $\alpha 1$ adrenoceptor agonist PE on cardiomyocyte hypertrophy and carried out an in depth assessment to determine whether the ET system mediates these hypertrophic responses. Our data strongly suggest an important role for ET-1 and its receptors, but especially the $ET_A$ receptor in mediating the actions of both AngII and PE through a mechanism involving increased synthesis of ET-1. However, our study also reveals that the ability of these agents to increase ET-1 production

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occurs via different mechanisms.

A role for ET-1 mediating the hypertrophy produced by PE and AngII is borne out by various lines of evidence which can be summarized as follows. The increased cell area produced by both agents was completely blocked by the $ET_A$ receptor antagonist BQ123. We also attempted to document changes in molecular markers. PE and AngII not only increased the cell area but also the expression of both ANP and MLC-2 (Chien et al., 1991). Moreover, both ET receptor antagonists blocked the upregulation of ANP and MLC-2 induced by PE and AngII. The ability of the $ET_B$ blocker BQ788 to inhibit both ANP and MLC upregulation by PE and AngII while leaving increased cell area produced by both agents unaffected was surprising but may reflect the complexity of the hypertrophic process and ET signalling. Indeed, $ET_B$ represents only 10% of the ET receptor in cardiomyocyte (Fareh et al., 1996) and it is possible that blocking of $ET_B$ is sufficient to inhibit the hypertrophic marker expression but not the overall hypertrophic effect.

The ability of PE and AngII to upregulate expression of both cardiac ET receptors $ET_A$ and $ET_B$ further supports the notion that the ET system could potentially mediate the effects of these agents. To assess whether PE and AngII directly upregulate these receptors or whether this reflects a secondary response via endogenous ET-1 we determined the effect of PPRD, an agent which inhibits the enzyme ECE-1 that catalyzes the conversion of big ET-1 to ET-1. PPRD blocked the upregulation of the $ET_A$ receptor by both PE and AngII whereas it had no effect on

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$ET_B$ upregulation by these agents. Moreover, PPRD surprisingly directly upregulated $ET_B$ expression in the absence of any other intervention. The studies with PPRD further support the concept of $ET_A$ receptor mediation of hypertrophic responses since the agent blocked the increased cell area produced by both PE and AngII.

Studies with PPRD further suggested that endogenous ET-1 mediates the hypertrophic response to both PE and AngII. We sought to explore this concept by direct measurements of ET-1 and components of the ET-1 synthesis pathways. Indeed, both PE and AngII increased release of ET-1 from cardiac cells although subsequent analysis revealed that this occurs via different mechanisms. Accordingly, AngII, but not PE increased big ET-1 levels. Moreover, PE, but not AngII stimulated ECE-1 expression, as previously reported by others (Kaburagi et al., 1999) whereas conversely, only AngII increased preproET-1 expression. The integration of these findings into our working hypothesis is summarized below.

As summarized in Figure 7, we also studied the potential cell signalling mechanisms in mediating the hypertrophic responses by concentrating on the MAPK family. MAPK, but particularly ERK1/2, is generally associated with cell growth and is strongly activated by ET-1, PE and AngII (Clerk et al., 1994; Miyata and Haneda, 1994), although it should be noted that ERK1/2 inhibition has been shown not to reduce the hypertrophic phenotype in various experimental settings (Clerk et al., 1998; Ono et al., 2000). In addition, both JNK and p38 can also be activated by PE and AngII in cardiac myocytes although these are more markedly

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activated by cytotoxic cellular stresses such as osmotic or oxidative stress (Bogoyevitch et al., 1995; Clerk et al., 1998). Our results showed that the early stimulation of all three components of the MAPK family by PE and AngII was unaffected by ET receptor blockade. Thus ET receptor blockade inhibits the hypertrophic response but leaves the MAPK activation unaffected. This finding is not completely surprising since 10 min treatment would not be expected to be sufficient time for either PE or AngII to increase synthesis of the ET-1 peptide. Overall, this finding suggests, but does not necessarily prove, that the obligatory role of ET-1 in mediating the hypertrophic response to either PE or AngII can be dissociated from MAPK activation.

**Summary and conclusions**

Our findings strongly support the hypothesis that ET-1 mediates the hypertrophic response to both PE and AngII and that both agents increase ET-1 synthesis by the cardiac cell. However, two recent studies using either pharmacological $ET_A$ receptor antagonism (De Smet et al., 2003) or cardiomyocyte-specific $ET_A$ knockout mice (Kedzierski et al., 2003) failed to demonstrate a pivotal role for this receptor against AngII-induced hypertrophy. We are unable at present to explain the divergent results but this may represent the type of experimental model. Moreover, as suggested by these authors it is possible under chronic in vivo conditions other mechanisms are activated to compensate for $ET_A$ inhibition such as the upregulation of other receptor subtypes. In view of the potential therapeutic relevance of this phenomenon further work is necessary in order to fully understand the importance of these findings particularly under in vivo

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conditions.

Our hypothesis regarding the nature of this activation based on our systematic evaluation of the ET system in response to the two agonists in cultured myocytes, is summarized in Figure 8. According to this scheme both PE and AngII increase the production of ET-1 although via separate mechanism: AngII by increasing expression of preproET-1 and PE by activating ECE-1 thus resulting in increased conversion of big ET-1 to ET-1 with the latter acting on the $ET_A$ receptor to produce the hypertrophic response. Hypertrophy is thus prevented by PPRD since this agent blocks the conversion of big ET-1 to ET-1. The ability of PPRD to upregulate the $ET_B$ receptor was surprising but may suggest that endogenous ET-1 serves as an inhibitory regulator of this receptor which is then reversed by ECE-1 inhibition or it may reflect a direct stimulatory effect of PPRD on the $ET_B$ receptor.

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This article has not been copyedited and formatted. The final version may differ from this version.

## References

Bogoyevitch MA, Ketterman AJ and Sugden PH (1995) Cellular stresses differentially activate c- Jun N-terminal protein kinases and extracellular signal-regulated protein kinases in cultured ventricular myocytes. *J Biol Chem* **270**:29710-29717.

Chien KR, Knowlton KU, Zhu H and Chien S (1991) Regulation of cardiac gene expression during myocardial growth and hypertrophy: molecular studies of an adaptive physiologic response. *FASEB J* **5**:3037-3046.

Clerk A, Bogoyevitch MA, Anderson MB and Sugden PH (1994) Differential activation of protein kinase C isoforms by endothelin-1 and phenylephrine and subsequent stimulation of p42 and p44 mitogen-activated protein kinases in ventricular myocytes cultured from neonatal rat hearts. *J Biol Chem* **269**:32848-32857.

Clerk A, Michael A and Sugden PH (1998) Stimulation of the p38 mitogen-activated protein kinase pathway in neonatal rat ventricular myocytes by the G protein-coupled receptor agonists, endothelin-1 and phenylephrine: a role in cardiac myocyte hypertrophy? *J Cell Biol* **142**:523-535.

Clerk A and Sugden PH (2000) Small guanine nucleotide-binding proteins and myocardial hypertrophy. *Circ Res* **86**:1019-1023.

Cullen JP, Bell D, Kelso EJ and McDermott BJ (2001) Use of A-192621 to provide evidence for involvement of endothelin ET(B)-receptors in endothelin-1-mediated cardiomyocyte hypertrophy. *Eur J Pharmacol* **417**:157-168.

JPET Fast Forward. Published on March 8, 2004 as DOI: 10.1124/jpet.104.065185
This article has not been copyedited and formatted. The final version may differ from this version.

De Smet HR, Menadue MF, Oliver JR and Phillips PA (2003) Endothelin ETA receptor antagonism does not attenuate angiotensin II-induced cardiac hypertrophy in vivo in rats. *Clin Exp Pharmacol Physiol* **30**:278-283.

Dorn GW, 2nd and Brown JH (1999) Gq signaling in cardiac adaptation and maladaptation. *Trends Cardiovasc Med* **9**:26-34.

Dzau VJ (1988) Cardiac renin-angiotensin system. Molecular and functional aspects. *Am J Med* **84**:22-27.

Ezra D, Goldstein RE, Czaja JF and Feuerstein GZ (1989) Lethal ischemia due to intracoronary endothelin in pigs. *Am J Physiol* **257**:H339-343.

Fareh J, Touyz RM, Schiffrin EL and Thibault G (1996) Endothelin-1 and angiotensin II receptors in cells from rat hypertrophied heart. Receptor regulation and intracellular $Ca^{2+}$ modulation. *Circ Res* **78**:302-311.

Gan XT, Chakrabarti S and Karmazyn M (2003) Increased endothelin-1 and endothelin receptor expression in myocytes of ischemic and reperfused rat hearts and ventricular myocytes exposed to ischemic conditions and its inhibition by nitric oxide generation. *Can J Physiol Pharmacol* **81**:105-113.

Grover GJ, Dzwonczyk S and Parham CS (1993) The endothelin-1 receptor antagonist BQ-123 reduces infarct size in a canine model of coronary occlusion and reperfusion. *Cardiovasc Res* **27**:1613-1618.

JPET Fast Forward. Published on March 8, 2004 as DOI: 10.1124/jpet.104.065185
This article has not been copyedited and formatted. The final version may differ from this version.

Ito H, Hirata Y, Adachi S, Tanaka M, Tsujino M, Koike A, Nogami A, Murumo F and Hiroe M (1993) Endothelin-1 is an autocrine/paracrine factor in the mechanism of angiotensin II-induced hypertrophy in cultured rat cardiomyocytes. *J Clin Invest* **92**:398-403.

Kaburagi S, Hasegawa K, Morimoto T, Araki M, Sawamura T, Masaki T and Sasayama S (1999) The role of endothelin-converting enzyme-1 in the development of alpha1-adrenergic-stimulated hypertrophy in cultured neonatal rat cardiac myocytes. *Circulation* **99**:292- 298.

Kedzierski RM, Grayburn PA, Kisanuki YY, Williams CS, Hammer RE, Richardson JA, Schneider MD and Yanagisawa M (2003) Cardiomyocyte-specific endothelin A receptor knockout mice have normal cardiac function and an unaltered hypertrophic response to angiotensin II and isoproterenol. *Mol Cell Biol* **23**:8226-8232.

Miyata S and Haneda T (1994) Hypertrophic growth of cultured neonatal rat heart cells mediated by type 1 angiotensin II receptor. *Am J Physiol* **266**:H2443-2451.

Molkentin JD and Dorn IG, 2nd (2001) Cytoplasmic signaling pathways that regulate cardiac hypertrophy. *Annu Rev Physiol* **63**:391-426.

Mulder P, Richard V, Derumeaux G, Hogie M, Henry JP, Lallemand F, Compagnon P, Mace B, Comoy E, Letac B and Thuillez C (1997) Role of endogenous endothelin in chronic heart failure: effect of long-term treatment with an endothelin antagonist on survival, hemodynamics, and cardiac remodeling. *Circulation* **96**:1976-1982.

Ono Y, Ito H, Tamamori M, Nozato T, Adachi S, Abe S, Marumo F and Hiroe M (2000) Role and relation of p70 S6 and extracellular signal-regulated kinases in the phenotypic

changes of hypertrophy of cardiac myocytes. *Jpn Circ J* **64**:695-700.

Oshima Y, Fujio Y, Funamoto M, Negoro S, Izumi M, Nakaoka Y, Hirota H, Yamauchi-Takihara K and Kawase I (2002) Aldosterone augments endothelin-1-induced cardiac myocyte hypertrophy with the reinforcement of the JNK pathway. *FEBS Lett* **524**:123-126.

Rajapurohitam V, Gan XT, Kirshenbaum LA and Karmazyn M (2003) The obesity-associated peptide leptin induces hypertrophy in neonatal rat ventricular myocytes. *Circ Res* **93**:277- 279.

Rubanyi GM and Polokoff MA (1994) Endothelins: molecular biology, biochemistry, pharmacology, physiology, and pathophysiology. *Pharmacol Rev* **46**:325-415.

Sakai S, Miyauchi T, Kobayashi M, Yamaguchi I, Goto K and Sugishita Y (1996) Inhibition of myocardial endothelin pathway improves long-term survival in heart failure. *Nature* **384**:353-355.

Shubeita HE, McDonough PM, Harris AN, Knowlton KU, Glembotski CC, Brown JH and Chien KR (1990) Endothelin induction of inositol phospholipid hydrolysis, sarcomere assembly, and cardiac gene expression in ventricular myocytes. A paracrine mechanism for myocardial cell hypertrophy. *J Biol Chem* **265**:20555-20562.

Simpson P, McGrath A and Savion S (1982) Myocyte hypertrophy in neonatal rat heart cultures and its regulation by serum and by catecholamines. *Circ Res* **51**:787-801.

Stewart DJ, Kubac G, Costello KB and Cernacek P (1991) Increased plasma endothelin-1 in the early hours of acute myocardial infarction. *J Am Coll Cardiol* **18**:38-43.

Yorikane R, Sakai S, Miyauchi T, Sakurai T, Sugishita Y and Goto K (1993) Increased production of endothelin-1 in the hypertrophied rat heart due to pressure overload. *FEBS Lett* **332**:31-34.

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## Figure Legends

**Figure 1**

Effect of ET receptor antagonists on PE and AngII induced cardiac hypertrophy as determined by cell surface area. Cells were pretreated with $ET_A$ antagonists BQ123 or $ET_B$ antagonists BQ 788 for 15 min after which PE or AngII was added for 24 hr. The $ET_A$ antagonist BQ123 but not $ET_B$ antagonist BQ788 inhibited PE (panel A) and AngII (panel B) induced cardiomyocytes hypertrophy. The bar shown on pictures equals to 10 μm. * $P<0.05$ vs control, †$P<0.05$ vs PE or AngII (N=6 group, more than 40 individual cells were counted in each group). A: angiotensin II

**Figure 2**

Effect of ET receptor antagonists on agonist induced hypertrophy as determined by levels of the molecular markers ANP and MLC-2. Cells were treated with agonists for 24 hr as shown in figure in the absence or presence of the $ET_A$ receptor antagonist BQ123 and the $ET_B$ receptor antagonist BQ788. Both RT-PCR (panel A) and real time PCR (panel B) showed the upregulation of ANP and MLC by PE and AngII which was blocked by both receptor antagonists. *$P<0.05$ vs control (N=6). Panel C shows the representative melting curve of the amplified genes where all samples demonstrated a specific product, indicating no primer-dimer formation. Melting temperature (Tm) values for ANP, MLC-2 and 18s RNA were $83^0$C, $88^0$C

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and $85^0$C, respectively.

**Figure 3**

Profiles of ET-1 receptor expression in neonatal cardiomyocytes determined by Western blotting. Cells were pretreated with ECE-1 inhibitor phosphoramidon (PPRD) for 15 min after which they were treated with PE or AngII for 24hr. Both PE (panel A) and AngII (panel B) upregulated $ET_A$ expression. PPRD prevented the effect of PE (panel A) and AngII (panel B). $ET_B$ expression was stimulated by PE (panel C) and AngII (panel D) as well as by PPRD itself in the absence of any agonist (panel C, D). ET-1 receptor expression was normalized with actin and compared to control. *P<0.05 vs control, †P<0.05 vs AngII (N=4). A: Angiotensin II.

**Figure 4**

Blockade of PE and AngII induced hypertrophy by the ECE-1 inhibitor phosphoramidon (PPRD). Cells were pretreated with PPRD for 15 min after which they were treated with PE or AngII for 24hr. The cell area data showed PPRD completely blocked hypertrophic response to both PE (Panel A) and AngII (panel B). *P<0.05 vs control. (N=5)

**Figure 5**

Comparative effects of PE and AngII on ECE-1 and preproET-1 expression. The myocytes were treated with PE or AngII for 24 hr. ECE and preproET-1 expression were measured by real time PCR and the data were normalized by 18s RNA and compared with control. PE significantly induced ECE expression in 24h (N=6, ***P<0.001) but had no effect on preproET-

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1. In contrast, AngII has no effect on ECE-1 expression but significantly increased expression of preproET-1 by three fold (N=6, †P<0.05).

**Figure 6**

Effect of PE and AngII on ET-1 and Big ET-1 levels in myocyte culture medium. Cells were first treated with the appropriate antagonist after which PE or AngII were added for 24 hr. Protein levels of ET-1 and big ET-1 were measured in the medium using Enzyme Immunometric Assay kit. Both PE and AngII significantly enhanced ET-1 levels (panel A) although only AngII increased big ET-1 levels (panel B). *P<0.05 vs control (N=6)

**Figure 7**

Representative Western blots and quantitative assessment of MAPK levels in response to agonists. Cells were treated with PE or AngII for 10 min after preincubated with ET receptor antagonists BQ 123 or BQ 788 for 15 min. Numbers indicate the following treatment groups: 1, control; 2, PE; 3, PE + BQ123; 4, PE + BQ788; 5, AngII; 6, AngII + BQ123; 7, AngII + BQ788. Note that MAPK activation occurred in response to PE and AngII but was unaffected by ET-1 receptor blockade. (N=3)

**Figure 8**

Proposed mechanism for ET-1 as a mediator of the hypertrophic response to PE and AngII in cardiomyocytes. PE stimulates the production of ET-1 by increasing ECE-1 expression resulting in increased ET-1 production. In contrast, AngII increases ET-1 production by

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stimulating preproET-1 expression. ET-1 then exerts its hypertrophic effect by acting primarily on $ET_A$ receptors and increasing their expression. The ability of PPRD to upregulate $ET_B$ receptor expression suggests a possible direct effect of the agent or by disinhibiting an inhibitory influence of endogenous ET-1.

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**Table 1A. Primer Sequences**

| Gene | Product size (bp) | Primers | |
|---|---|---|---|
| ANP | 320 | Sense | 5'-CTGCTAGACCACCTGGAGGA-3' |
| | | Antisense | 5'-AAGCTGTTGCAGCCTAGTCC-3' |
| MLC-2 | 332 | Sense | 5'-CCATGTTTGAGCAGACCCAGA-3' |
| | | Antisense | 5'-GCTGCGAACATCTGGTCGATC-3' |
| PreproET-1 | 543 | Sense | 5'- AAGTCCCAGCCAGCATGGAGAGCG-3' |
| | | Antisense | 5'- CGTTGCTCCTGCTCCTCCTTGATGG-3' |
| ECE-1 | 648 | Sense | 5'-TCTCCACCCTCATCAACAGCAC-3' |
| | | Antisense | 5'-CATTGGGTGAAGAGCGGGTGTA-3' |
| 18S | 150 | Sense | 5'- GTAACCCGTTGAACCCCATT-3' |
| | | Antisense | 5'- CCATCCAATCGGTAGTAGCG-3' |

**Table 1B. Real time PCR Program Descriptions**

| Gene | Initial Denaturation | Cycles | Denaturation | Annealing | Extension | Prolonged extension |
|---|---|---|---|---|---|---|
| ANP | $94^0$C 15 min | 34 | $94^0$C 20 sec | $59^0$C20sec | $72^0$C30sec | $72^0$C5min |
| MLC-2 | | 36 | | $60^0$C25sec | $72^0$C30sec | |
| PreproET-1 | | 44 | | $54^0$C20sec | $72^0$C45sec | |
| ECE-1 | | 44 | | $54^0$C20sec | $72^0$C45sec | |
| 18S | | 34 | | $55^0$C20sec | $72^0$C30sec | |

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**Table 2. Effect of Receptor Antagonists on Hypertrophic Responses to PE, AngII and ET-1.**

**D**ata indicate the relative cell surface area compared to control cells. Cells were preincubated with different antagonists for 15 min, after which the appropriate agonists were added for 24h. Only the $ET_A$ antagonist BQ123 inhibited the hypertrophic effect of all three agonists. *P<0.05 vs control, N=6

| **Antagonists** | **Agonists** | | | |
|---|---|---|---|---|
| | **control** | **PE** | **AngII** | **ET-1** |
| **control** | 1 | 1.42±0.06* | 1.53±0.06* | 1.57±0.06* |
| **Prazosin (α1 antagonist)** | 1.16±0.09 | 0.95±0.03 | 1.57±0.05* | 1.63±0.08* |
| **[$Sar^1$-$Ile^8$] ($AT_1$ antagonist)** | 1.08±0.05 | 1.33±0.05* | 1.18±0.03 | 1.49±0.05* |
| **PD123319 ($AT_2$ antagonist)** | 1.02±0.03 | 1.32±0.06* | 1.32±0.04* | 1.67±0.09* |
| **BQ123 ($ET_A$ antagonist)** | 0.97±0.03 | 1.03±0.03 | 1.06±0.05 | 1.02±0.04 |
| **BQ788 ($ET_B$ antagonist)** | 1.11±0.04 | 1.43±0.09* | 1.45±0.03* | 1.40±0.08* |

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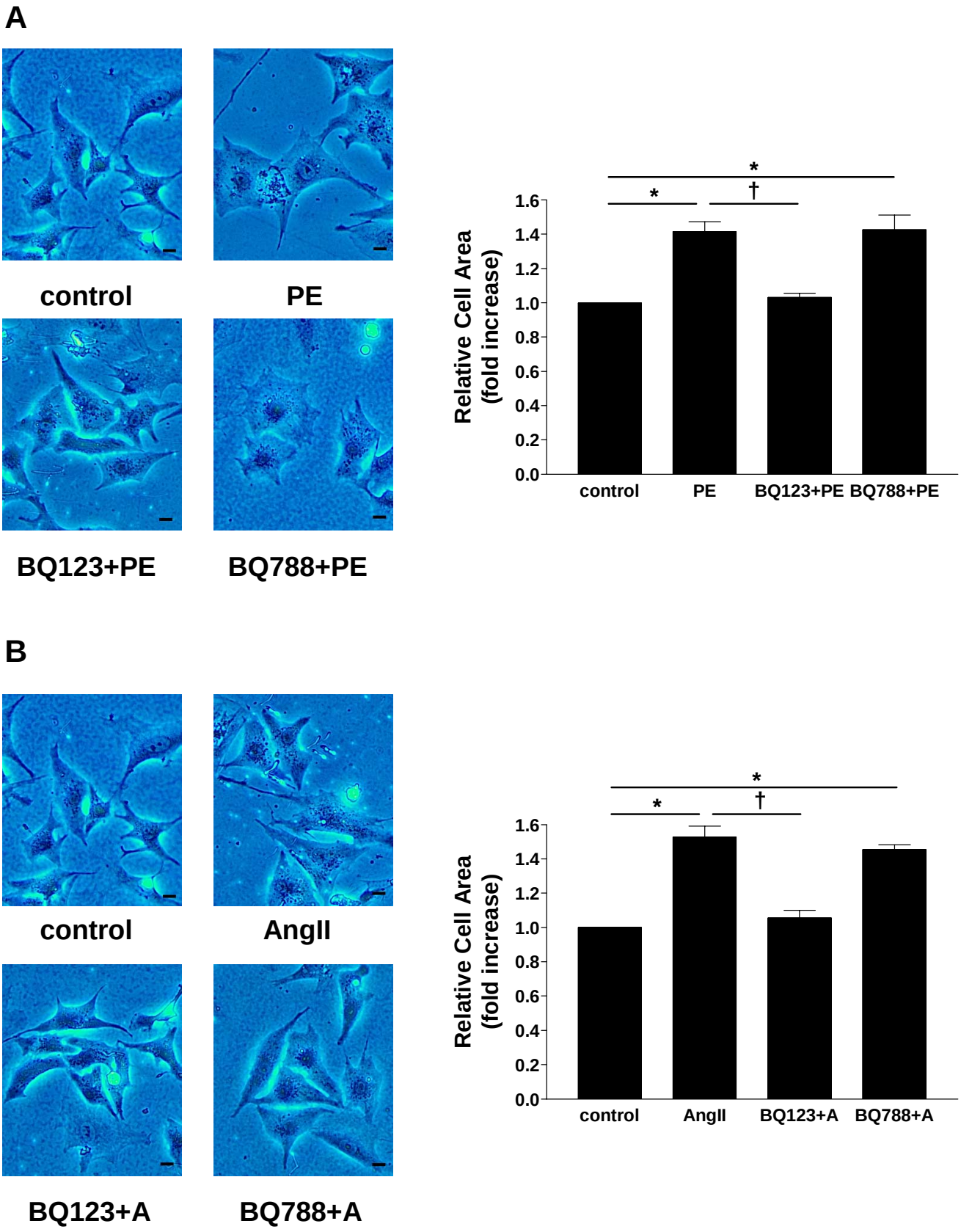


Fig. 1

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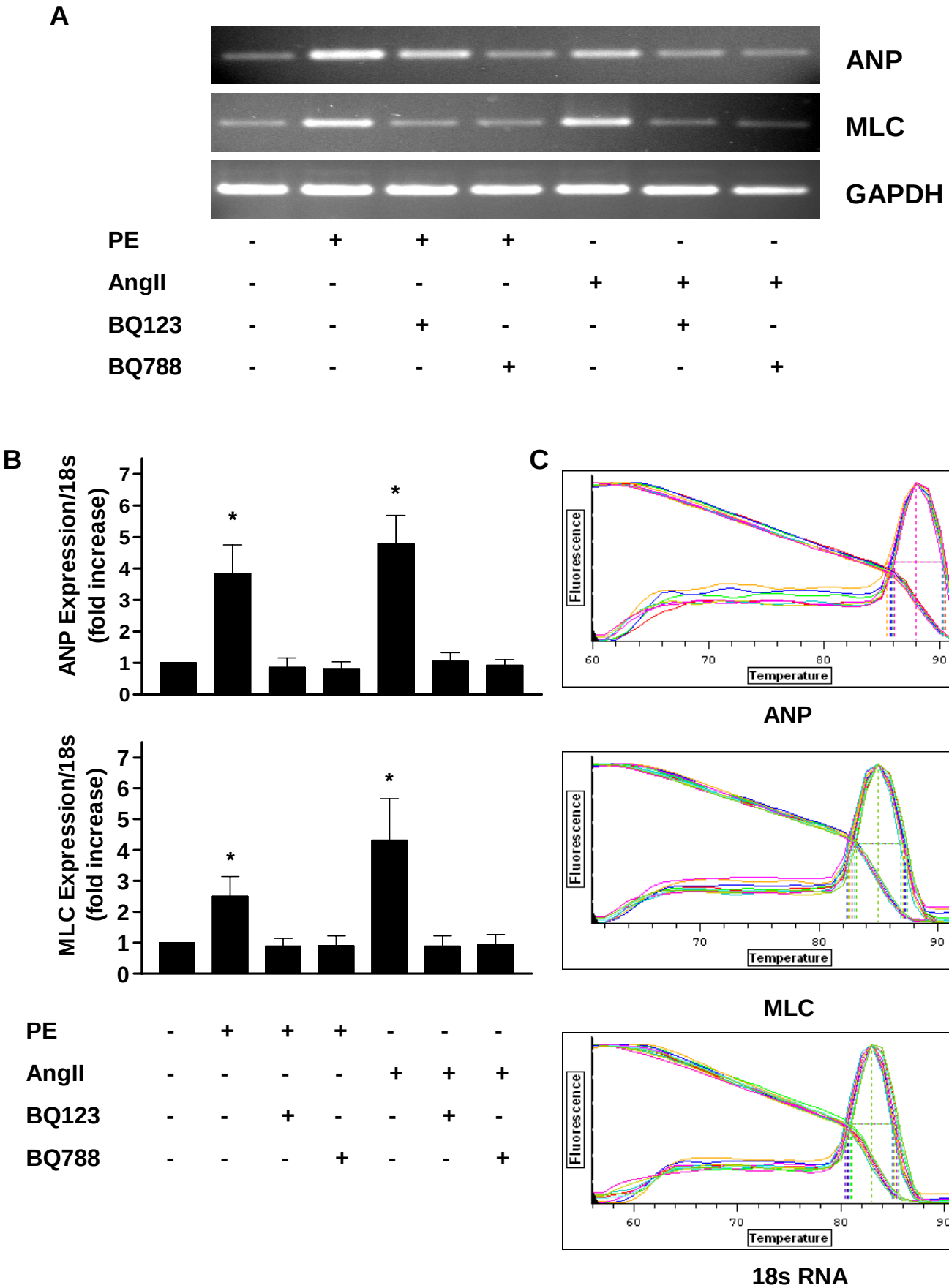


Fig. 2

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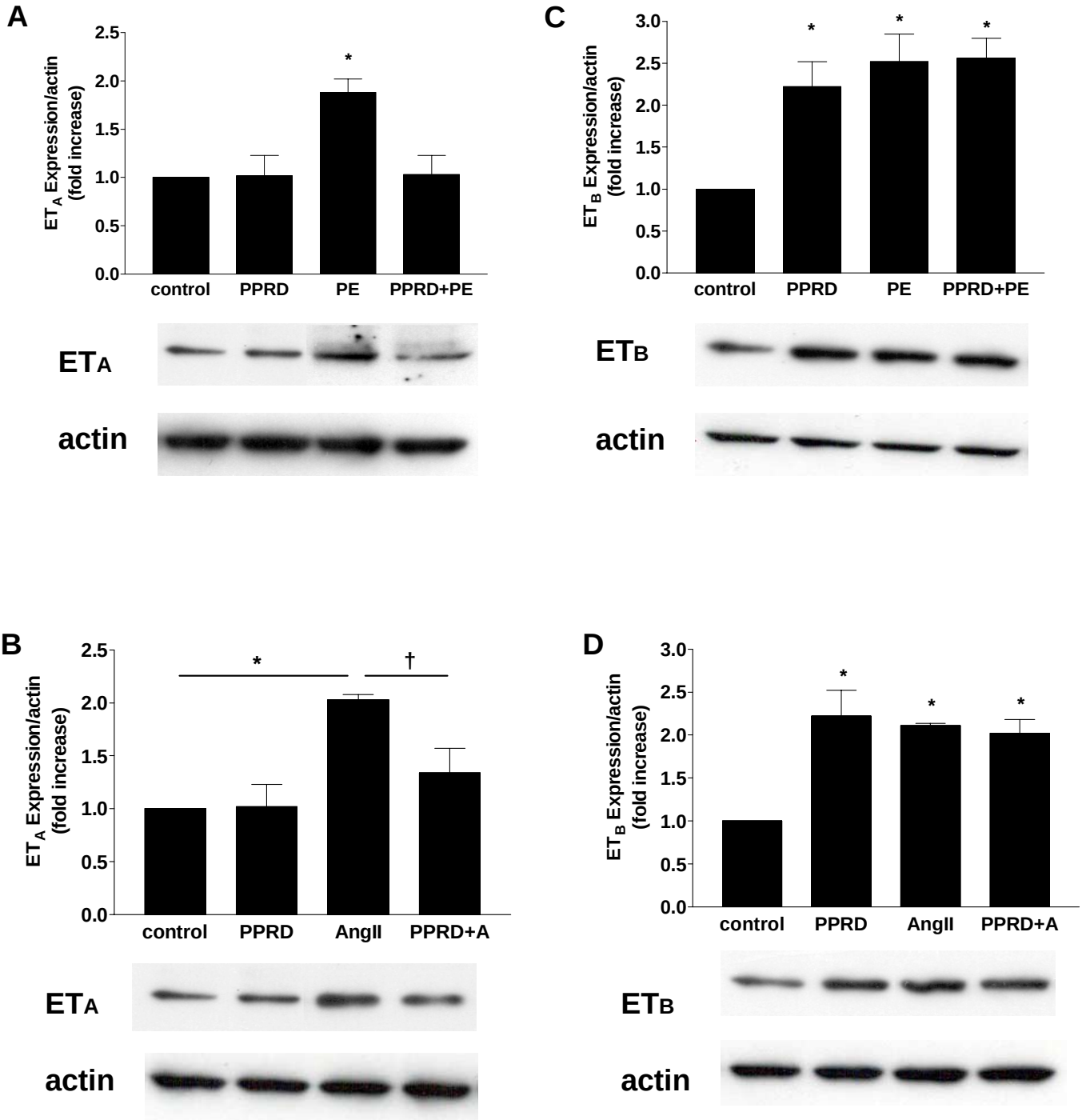


**Fig. 3**

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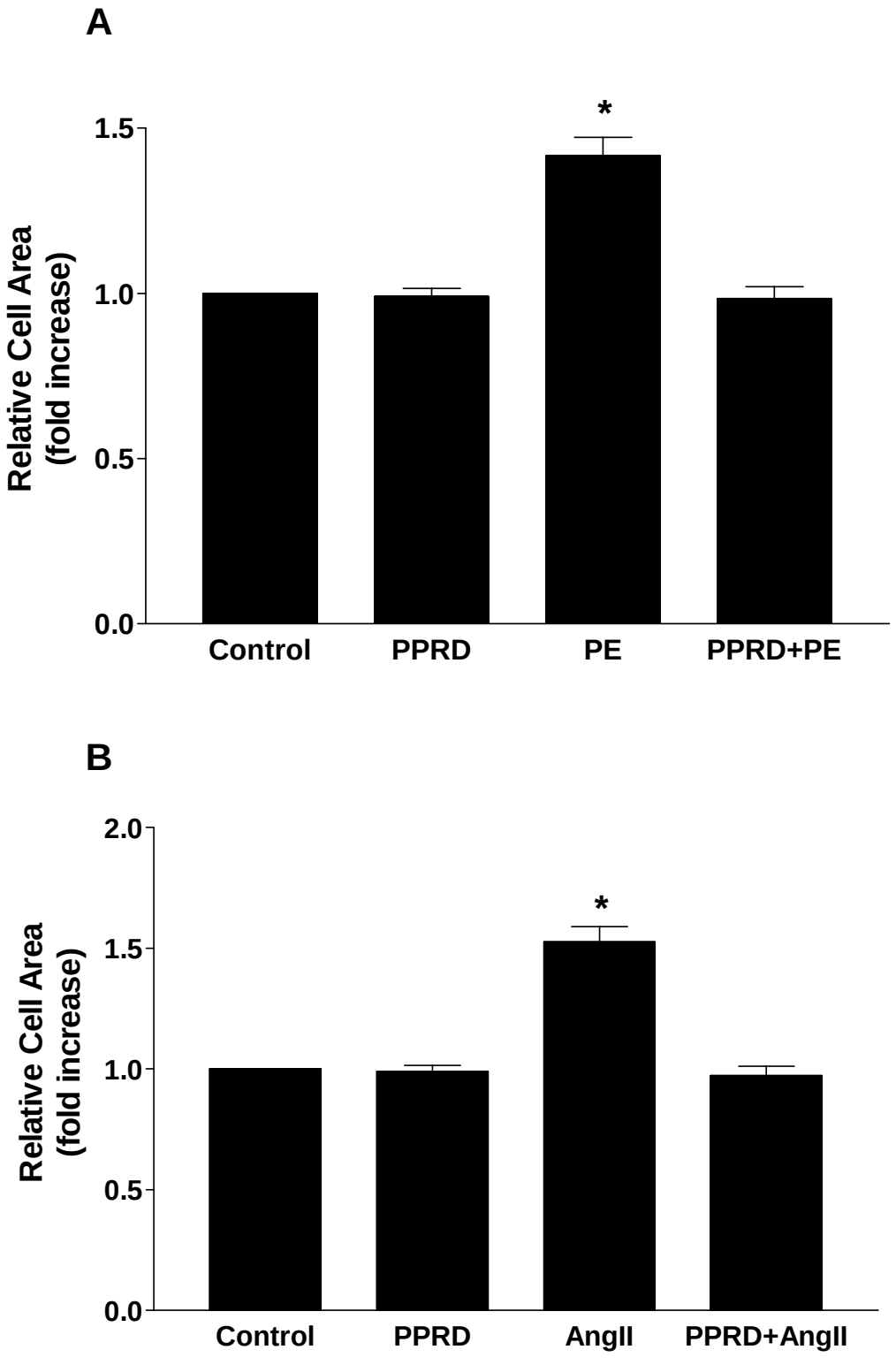


**Fig.4**

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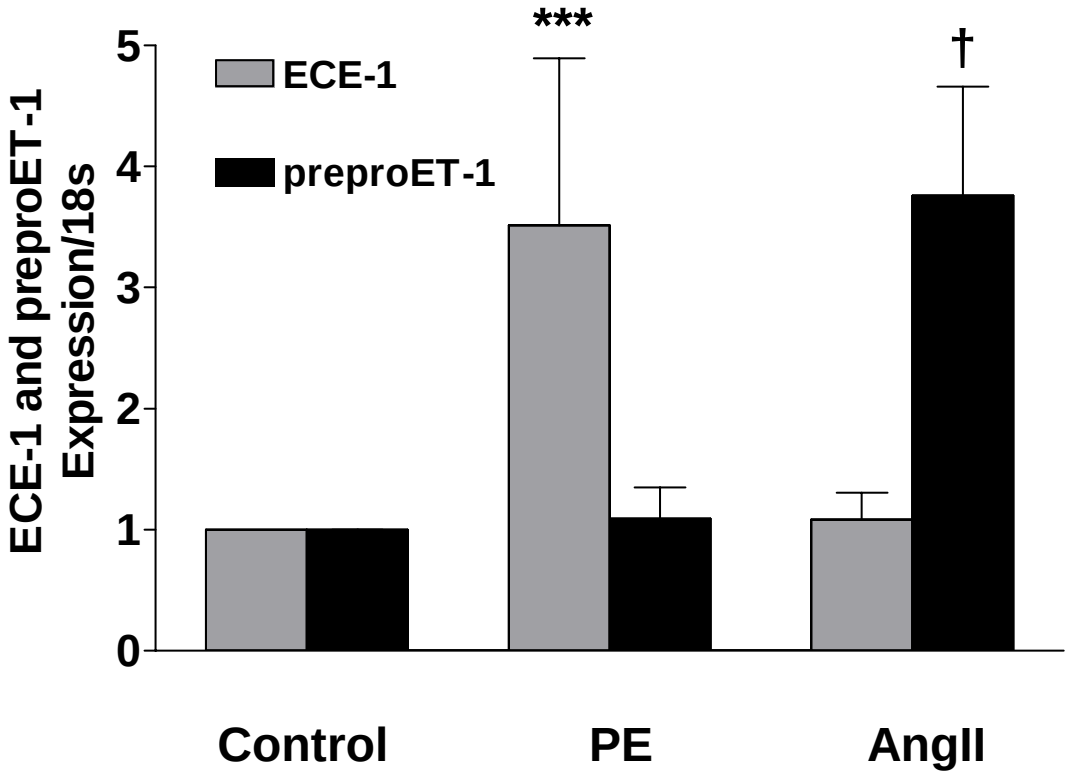


**Fig.5**

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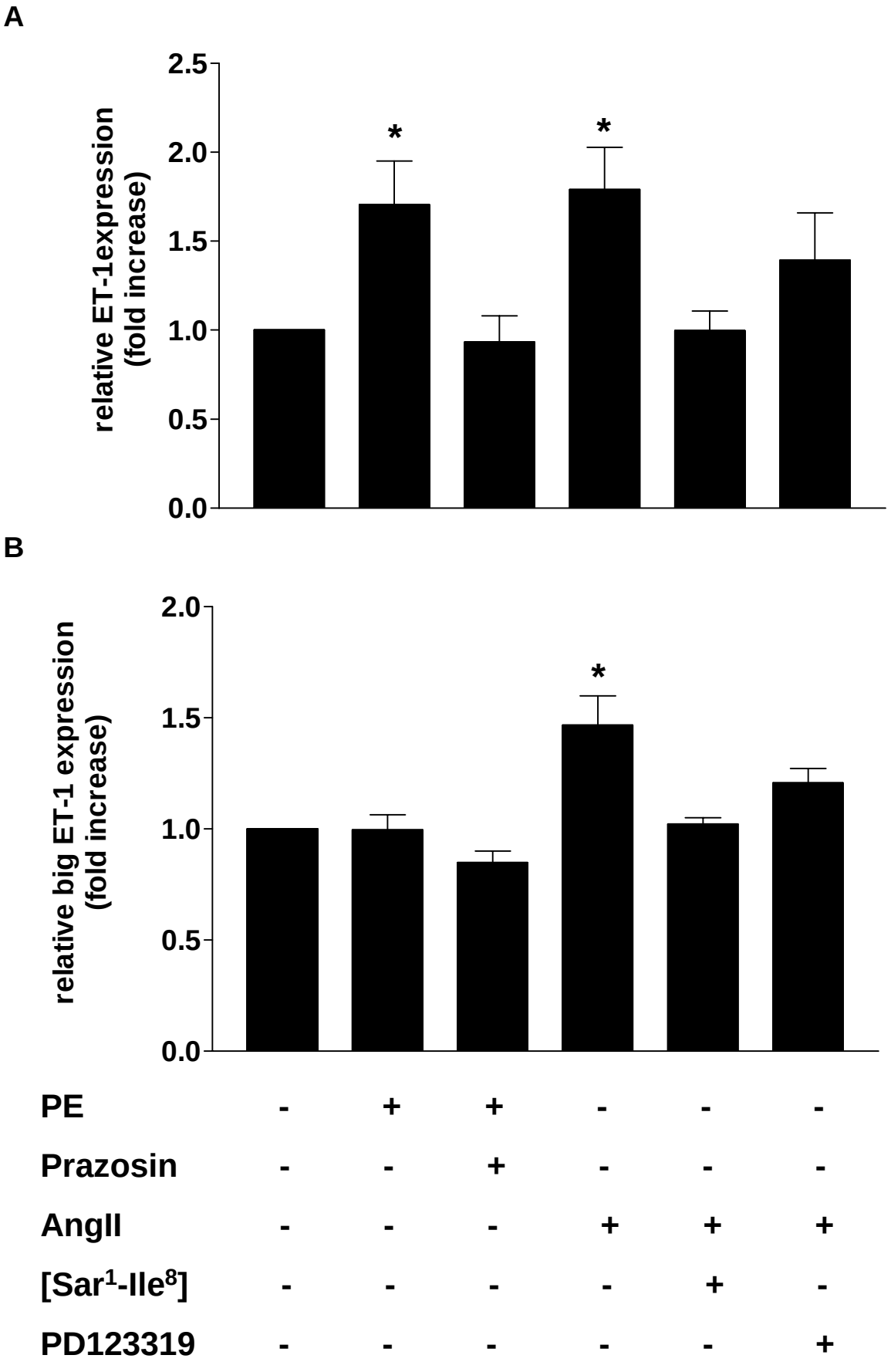


Fig.6

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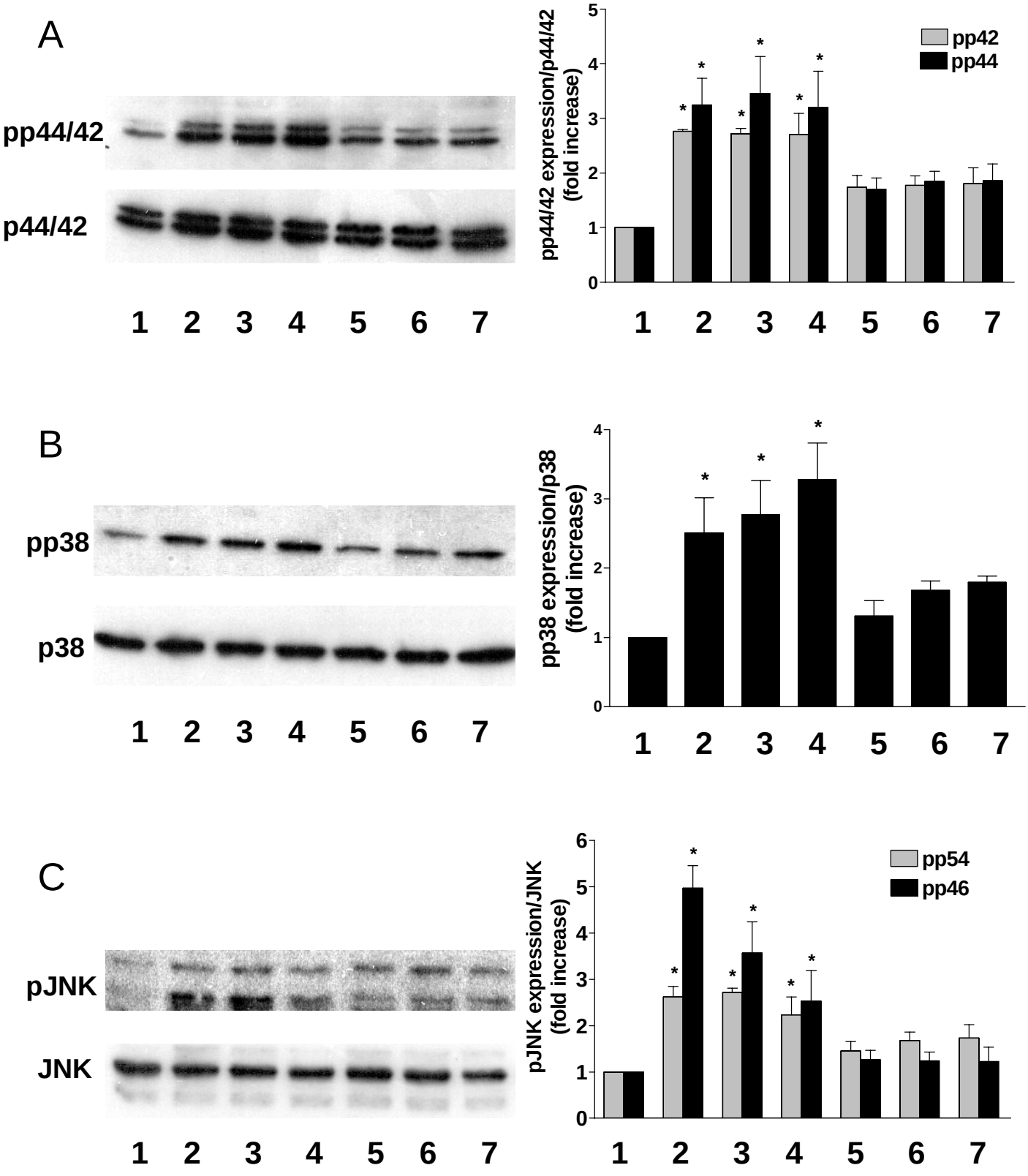


Fig.7

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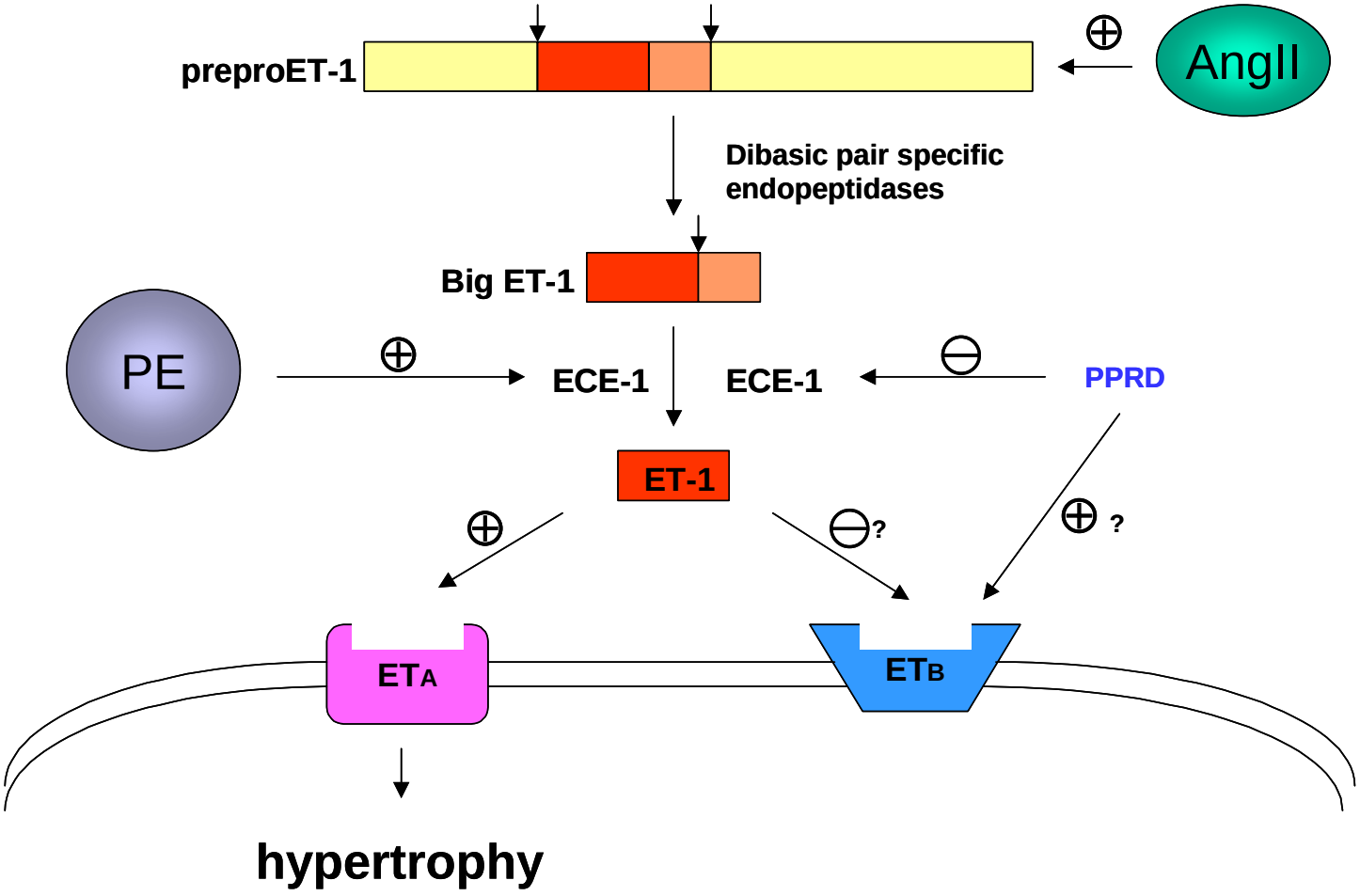


Fig.8