Hypoxic activation of the atrial natriuretic peptide gene promoter through direct and indirect actions of hypoxia-inducible factor-1

Yang-Sook CHUN*, Ju-Yeon HYUN†, Yong-Geun KWAK‡, In-San KIM§, Chan-Hyung KIM†, Eunjoo CHOI†, Myung-Suk KIM† and Jong-Wan PARK†[1]

*Human Genome Research Institute and Cancer Research Institute, BK21 Human Life Sciences, Seoul National University College of Medicine, 28 Yongon-dong, Chongno-gu, Seoul 110-799, South Korea, †Department of Pharmacology, Seoul National University College of Medicine, 28 Yongon-dong, Chongno-gu, Seoul 110-799, South Korea, ‡Department of Pharmacology, Chonbuk National University College of Medicine, Chonju, South Korea, and §Department of Biochemistry, Kyungpook National University, College of Medicine, Taegu, South Korea

Atrial natriuretic peptide (ANP) is a cardiac peptide, the transcription of which is up-regulated in the ischaemic ventricle. However, the molecular mechanism of ANP induction is unclear. This study demonstrated that ANP mRNA expression in rat ventricular myocardium is induced in an early phase of ischaemia, preceded by hypoxia-inducible factor-1 (HIF-1) α expression. The ANP gene was also induced by hypoxia or HIF-1 inducers such as $CoCl_2$ and desferrioxamine in H9c2 and neonatal cardiomyocytes. The 2307 bp 5′-flanking region of the rat ANP gene was cloned and fused to the luciferase gene. Evidence of the promoter activity was only apparent in the myocytes and was induced by hypoxia and HIF-1 inducers. The overexpression of HIF-1α markedly enhanced ANP promoter activity, and a dominant-negative isoform completely suppressed it. We demonstrated that the promoter regions are essential for hypoxic ANP induction. One promoter region, containing the HIF-1-binding sequence, is regulated directly by HIF-1. The other region is also activated by HIF-1 despite having no HIF-1-binding sequence. These results suggest that HIF-1 enhances the transactivation of the ANP gene in hypoxic myocytes, implying that stimulation of the ANP promoter by HIF-1 may in fact be responsible for the induction of the ANP gene in ischaemic ventricular myocardium.

Key words: atrial natriuretic peptide, cardiomyocyte, hypoxia-inducible factor-1, ischaemia.

INTRODUCTION

Restricted oxygen availability is a feature of many physiological and pathological conditions. For the adaptation to hypoxia, mammalian cells induce hypoxia-inducible genes, such as vascular endothelial growth factor (VEGF), various glycolytic enzymes and glucose transporter 1 [1]. The expression of these genes is regulated by hypoxia-inducible factor-1 (HIF-1), a heterodimeric transcription factor of HIF-1α and HIF-1β [2], which binds to the hypoxia-response element (HRE) in the 5′-flanking regions of the genes. Normally, HIF-1 activity depends on the amount of HIF-1α protein. Under normoxic conditions, von Hippel-Lindau tumour suppressor protein (pVHL), one of the multiprotein ubiquitin E3 ligase complexes, mediates the ubiquitylation of HIF-1α, which becomes the target of proteasomal proteolysis [3]. In this process, the hydroxylation in two proline residues of HIF-1α determines the binding of pVHL to HIF-1α [4–6]. However, under hypoxic conditions, proline hydroxylation is inhibited and then HIF-1α becomes stable.

Compared with cancer cells, few studies on HIF-1α in cardiac myocytes have been reported. Recently, the induction of HIF-1α protein was observed in biopsy specimens obtained from ischaemic and infarcted human myocardium [7]. This suggests that the early induction of HIF-1α in the ischaemic myocardium mediates the transcription of the VEGF [8], adrenomedullin [9] and endothelin-1 [10] genes, all of which have HIF-1-binding HREs in their 5′-flanking regions. Atrial natriuretic peptide (ANP) is also known to be a cardiac peptide, the transcription of which is up-regulated in the ischaemic ventricular myocardium [11,12]. However, whether the hypoxic induction of ANP in the heart is mediated by HIF-1 is unclear.

ANP is a potent diuretic, natriuretic and vasorelaxant peptide that is synthesized and released primarily from atrial myocytes [13,14], but is also found in the ventricular tissue [11]. The plasma levels of ANP are elevated in patients with pressure or volume overload, such as hypertension or congestive heart failure, which is secondary to increased atrial stretch [15]. In addition, many studies have demonstrated that the expression of the ANP gene is elevated in the ischaemic ventricular tissues, suggesting the possible role of ANP in the myocardial adaptation to ischaemia or hypoxia [11,12]. Previously, Chen et al. [16] analysed the 5′-flanking region of the rat ANP gene to determine the *cis*-element responsible for its hypoxic induction by using AT-1 cells, which are tumour cells originating from atrial myocytes. They concluded that the promoter of the ANP gene has no HIF-1-binding sequence and may be regulated by unidentified transcription factors. However, in the present study we found three possible *cis*-elements of the HIF-1-binding consensus sequence, 5′-RCGTG-3′, in the 5′-flanking region of the rat ANP gene. We demonstrated that one of them binds to HIF-1 and activates the transcription of the ANP gene under hypoxia. We also found the other promoter region which is regulated indirectly by HIF-1. Thus HIF-1 appears to be responsible for the hypoxic induction of the ANP gene in ventricular myocytes.

Abbreviations used: ANP, atrial natriuretic peptide; EMSA, electrophoretic mobility-shift assay; EPO, erythropoietin; HIF-1, hypoxia-inducible factor-1; HRE, hypoxia-response element; PHRE, putative HRE; hPHRE, human PHRE; RT-PCR, reverse transcriptase PCR; VEGF, vascular endothelial growth factor; VEGF-E, VEGF enhancer.

[1] To whom correspondence should be addressed (e-mail parkjw@plaza.snu.ac.kr).

EXPERIMENTAL

Cell culture and hypoxic induction

Neonatal cardiac myocytes were isolated from the ventricles of 2-day old Sprague–Dawley rats and established in primary culture as described in [17]. The ventricles were excised and minced in a solution containing 116 mM NaCl, 20 mM Hepes, 10 mM NaH_2PO_4, 5.5 mM glucose, 5 mM KCl and 0.8 mM $MgSO_4$, buffered at pH 7.4. The minced tissues was placed in a 50 ml flask containing an enzyme digestion solution with 65 units/ml collagenase type II and 0.6 mg/ml pancreatin, and incubated at 37 °C for a series of 20 min periods. After each 20 min interval, the cell-containing supernatant was centrifuged, and the cell pellet was resuspended in 10 % fetal bovine serum. Pooled cells were washed and then subjected to centrifugation through a discontinuous Percoll gradient of 1.05, 1.062 and 1.082 g/ml. The interface band between 1.062 and 1.082 g/ml was collected and used as the sole source of purified myocytes. These myocytes were resuspended in Dulbecco's modified Eagle's medium supplemented with 10 % fetal bovine serum, and plated at a density of 200 cells/mm^2 on collagen-coated dishes.

The neonatal ventricular myocytes, the rat ventricular myoblast cell line H9c2 (ATCC) and the HEK-293 cell line were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL), supplemented with 10 % heat-inactivated fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere containing 5 % CO_2 at 37 °C. Oxygen tensions in the incubator (model 9108MS2; Vision Sci Co., Seoul, South Korea) were either 140 mmHg (20 % O_2, v/v; normoxia) or 7 mmHg (1 % O_2, v/v; hypoxia).

Regional ischaemia of the rat heart

The investigation conformed to the Guide for the Care and Use of Laboratory Animals protocol, published by the US National Institutes of Health (NIH Publication No. 85-23, revised in 1985). Specific pathogen-free adult male Sprague–Dawley (250–300 g) rats were anaesthetized with sodium pentobarbital (50 mg/kg), incubated and mechanically ventilated by a rodent ventilator with room air. An anterolateral thoracotomy was performed and the heart was rapidly exteriorized. A 6-0 silk suture was snared around the proximal left anterior descending coronary artery, and tightly ligated to occlude the vessel. The heart was then placed back and the chest was closed in two layers with 4-0 silk sutures. The animal was then allowed to recover. Sham-operated rats were treated in the same manner but the coronary artery was not ligated. Ventricular tissues were prepared from two different areas: the ischaemic area in the centre of the territory of the left anterior descending coronary artery, and the non-ischaemic area in the posterior part of the left ventricle, far from the ischaemic area. The tissues were then washed with cold saline and processed as outlined below. Peri-ischaemic tissue around the ischaemic zone was removed and not used in this study.

Semi-quantitative reverse transcriptase PCR (RT-PCR) assay

ANP, VEGF and β-actin mRNA expression were measured by a semi-quantitative RT-PCR method using [α-^{32}P]CTP as described previously [18]. Total RNA was extracted with Trizol reagent (Gibco-BRL) according to manufacturer's instructions. RNA (1 μg) was added to a 50 μl RT-PCR reaction (PCR-Access; Promega). The reaction master mix was prepared according to the manufacturer's instructions to give final concentrations of 1 × AMV/*Tfl* reaction buffer of 0.2 mM dNTP, 5 μCi [α-^{32}P]CTP, 1.5 mM $MgSO_4$, 0.1 unit/μl avian myeloblastosis virus ('AMV') reverse transcriptase, 0.1 unit/μl *Thermus flavus* ('*Tfl*') DNA polymerase and 250 nM primers. To quantify ANP, VEGF and β-actin mRNAs, specific primers were used in a reaction involving one cycle of reverse transcription at 48 °C for 45 min and 24 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s, elongation at 68 °C for 1 min and a final extension at 68 °C for 5 min. The resulting PCR fragments (5 μl) underwent electrophoresis on 4 % polyacrylamide gels at 100 V in 0.3 × Tris/borate/EDTA buffer (15 mM Tris, 30 mM borate and 0.06 mM EDTA, pH 7.5) at 4 °C, and the dried gels underwent autoradiography. The nucleotide sequences of the primers used were 5′-AGCATGGGCTCCTTCTCCA-3′ and 5′-TTATCTTCAGTACCGGAAGCT-3′ for ANP; 5′-TCTACCAGCGCAGCTATTG-3′ and 5′-TCACCGCCTTGGCTTGTC-3′ for VEGF; and 5′-GGTGGGTATGGGTCAGAA-3′ and 5′-TGCATCCTGTCAGCGATG-3′ for β-actin.

Transient transfections and reporter gene assays

The rat ANP promoter 5′-flanking region (GenBank® accession number J03267) from −2307 to +53 was amplified by PCR from rat genomic DNA using the oligonucleotide 5′-TGATGGTTGGGTTGAATGACC-3′ as the forward primer and 5′-CTGTCTCGGCTCACTCTCT-3′ as the reverse primer, and cloned using a pCR2.1-TOPO cloning kit (Invitrogen). The insert DNA was digested with *Sac*I and *Xho*I and ligated into the *Sac*I and *Xho*I sites of pGL3-Basic (Promega), which was designated −2307ANP. Deletion mutants of −2307ANP containing regions −2108 to +53, −1765 to +53, −619 to +53, −512 to +53 and −34 to +53 were constructed using the ExSite Mutagenesis kit (Stratagene) and designated −2108ANP, −1765ANP, −619ANP, −512ANP and −34ANP, respectively. Mutation of the putative HIF-1-binding sequence 5′-GACGTG-3′ at position −1771 to −1766 in the sense DNA strand of the −2307ANP plasmid, by replacement of bases CGT with AAA to construct −2307ANP*m*, was introduced by using the QuikChange™ site-directed mutagenesis kit (Stratagene). All constructs were verified by DNA sequencing. Myocytes were seeded at an initial plating density of 200 cells/mm^2 in a medium supplemented with 10 % fetal bovine serum and 50 μg/ml gentamycin or 100 μg/ml penicillin/streptomycin. After an overnight recovery period, the cells were co-transfected with 0.6 μg each of luciferase reporter gene and plasmid cytomegalovirus-β-gal and/or plasmids of HIF-1 isoforms (HIF-1α, HIF-1β and a dominant-negative isoform HIF-1α^{516}) for each dish, using the calcium phosphate method. pcDNA was added to ensure that the final DNA concentrations in both control and experimental dishes were treated at the same level. After stabilizing, the cells were incubated for 16 h at either 20 % or 1 % O_2, in either the absence or presence of 100 μM $CoCl_2$ or 130 μM desferrioxamine. The cells were lysed and assayed for luciferase activity using a Biocounter M1500 luminometer (Lumac). β-Galactosidase assays were performed for normalization of transfection efficiency.

Nuclear extract preparation and electrophoretic mobility-shift assay (EMSA)

The cells were quickly washed twice in ice-cold PBS, and then removed by scraping. The cells were centrifuged at 1000 ***g*** for

5 min at 4 °C, washed twice with ice-cold PBS, and then resuspended in three packed cell volumes of lysis buffer consisting of 10 mM Tris, pH 7.8, 10 mM KCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 0.2 % Nonidet P-40, 0.5 mM dithiothreitol, 1 mM Na_3VO_4 and 0.4 mM PMSF. The cells were then vortex-mixed at medium speed for 10 s and incubated on ice for 5 min. The nuclei were then pelleted at 1000 ***g*** for 5 min at 4 °C. One packed cell volume of extract buffer, consisting of 20 mM Tris, pH 7.8, 420 mM NaCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 20 % glycerol, 0.5 mM dithiothreitol, 1 mM Na_3VO_4 and 0.4 mM PMSF, was added to the nuclei and the whole mixture vortex-mixed at medium speed for 5 s. The nuclear extracts were then centrifuged at 10000 ***g*** for 5 min, and divided into aliquots in chilled tubes, frozen quickly in liquid nitrogen, and stored at −70 °C.

EMSA probes for putative HRE (PHRE) and human PHRE (hPHRE) sites were synthesized based on the sequences of the rat (GenBank accession number J03267) and human (GenBank accession number S77079) ANP gene provide by National Center for Biotechnology Information (NCBI). Sequences of the sense strands of the oligonucleotide probes used for EMSA were as follows: 5′-CTGTCTCCCAGCGTGCACCGAATG-3′ (PHRE1), 5′-AGGTATGGAGACGTGCAGAGCATC-3′ (PHRE2), 5′-TGGTGTTTACACGTGTAGCAGAAT-3′ (PHRE3) and 5′-GGGATTATACGTGTGAGCCACTGCA-3′ (hPHRE). Oligonucleotides of erythropoietin (EPO) HRE (5′-ACCGGCCCTACGTGCTGTCTCAC-3′) and mutated EPO HRE (5′-ACCGGCCCTA*AAA*GCTGTCTCAC-3′; the mutated residue is in italic) were used for competition. A ^{32}P-labelled double-stranded probe was prepared and EMSA was performed as described previously [18]. DNA–protein binding reactions were carried out for 20 min at 4 °C and run on a 5 % non-denaturing polyacrylamide gel. For supershift analysis, 1 μl of HIF-1α antiserum was added to the completed EMSA reaction mixture and incubated for 2 h at 4 °C prior to loading.

Western blot analysis

The expression of HIF-1α was determined by Western blot analysis as described previously [19]. The cells were harvested with a lysis buffer (10 mM Tris/HCl, pH 7.8, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM dithiothreitol, 0.4 mM PMSF, 1 mM Na_3VO_4 and and 2 % Nonidet P-40) containing a protease inhibitor cocktail (Sigma). Cell extract (20 μg) was separated by SDS/PAGE (6.5 % gel) and transferred to an Immobilon-P membrane (Millipore). Immobilized proteins were incubated overnight at 4 °C with a rabbit polyclonal anti-HIF-1α antibody [19], diluted 1:1000 in 5 % non-fat milk in Tris-buffered saline/0.1 % Tween-20 (TTBS). Horseradish peroxidase-conjugated goat anti-rabbit antiserum (Santa Cruz Biotechnology) was used as a secondary antibody (1:5000). After extensive washing with TTBS, the complexes were visualized using enhanced chemiluminescence plus reagent (Amersham Biosciences) according to the manufacturer's instructions.

Immunohistochemistry

Myocardial expression of HIF-1α was determined using immunohistochemistry as described previously [20]. The hearts were frozen in Tissue-Tek optimal cutting temperature compound (Sakura Fintek). Sections of 8 μm thickness were used for immunostaining HIF-1α. Endogenous peroxidase activity was blocked by treating the sections with 0.3 % H_2O_2 in PBS for 30 min. After several washes with PBS, the sections were incubated in four stages, firstly with 5 % normal goat serum in PBS for 1 h to block non-specific binding, secondly overnight at 4 °C with a rabbit polyclonal anti-HIF-1α antibody (1:100), thirdly with a biotinylated anti-rabbit IgG antibody (1:200, Vector Laboratories) for 2 h and fourthly in an avidin–biotin-peroxidase complex (Vectastain ABC Kit; Vector Laboratories) for 1 h. Finally, the sections were developed in diaminobenzidine and H_2O_2 solution. Rabbit pre-immune serum was used in place of HIF-1α antibody as the negative staining control.

RESULTS

Expression of the ANP gene in the ischaemic myocardium

Myocardial tissues were obtained from ischaemic and non-ischaemic regions in the left ventricles of regionally ischaemic rat hearts. Temporal changes in the levels of ANP mRNA were observed in these tissues (Figure 1, top panel). In the ischaemic region, an initial increase in the ANP mRNA level relative to that of the sham-operated rats was observed as early as 6 h after regional ischaemia, with a further increase after 8 h. In the non-ischaemic region, the ANP mRNA level was not elevated until 8 h after ischaemia, but significantly increased 24 h after ischaemia. The expression of ANP mRNA in the non-ischaemic myocardium after 24 h regional ischaemia seems to be enhanced by diastolic wall stress occurring in failing hearts [12]. For a reliable comparison of the ANP and β-actin mRNA levels, primers for β-actin were co-incubated with primers for ANP in the RT-PCR reaction mixtures, and the β-actin cDNA then amplified with the ANP cDNA for the same time, under the same conditions. For quantification of these PCR fragments, each radioactive band was cut out of the dried gel, and the radioactivities of the gel bands measured using a β-counter. The relative amount of ANP mRNA was assessed by dividing the c.p.m. value of the ANP band by that of the corresponding β-actin band. Figure 1 (middle panel) shows markedly elevated levels of the ANP mRNA in the ischaemic myocardium from 6 h ischaemia, compared with that in the sham control and non-ischaemic myocardium. These results suggest that the ANP mRNA expression in rat ventricular myocardium is induced in an early phase of ischaemia.

Early expression of HIF-1α in the ischaemic myocardium

Normally, the elevation in the mRNA levels of hypoxia-inducible genes is preceded by the nuclear accumulation of HIF-1α. To test the possibility that the ANP mRNA expression is regulated by HIF-1, the expression level and localization of HIF-1α protein in the ischaemic myocardium were examined. Immunohistochemical analysis revealed that HIF-1α accumulated in the nuclei of myocytes in the ischaemic region to an extent measurably greater than that in the sham-operated rats as early as 2 h after regional ischaemia (Figure 1, bottom panel). Since the number of HIF-1α-positive nuclei did not exhibit any further increase 4 h after ischaemia, HIF-1α appears to be maximally induced 4 h prior to the induction of the ANP gene expression (6 h after ischaemia). These results indicate that the expression of HIF-1α precedes the expression of ANP mRNA in the ischaemic ventricular myocardium.

Expression of the ANP gene in response to hypoxia

To examine whether ANP gene expression is regulated by HIF-1, H9c2 cells and neonatal ventricular myocytes were used. The cells were exposed to hypoxia for 18 h, or treated with HIF-1 agonists, $CoCl_2$ and desferrioxamine for 18 h, followed by

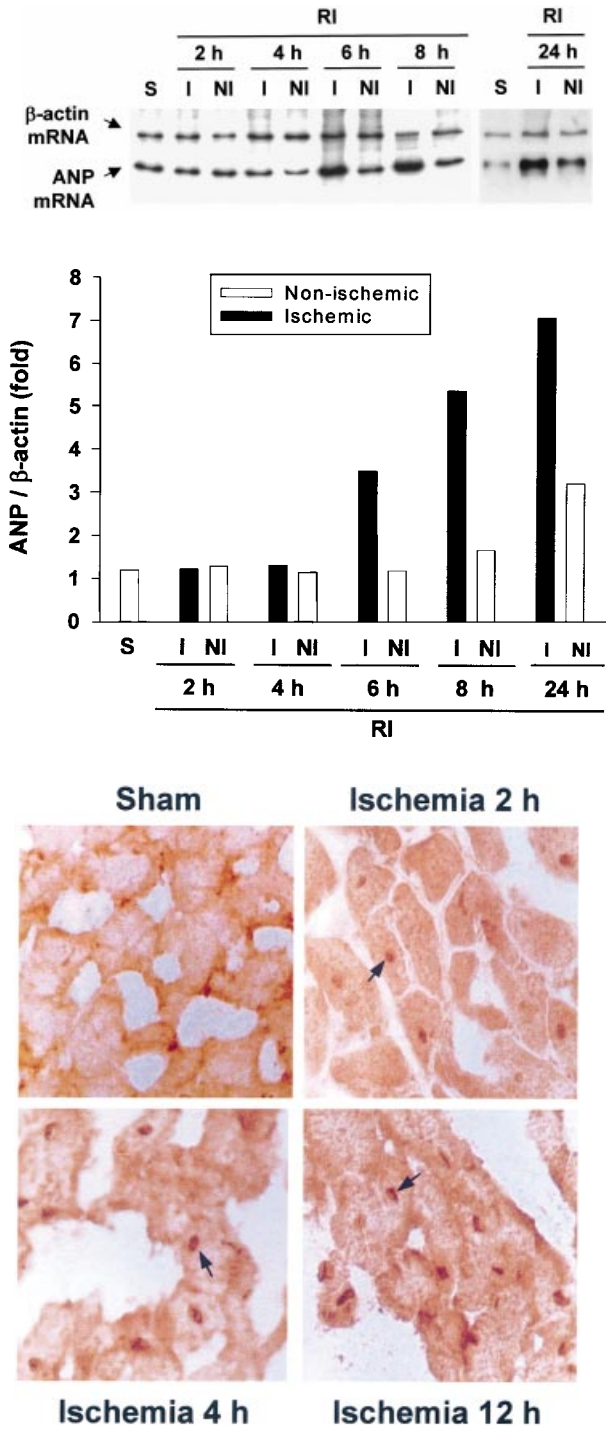

Figure 1 Expressions of ANP mRNA and HIF-1α protein in the ischaemic ventricular myocardium

Top panel: ANP mRNA expression. RNA was isolated from the ventricular tissues obtained from ischaemic (I) and non-ischaemic (NI) regions 2, 4, 6, 8 and 24 h after regional ischaemia (RI), and analysed by semi-quantitative RT-PCR using specific primers for ANP and β-actin. β-Actin mRNA was concomitantly amplified with the ANP mRNA for the reliable comparison of the ANP and β-actin mRNA levels. The data are representative of three separate experiments and S represents the sham-treated group. Middle panel: relative quantification of ANP mRNA. Each radioactive band was cut out from the dried gel and the radioactivities of the gel bands were measured using a β-counter. The relative amount of ANP mRNA was assessed by dividing the c.p.m. value of the ANP band by that of the corresponding β-actin band. Lower panel: HIF-1α expression. The expression level and localization of HIF-1α protein in the ischaemic myocardium were examined in the ventricular tissues obtained from ischaemic regions 2 and 4 h after ischaemia by immunohistochemistry using anti-HIF-1α antibody. Frozen sections of the ischaemic ventricles were incubated with HIF-1α antiserum and then stained with an avidin–biotin-peroxidase. The arrows indicate immunopositive nuclei. The data are representative of three separate experiments.

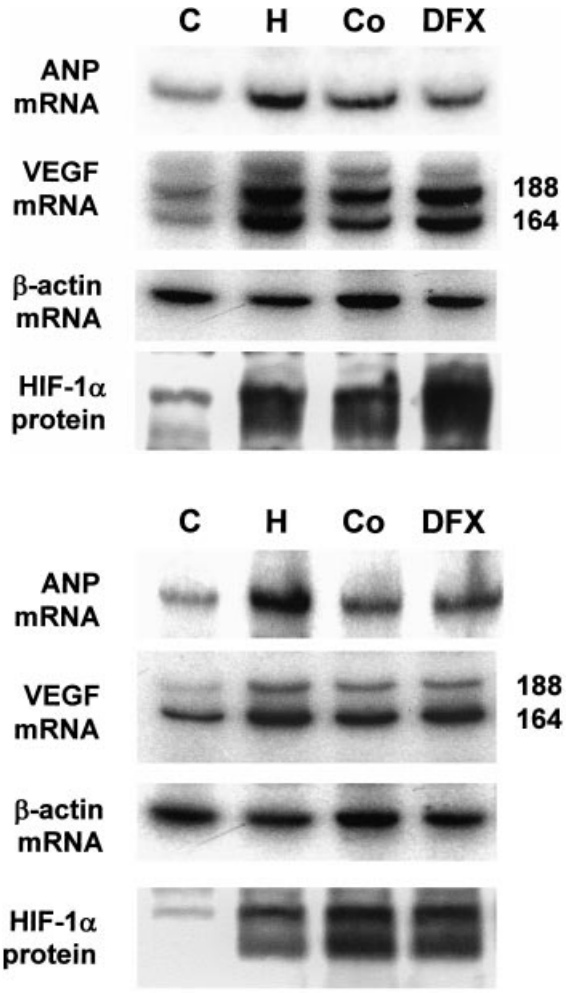

Figure 2 Induction of ANP mRNA by hypoxia and HIF-1 inducers

ANP and VEGF mRNA and HIF-1α protein expression in H9c2 cells (top panel) and neonatal ventricular myocytes (bottom panel). The cells were subjected to normoxia (N) or 18 h of hypoxia (H), or treated with 100 μM $CoCl_2$ (Co) or 130 μM desferrioxamine (DFX) for 18 h. ANP, VEGF and β-actin mRNAs were analysed by semi-quantitative RT-PCR. Two major bands of VEGF mRNA represent RT-PCR fragments amplified from VEGF188 and VEGF164 mRNAs. The HIF-1α protein in the total lysates (20 μg of protein each) was analysed by Western blot using HIF-1α antiserum. The data are representative of three separate experiments.

isolation of the total cellular RNA for semi-quantitative RT-PCR. The levels of VEGF mRNA, which is known to be induced by HIF-1α, were increased by hypoxia and HIF-1 agonists as expected. The two major bands of rat VEGF mRNA were identified as the VEGF188 and VEGF164 isoforms based on the molecular size of amplified DNA. The expression of ANP mRNA was also induced by hypoxia and HIF-1 agonists, expression that is comparable with the hypoxic induction of VEGF mRNA (Figure 2). Under the same conditions, the expression of HIF-1α protein was elevated in both myogenic cells. These results support the possible involvement of HIF-1 in the hypoxic induction of ANP.

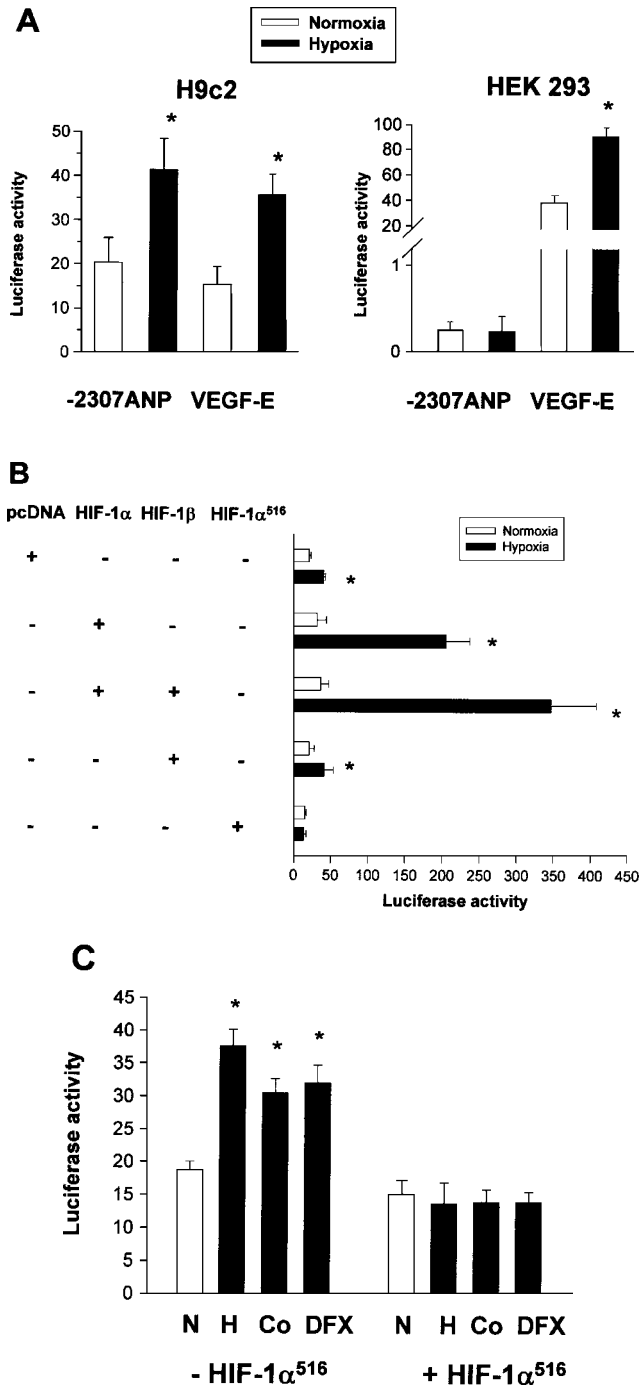

Figure 3 Regulation of the ANP promoter by HIF-1

(**A**) Hypoxic activation of the ANP promoter. pGL3-Basic vectors containing the full-length ANP promoter (−2307ANP) or the VEGF enhancer (VEGF-E) were transiently transfected into H9c2 and HEK-293 cells with a plasmid containing β-galactosidase to correct for transfection efficiency. Transfected cells were incubated under 20% (white bars) or 1% (black bars) oxygen for 18 h. Luciferase activities in the cell lysate were measured by chemiluminescence and normalized for β-galactosidase activity. (**B**) Stimulation of the ANP promoter by HIF-1. The ANP promoter-luciferase reporter plasmid was co-transfected into H9c2 cells with HIF-1α and/or HIF-1β expression vectors, or co-transfected with a dominant-negative isoform of HIF-1α, the HIF-1α^{516} expression vector. The cells were incubated under 20 or 1% oxygen for 18 h, and then were prepared for luciferase assay. (**C**) Role of endogenous HIF-1 in hypoxic activation of the ANP promoter. The ANP promoter-luciferase reporter plasmid was co-transfected with HIF-1α^{516} expression vector (+HIF-1α^{516}) or pcDNA (−HIF-1α^{516}). The cells were incubated with 100 μM $CoCl_2$ (Co) or 130 μM desferrioxamine (DFX) for 18 h, and then were prepared for luciferase assay. N, normoxia; H, hypoxia. Each bar represents the mean ± S.D. from nine experiments. $^*P < 0.05$ versus the normoxic control.

Regulation of the ANP promoter activity by HIF-1

To determine whether the rat ANP gene contains a regulatory sequence responsive to hypoxia and HIF-1 agonists, the 5′-flanking region of the rat ANP gene was cloned and tested for activation in a luciferase reporter gene system. The full-length promoter region (−2307ANP) conferred basal promoter activity under normoxic conditions in H9c2 cells. Exposure of H9c2 cells to hypoxia resulted in a 2.1-fold increase in promoter activity (Figure 3A). A VEGF enhancer (VEGF-E) reporter gene constructed previously [21] was used as a reference to estimate the endogenous HIF-1 activity. The VEGF enhancer activity showed a 2.4-fold increase in hypoxic H9c2 cells. In contrast, the promoter activity of −2307ANP was negligible in HEK-293 cells under normoxic conditions and was not induced by hypoxia, even though the activities of VEGF-E under normoxic and hypoxic conditions were comparable with those in H9c2 cells. This indicates the cardiac-specific activity of the rat ANP promoter activity. To confirm the regulation of the rat ANP promoter activity by HIF-1, the −2307ANP reporter vector was co-transfected into H9c2 cells with expression vectors encoding human HIF-1α or HIF-1β. In cells overexpressing HIF-1α, the ANP promoter activity was increased by about 1.8-fold under normoxic conditions. Hypoxia and co-transfected HIF-1α synergistically enhanced the ANP promoter activity to a level about 5.3-fold greater than that of the pcDNA-transfected cells,

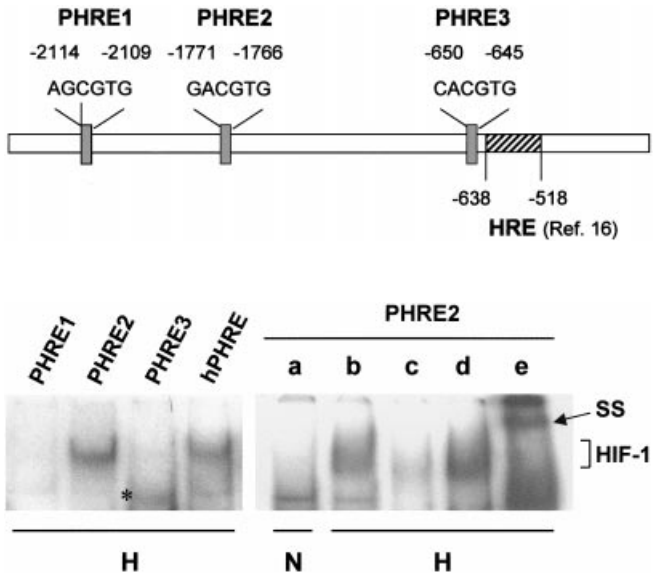

Figure 4 Identification of HRE in the ANP promoter

Top panel: positions of three PHREs in the rat ANP promoter. The hatched bar represents the HRE reported previously by Chen et al. [16]. Bottom panel: binding of HIF-1 to the putative HREs. Nuclear extracts were obtained from H9c2 cells cultured under 1% oxygen for 8 h (hypoxia, H), and incubated with a ^{32}P-labelled oligonucleotide probe containing PHREs (PHRE 1, 2 or 3) of the rat ANP gene or hPHRE of the human ANP gene. The complexes formed were resolved by non-denaturing PAGE and visualized by autoradiography (left-hand panel). Nuclear extracts were obtained from H9c2 cells cultured under 20% (a) or 1% (b) oxygen (right-hand panel). To examine the specificity of HIF-1 binding to the PHRE2 oligomer, a 10-fold molar excess of unlabelled, annealed oligonucleotide competitor representing the wild-type EPO HRE (c) or the mutated EPO HRE (d) was added to the EMSA reaction mixture containing the hypoxic nuclear extract just prior to the addition of a radiolabelled HRE2 probe. For gel supershift analysis, 1 μl of HIF-1α antiserum was added to the completed EMSA reaction mixture and incubated for 2 h at 4 °C prior to loading (e). HIF-1 binding (HIF-1) and supershifted bands (SS) are indicated.

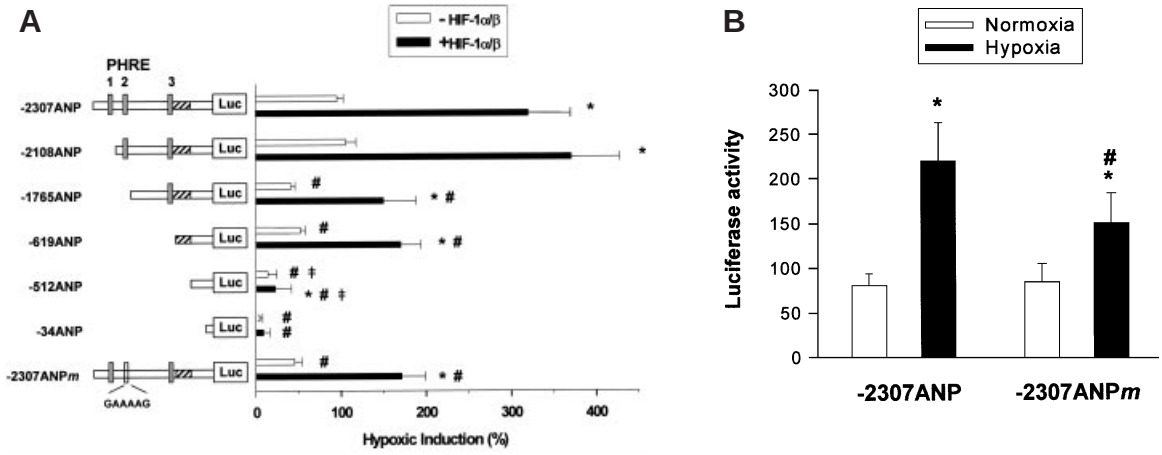

Figure 5 Deletion and mutation analysis of the ANP promoter

(**A**) Analysis of the ANP promoter in H9c2 cells. The ANP promoter region in the −2307ANP reporter vector was progressively deleted using PCR-based mutagenesis (−2108ANP, −1765ANP, −619ANP, −512ANP and −34ANP). The nucleotide sequence in PHRE2, 5′-ACGTG-3′, was mutated to 5′-AAAAG-3′ using the QuikChange™ site-directed mutagenesis method (−2307ANP*m*). The cells transfected with the deleted or mutated reporter vectors were incubated under 20 or 1% oxygen for 18 h, and then were prepared for luciferase assay. The percentage of hypoxic induction was calculated using the equation $[(h-n)/n] \times 100\%$, where h is the luciferase activity in hypoxic conditions and n is that in normoxic conditions. Each bar represents the mean ± S.D. from 12 experiments. $^*P < 0.05$ versus the non-transfected control, $\#P < 0.05$ versus −2307ANP at the corresponding oxygen level, and $\ddagger P < 0.05$ versus −1765ANP at the corresponding oxygen level. (**B**) Analysis of the ANP promoter in ventricular myocytes. Myocytes transfected with −2307ANP and −2307ANP*m* reporter vectors were incubated under normoxic or hypoxic conditions, and then were prepared for luciferase assay. Each bar represents the mean ± S.D. of luciferase activities. $^*P < 0.05$ versus the normoxic control and $\#P < 0.05$ versus −2307ANP.

whereas HIF-1β had no effect. In cells co-transfected with HIF-1α and HIF-1β, the ANP promoter activity was further stimulated by hypoxia (Figure 3B). To determine the role of endogenous HIF-1 in the hypoxic stimulation of the ANP promoter, endogenous HIF-1 activities in H9c2 cells were blocked by transfection with a dominant-negative isoform of HIF-1α, HIF-1α^{516}, as we reported previously [21]. HIF-1α^{516} completely suppressed the hypoxic stimulation of the ANP promoter activity (Figure 3B). Moreover, HIF-1α^{516} inhibited the activation of the ANP promoter induced by $CoCl_2$ and desferrioxamine to the normoxic level (Figure 3C). These results suggest that the 5′-flanking region of the ANP gene contains functional HRE regulated by HIF-1.

Identification of HRE in the rat ANP gene 5′-flanking region

Based on the consensus sequence for HIF-1-binding, we found three PHRE sites, which were designated PHRE1, PHRE2 and PHRE3, in the rat ANP promoter as shown in Figure 4 (top panel). To determine which PHRE site is an authentic HIF-1-binding site, EMSA was performed using nuclear extracts prepared from H9c2 cells incubated under hypoxic and normoxic conditions. The PHRE2 oligomer alone displayed a binding activity induced by hypoxia, but such a binding activity was undetectable in the PHRE1 and PHRE3 oligomers (Figure 4, bottom panel). The DNA-binding of PHRE2 compete effectively with an unlabelled oligomer containing the HIF-1-binding sequence of the EPO enhancer [18], but did not competed with an unlabelled oligomer mutated in the HIF-1-binding sequence [18]. Moreover, the observed supershift by the anti-HIF-1α antibody confirms that the PHRE2 oligomer bound to HIF-1. These results indicate that PHRE2 is an authentic HIF-1-binding site. Based on the PHRE sequence of the rat ANP gene 5′-flanking region, we also found a putative HRE (−1259 to −1239) in the human ANP gene 5′-flanking region. The oligomer of the hPHRE produced a binding band with the same mobility as that of rat PHRE2 (Figure 4, bottom panel). On the other hand, the PHRE3 displayed a faint band at a lower position than that of the PHRE2 (indicated by an asterisk in Figure 4, bottom panel). Although the protein binding with the PHRE3 was not identified, the PHRE3 might bind with protein(s) other than HIF-1 because its position on the gel is quite different from that of the HIF-1–DNA complex.

To determine whether PHRE2 is responsible for the hypoxic induction of the rat ANP promoter activity, the 5′-flanking region in the ANP gene was progressively deleted in the −2307ANP reporter vector, which was transfected into H9c2 cells. Figure 5 shows that the region between −2108 and −1765 is responsible for the hypoxic induction of the ANP promoter activity in part. Since this region contains PHRE2, it seems to be the HRE that is responsive to the hypoxic induction of ANP. To test this possibility, the putative HIF-1-binding sequence in PHRE2, 5′-ACGTG-3′, was mutated to 5′-AAAAG-3′, designated −2307ANP*m*. The mutation of PHRE2 attenuated the hypoxic induction of the reporter (Figure 5). These results indicate that PHRE2 is responsible for the hypoxic induction of the rat ANP promoter activity in part. Although −1765ANP and −2307ANP*m* have no PHRE2, they still showed partial induction in response to hypoxia, suggesting there is other HRE without the HIF-1-binding sequence next to the PHRE2 site. To identify the HRE remaining on −1765ANP, the 5′ site of the promoter was further deleted to make −619ANP, −512ANP and −34ANP. The loss of the segment between −619 and −512 attenuated the hypoxic activation of the ANP promoter remaining after removing PHRE2, whereas the loss of PHRE3 did not affect the promoter activity. To confirm that the PHRE2 promoter region is activated by HIF-1, we examined the responses of the ANP reporter vectors to the co-expression of HIF-1α and β. The −2307ANP and −2108ANP vectors containing PHRE2 showed a remarkable induction in luciferase activity. Unexpectedly, however, −1765ANP and −2307ANP*m* vectors also showed a significant induction by HIF-1. The loss of the

segment between −619 and −512 further attenuated the HIF-1-dependent activation of the ANP. This suggests the unidentified HRE located between −619 and −512 is activated indirectly by HIF-1 and, in part, participates in the hypoxic expression of the rat ANP gene.

To confirm the contribution of PHRE2 to hypoxic induction of ANP, we examined whether the wild-type and mutated ANP reporter vectors responded to hypoxia in cardiac myocytes as revealed in H9c2 cells. The −2307ANP reporter vector showed a 2.8-fold increase in hypoxic cardiac myocytes. As expected, the hypoxic induction of the −2307ANP*m* promoter was significantly decreased compared with that of the wild-type ANP promoter. However, the activity of the mutated promoter was partially induced by hypoxia. Since these findings are comparable with those observed in H9c2 cells, it is suggested that there are no differences in cellular responses to hypoxia of primarily cultured myocytes and the H9c2 cell-line.

Taken together, these results demonstrate the requirement for HIF-1 in the activation of the ANP gene in cardiac myocytes under hypoxic conditions, and this may in fact be the molecular mechanism of ANP gene induction in the ischaemic ventricular myocardium.

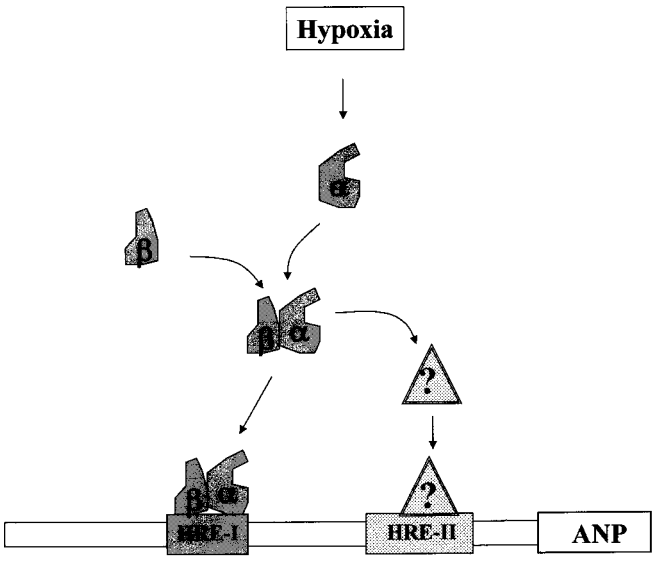

Figure 7 Hypothesis of the mechanism of hypoxic ANP induction

α, HIF-1α; β, HIF-1β; ?, unidentified *trans*-acting factor; HRE-I (−1771 to −1753), the *cis*-element binding to HIF-1; HRE-II (−619 to −512), the *cis*-element regulated indirectly by HIF-1.

DISCUSSION

This study demonstrated that ANP mRNA expression in rat ventricular myocardium was induced in an early phase of ischaemia, preceded by the expression of HIF-1α. The ANP gene was also induced by hypoxia, $CoCl_2$ and desferrioxamine in H9c2 cells and ventricular myocytes, which was also accompanied by the expression of HIF-1α. The 2307 bp 5′-flanking region of the rat ANP gene was cloned and fused to the luciferase gene. The promoter activity was only shown in myogenic cells and was induced by hypoxia, $CoCl_2$ and desferrioxamine. The overexpression of HIF-1α markedly enhanced the promoter activity under hypoxic conditions. Moreover, a dominant-negative isoform of HIF-1α completely suppressed the ANP promoter activity induced by hypoxia, $CoCl_2$ and desferrioxamine. We demonstrated that the region from −1771 to −1766 is responsible for the hypoxic induction of the ANP promoter activity in part. EMSA confirmed that this region is an authentic HIF-1-binding site. Since the HREs found in many HIF-1-regulated genes include a HIF-1-binding sequence, 5′-ACGTG-3′ and a downstream HIF-1 ancillary sequence, 5′-CACAG-3′, we compared the ANP HRE with HREs of other hypoxia-inducible genes (Figure 6). The ANP HRE also contains the very well conserved sequences, i.e. the HIF-1-binding sequence and HIF-1 ancillary sequence. Other than this HRE, a particular promoter region between −619 and −512 was also regulated by HIF-1, and contributed, in part, to the hypoxic activation of the ANP promoter. These results suggest that HIF-1, directly and indirectly, enhances the transactivation of the ANP gene in hypoxic cardiac myocytes, as summarized in Figure 7. Therefore, this implies that HIF-1 stimulation of the ANP promoter may be responsible for the induction of the ANP gene in ischaemic ventricular myocardium.

	HBS	HAS
Rat ANP (-1771 to -1753)	GAGACGTGCAGAGCATCACAG	
Human ANP (-1255 to -1240)	TATACGTGTGAGCCACTG	
Human EPO	CCTACGTGCTGTCTCACACAG	
Human GLUT-1	CAGGCGTGCCGTCTGACACGC	
Human VEGF	CATACGTGGGCTCCAACAG	
Mouse iNOS	ACTACGTGCTGCCTAGGGGCCACTG	

Figure 6 Comparison of *cis*-acting sites in HREs

The HRE structure in the ANP promoter was compared with those of known HIF-1-responsive genes. HBS and HAS represent the HIF-1-binding sequence (underlined) and the HIF-1 ancillary sequence (underlined), respectively. iNOS, inducible nitric oxide synthase.

An increasing number of mammalian genes that are induced by hypoxia have been identified. A vasodilation-related group of these genes includes VEGF, inducible nitric oxide synthase [22], haem oxygenase [23] and adrenomedullin [9]. The hypoxic induction of these genes is believed to contribute to increasing the oxygen tension in hypoxic tissues. In this respect, our results suggest that ANP can be included in this group of HIF-1-mediated vasodilators. However, this compensatory mechanism by ANP might be limited to the heart due to the tissue-specific activity of the ANP promoter [24]. We also demonstrated the cardiac cell-specific activity of the ANP promoter by comparing its activity in myocytes and HEK-293 cells. Moreover, the hypoxic activation of the ANP promoter was observed only in myocytes.

Chen et al. [16] previously analysed the *cis*-element of the rat ANP gene responsive to hypoxia. According to their results, the levels of ANP mRNA did not exhibit any change until 40 h after hypoxia, but were significantly elevated later in AT-1 cells, which are atrial tumour cells. They also analysed the −3003/+62 sequence of the rat ANP promoter to find the HRE, using a luciferase reporter system. Deletion analysis in their study demonstrated that the promoter region, from −638 to −518, produced a significant induction of the promoter activity under hypoxic conditions. Moreover, EMSA, using the corresponding

120 bp oligonucleotide probe, revealed that multiple DNA–protein complexes were formed in the nuclear extracts of cells cultured under hypoxic conditions for 40 h. However, they did not examine the possible role of HIF-1 in the hypoxic induction of ANP. In this study, we also examined the role of the promoter region identified by Chen et al. [16] on the hypoxic induction of ANP. However, the promoter region partly contributed to the hypoxic activation of the ANP promoter, as shown in Figure 5. Moreover, we found that that region and PHRE2 are regulated by HIF-1. Why do their results differ from those of the promoter analysis undertaken in the present study? One possible explanation is that the cell lines used for promoter analysis were different. They used the AT-1 myoblast cell line originating from the rat atrium [25], whereas we used the H9c2 myoblast cell line originating from the rat ventricle [26] and ventricular myocytes. In the present study, we confirmed that HIF-1α protein is expressed and regulated by the oxygen tension in both H9c2 and neonatal ventricular cells. However, whether HIF-1α can be expressed in hypoxic AT-1 cells is unclear. Therefore, the question of whether AT-1 is the proper cell for finding the HIF-1-responsive element remains unanswered.

To know which transcription factor mediates the hypoxic induction of ANP, it is important to understand the time course for its transcriptional activation by hypoxia. In general, the transcription of the HIF-1-induced genes is activated in the early phase of ischaemia or hypoxia because HIF-1 is activated within several hours of hypoxia. For this reason, we meticulously checked the time course of the ANP mRNA expression in the ischaemic myocardium and compared it with the equivalent time course for HIF-1α expression. Surprisingly, HIF-1α in rat ventricular myocardium was fully induced as early as 2 h after ischaemia, as shown in Figure 1, which suggests that the expression of HIF-1α commenced within 2 h. Then, 4 h later, at 6 h after ischaemia, ANP mRNA induction started in the ischaemic myocardium. This 4 h time difference is very reasonable for the HIF-1-mediated induction of ANP because the induction of VEGF, which is one of the HIF-1-regulated genes, occurs about 4 h after the expression of HIF-1α in the stretched ventricle [20]. However, factor(s) other than HIF-1 may be required for the induction of ANP to be delayed for as long as 7 days following ischaemia as previously reported [12,27]. The myocardial stretch caused by ischaemia-induced heart failure is partly responsible for the delayed induction of ANP mRNA. However, a previous study, demonstrating that ANP mRNA is induced in the ischaemic left ventricle to a much greater extent than the right ventricle or the atrium [27], suggests the possibility that ischaemia *per se* may contribute to the long-term induction of ANP mRNA. Chen et al. [16] have also suggested that the promoter region (-638 to -518) of the rat ANP gene may be related to this incidence of ANP induction in delayed response to ischaemia. Taken together, it is apparent that the two elements of the ANP gene 5′-flanking region may be activated by hypoxia under different time courses: the segment from -1771 to -1766 for the early hypoxic response and the segment from -638 to -518 for the delayed hypoxic response.

Our results from the promoter analysis suggest the possible presence of *trans*-acting factor(s) which may be activated by HIF-1, and enhance the hypoxic expression of the ANP gene. Although the *trans*-acting factor has not been identified, it might interact with some promoter region located between -619 and -512. Since this promoter region includes the HRE demonstrated previously by Chen et al. [16], it is possible that the putative transcription factor(s) suggested by them mediates the indirect action of HIF-1 on the ANP promoter. This possibility remains to be clarified.

This work was supported by a Korean Research Foundation grant (KRF-01-015-FP0096).

REFERENCES

1 Semenza, G. L. (2000) HIF-1 and human disease: one highly involved factor. Genes Dev. **14**, 1983–1991

2 Wang, G. L. and Semenza, G. L. (1995) Purification and characterization of hypoxia-inducible factor 1. J. Biol. Chem. **270**, 1230–1237

3 Maxwell, P. H., Wiesener, M. S., Chang, G. W., Clifford, S. C., Vaux, E. C., Cockman, M. E., Wykoff, C. C., Pugh, C. W., Maher, E. R. and Ratcliffe, P. J. (1999) The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature (London) **399**, 271–275

4 Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J. M., Lane, W. S. and Kaelin, Jr, W. G. (2001) HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O_2 sensing. Science **292**, 464–468

5 Jaakkola, P., Mole, D. R., Tian, Y. M., Wilson, M. I., Gielbert, J., Gaskell, S. J., Kriegsheim, Av., Hebestreit, H. F., Mukherji, M., Schofield, C. J. et al. (2001) Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation. Science **292**, 468–472

6 Masson, N., Willam, C., Maxwell, P. H., Pugh, C. W. and Ratcliffe, P. J. (2001) Independent function of two destruction domains in hypoxia-inducible factor-alpha chains activated by prolyl hydroxylation. EMBO J. **20**, 5197–5206

7 Lee, S. H., Wolf, P. L., Escudero, R., Deutsch, R., Jamieson, S. W. and Thistlethwaite, P. A. (2000) Early expression of angiogenesis factors in acute myocardial ischemia and infarction. N. Engl. J. Med. **342**, 626–633

8 Forsythe, J. A., Jiang, B. H., Iyer, N. V., Agani, F., Leung, S. W., Koos, R. D. and Semenza, G. L. (1996) Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol. Cell. Biol. **16**, 4604–4613

9 Garayoa, M., Martinez, A., Lee, S., Pio, R., An, W. G., Neckers, L., Trepel, J., Montuenga, L. M., Ryan, H., Johnson, R., Gassmann, M. and Cuttitta, F. (2000) Hypoxia-inducible factor-1 (HIF-1) up-regulates adrenomedullin expression in human tumor cell lines during oxygen deprivation: a possible promotion mechanism of carcinogenesis. Mol. Endocrinol. **14**, 848–862

10 Hu, J., Discher, D. J., Bishopric, N. H. and Webster, K. A. (1998) Hypoxia regulates expression of the endothelin-1 gene through a proximal hypoxia-inducible factor-1 binding site on the antisense strand. Biochem. Biophys. Res. Commun. **245**, 894–899

11 Drexler, H., Hanze, J., Finckh, M., Lu, W., Just, H. and Lang, R. E. (1989) Atrial natriuretic peptide in a rat model of cardiac failure. Atrial and ventricular mRNA, atrial content, plasma levels, and effect of volume loading. Circulation **79**, 620–633

12 Loennechen, J. P., Stoylen, A., Beisvag, V., Wisloff, U. and Ellingsen, O. (2001) Regional expression of endothelin-1, ANP, IGF-1, and LV wall stress in the infarcted rat heart. Am. J. Physiol. Heart Circ. Physiol. **280**, H2902–H2910

13 de Bold, A. J. (1985) Atrial natriuretic factor: a hormone produced by the heart. Science **230**, 767–770

14 Brenner, B. M., Ballermann, B. J., Gunning, M. E. and Zeidel, M. L. (1990) Diverse biological actions of atrial natriuretic peptide. Physiol. Rev. **70**, 665–699

15 Fyhrquist, F., Sirvio, M. L., Helin, K., Saijonmaa, O., Metsarinne, K., Paakkari, I., Jarvinen, A. and Tikkanen, I. (1993) Endothelin antiserum decreases volume-stimulated and basal plasma concentration of atrial natriuretic peptide. Circulation **88**, 1172–1176

16 Chen, Y. F., Durand, J. and Claycomb, W. C. (1997) Hypoxia stimulates atrial natriuretic peptide gene expression in cultured atrial cardiocytes. Hypertension **29**, 75–82

17 Simpson, D. G., Terracio, L., Terracio, M., Price, R. L., Turner, D. C. and Borg, T. K. (1994) Modulation of cardiac myocyte phenotype *in vitro* by the composition and orientation of the extracellular matrix. J. Cell Physiol. **161**, 89–105

18 Chun, Y. S., Choi, E., Kim, G. T., Choi, H., Kim, C. H., Lee, M. J., Kim, M. S. and Park, J. W. (2000) Cadmium blocks hypoxia-inducible factor (HIF)-1-mediated response to hypoxia by stimulating the proteasome-dependent degradation of HIF-1alpha. Eur. J. Biochem. **267**, 4198–4204

19 Chun, Y. S., Choi, E., Kim, G. T., Lee, M. J., Lee, M. J., Lee, S. E., Kim, M. S. and Park, J. W. (2000) Zinc induces the accumulation of hypoxia-inducible factor (HIF)-1alpha, but inhibits the nuclear translocation of HIF-1beta, causing HIF-1 inactivation. Biochem. Biophys. Res. Commun. **268**, 652–656

20 Kim, C. H., Cho, Y. S., Chun, Y. S., Park, J. W. and Kim, M. S. (2002) Early expression of myocardial HIF-1alpha in response to mechanical stresses: regulation by stretch-activated channels and the phosphatidylinositol 3-kinase signaling pathway. Circ. Res. **90**, E25–E33

21 Chun, Y. S., Choi, E., Kim, T. Y., Kim, M. S. and Park, J. W. (2002) A dominant-negative isoform lacking exons 11 and 12 of the human hypoxia-inducible factor-1α gene. Biochem. J. **15**, 71–79

22 Jung, F., Palmer, L. A., Zhou, N. and Johns, R. A. (2000) Hypoxic regulation of inducible nitric oxide synthase via hypoxia inducible factor-1 in cardiac myocytes. Circ. Res. **86**, 319–325
23 Lee, P. J., Jiang, B. H., Chin, B. Y., Iyer, N. V., Alam, J., Semenza, G. L. and Choi, A. M. (1997) Hypoxia-inducible factor-1 mediates transcriptional activation of the heme oxygenase-1 gene in response to hypoxia. J. Biol. Chem. **272**, 5375–5381
24 Wu, J. P., Kovacic-Milivojevic, B., Lapointe, M. C., Nakamura, K. and Gardner, D. G. (1991) Cis-active determinants of cardiac-specific expression in the human atrial natriuretic peptide gene. Mol. Endocrinol. **5**, 1311–1322
25 Kline, R. P., Sorota, S., Dresdner, K. P., Steinhelper, M. E., Lanson, Jr, N. A., Wit, A. L., Claycomb, W. C. and Field, L. J. (1993) Spontaneous activity in transgenic mouse heart: comparison of primary atrial tumor with cultured AT-1 atrial myocytes. J. Cardiovasc. Electrophysiol. **4**, 642–660
26 Kimes, B. W. and Brandt, B. L. (1976) Properties of a clonal muscle cell line from rat heart. Exp. Cell Res. **98**, 367–381
27 Drexler, H., Hanze, J., Finckh, M., Lu, W., Just, H. and Lang, R. E. (1989) Atrial natriuretic peptide in a rat model of cardiac failure. Atrial and ventricular mRNA, atrial content, plasma levels, and effect of volume loading. Circulation **79**, 620–633

Received 10 July 2002/30 October 2002; accepted 4 November 2002
Published as BJ Immediate Publication 4 November 2002, DOI 10.1042/BJ20021087