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Hypoxia Regulates the Expression of the Adrenomedullin and HIF-1 Genes in Cultured HL-1 Cardiomyocytes

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Adrenomedullin (AM) is a hypotensive protein expressed in a variety of cells and tissues. We observed previously that the expression of the adrenomedullin gene increases substantially in the developing rat heart and in cultured adult rat ventricular cardiac myocytes in response to hypoxia as a function of time. An adrenomedullin promoter–luciferase reporter construct was used to show that this increase in adrenomedullin mRNA resulted from increased transcription in response to hypoxia. We report here additional evidence documenting that this hypoxia-induced transcription of the adrenomedullin gene is regulated by the hypoxia-inducible factor-1 (HIF-1) transcription factor. We used Northern blot analysis to show an increase in the levels of *AM* and *HIF-1α* mRNA but not *HIF-1β* mRNA in the HL-1 cardiac myocyte cell line in response to hypoxia. Furthermore, Western blot analysis revealed that the levels of both HIF-1α and HIF-1β protein increased under hypoxic conditions. Data from electrophoretic mobility shift assays indicate that the heterodimeric HIF-1 complex binds to the HIF-1-responsive elements. Combined data from these studies demonstrate that the *AM* gene is regulated by hypoxia-responsive elements localized in the *AM* promoter region. © 1999 Academic Press

Key Words: **adrenomedullin; cardiomyocytes; HIF-1; hypoxia.**

Adrenomedullin (AM) is a hypotensive protein that was first identified in human pheochromocytoma tissue in 1993 (1). The AM protein was found to be expressed in a variety of rat tissues including the heart, adrenal medulla, brain, kidney, pancreas, lung, spleen, thyroid, and liver (2). Developmentally, the heart was the first organ shown to express the AM protein in both the mouse and the rat, with expression first observed as early as embryonic day eight (3). The AM protein has been implicated as an important regulator in the cardiovascular as well as the renal systems, where it has been observed to produce a dose-dependent increase in vasodilation (1,4).

Previously, we reported that the expression of the adrenomedullin gene increases significantly in the developing rat heart, while *AM* receptor mRNA levels remained relatively constant throughout development (5). Furthermore, *AM* mRNA levels increased in cultured adult rat ventricular cardiac myocytes in response to hypoxia as a function of time. Studies using an *AM* promoter-luciferase reporter construct indicated that the increase in *AM* mRNA occurred as a result of increased transcription in response to hypoxia. In addition, we demonstrated through site-directed mutagenesis that the hypoxia-inducible factor-1 (HIF-1) binding site, at −1095 bp from the transcriptional start site, was largely responsible for the observed hypoxic response.

In this study, we provide additional evidence documenting that the *AM* gene is regulated by hypoxia via the HIF-1 system. Northern analysis data show that *AM* and *HIF-1α* mRNA levels increase in cardiomyocytes under hypoxic conditions. Furthermore, western blot analysis reveals that levels of both HIF-1α and HIF-1β protein increase in response to hypoxia. Data from electrophoretic mobility shift assays show that the heterodimeric HIF-1 complex binds to the HIF-1 responsive elements localized in the *AM* promoter.

MATERIALS AND METHODS

Culture of HL-1 cells. HL-1 cells are a differentiated yet dividing cardiac myocyte cell line (6). They were cultured as described (6), in HL-1 medium (ExCell 320 medium part A and B [JRH Biosciences], 10% fetal bovine serum, 15 μg/ml insulin [Life Technologies, Inc.], 1 × non-essential amino acids [Life Technologies, Inc.], 50 μg/ml endothelial cell growth supplement [Upstate Biotechnology], 1 μM retinoic acid [Sigma], and 0.1 mM norepinephrine [Sigma]).

Northern blot analysis. Total RNA was isolated from hypoxic and normoxic HL-1 cells using TRIzol reagent (Life Technologies, Inc.). At 75% confluency, the HL-1 cells were exposed to normoxic (95% air: 5% CO_2) or hypoxic (1% O_2: 5% CO_2: 94% N_2) conditions at 37°C for

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12 or 24 h. Cells were washed twice with Dulbecco's phosphate buffered saline (PBS) and lysed with TRIzol reagent. Chloroform (0.2 ml/ml TRIzol) was added and the sample was centrifuged at 12,000 × g for 15 minutes at 4°C. The aqueous phase containing the RNA, was removed and the RNA recovered by precipitation with isopropanol. The RNA pellets were washed with 75% ethanol and dissolved in TLE (10 mM Tris-HCl [pH 8.5], 0.1 mM EDTA). Total RNA was treated with RNase-free DNase I according to standard protocol.

Total RNA (15 μg) was fractionated through a 1.2% agarose/2.2 M formaldehyde gel by denaturing electrophoresis. The RNA was then transferred via capillary action to a Hybond-N membrane (Amersham Life Science), followed by UV cross-linking. The nylon membrane was pre-hybridized in a solution containing 50% formamide, 1 mM EDTA [pH 7.2], 7% SDS, 0.25 M $NaHPO_4$ [pH 7.0], 0.25 M NaCl, and 0.1 mg/ml single-stranded herring testes DNA for 1 h at 42°C. The cDNAs for four probes: mouse adrenomedullin (1.2 kb PCR product) (5); human HIF-1α (sequence 1-3900 cDNA) (7); mouse HIF-1β (mouse ARNT cDNA) (8) and mouse GAPDH (9), were random prime labeled with [α-^{32}P]dCTP (Amersham Pharmacia Biotech) using the Prime-a-Gene labeling system (Promega). The probe was denatured by boiling for 5 minutes, added to the blot in prehybridization solution, and incubated overnight at 42°C. Washes were performed in succession using 2 × SSC 0.1% SDS, 1 × SSC 0.1% SDS, and 0.1 × SSC 0.1% SDS at temperatures increasing from 25°C to 42°C until detectable counts fell below 50 counts per s. The hybridization signals were quantitated using a PhosphorImager (Molecular Dynamics) and normalized against GAPDH.

Preparation of nuclear extract. Nuclear extracts were prepared from hypoxic and normoxic HL-1 cells according to the Semenza and Wang nuclear extraction protocol (10). Cells were harvested by washing with cold Dulbecco's PBS and pelleted by centrifugation at 1,850 × g for 5 minutes. The cell pellet was washed with and resuspended in buffer A (10 mM Tris-HCl [pH 7.8], 1.5 mM $MgCl_2$, and 10 mM KCl), and incubated on ice for 10 minutes. The cell suspension was then homogenized with a dounce homogenizer and nuclei were pelleted by centrifugation at 3,500 × g. The pellet was resuspended in buffer C (0.42 M KCl, 20 mM Tris-HCl [pH 7.8], 1.5 mM $MgCl_2$, and 20% glycerol) and mixed on a rotating platform at 4°C for 30 minutes. Both buffer A and C also contain the following: 0.5 mM DTT, 0.4 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml pepstatin, 2 μg/ml aprotinin, and 1 mM sodium vanadate. The nuclear debris was pelleted by centrifugation for 30 minutes at 25,000 × g and the supernatant was dialyzed against buffer D (20 mM Tris-HCl [pH 7.8], 0.1 M KCl, 0.2 mM EDTA, and 20% glycerol) at 4°C for 4 h. The dialysate was centrifuged at 25,000 × g for ten minutes and the supernatant was flash frozen with liquid N_2 and stored at −80°C. All centrifugation steps were carried out at 4°C. Protein concentration was determined using the BioRad *DC* Protein Assay with BSA standards.

Western blot analysis. Analysis of HIF-1α and HIF-1β protein levels were carried out using nuclear extracts from HL-1 cells that had been exposed to hypoxic and normoxic conditions. Protein samples were resuspended in sample buffer containing 2% SDS, 100 mM dithiothreitol, 60 mM Tris-HCl [pH 6.8], and 0.01% bromophenol blue. The protein mixture was then placed in a boiling water bath for 7 minutes and briefly centrifuged at low speed to collect the denatured proteins. Protein samples were resolved on a 6% Tris/glycine SDS-polyacrylamide gel in running buffer containing 25 mM Tris, 192 mM glycine, and 0.1% SDS. The proteins were then transferred to a nitrocellulose membrane (Hybond-C, Amersham Life Science) overnight at 4°C at 80 mA using a transfer buffer containing 20 mM Tris-HCl [pH 8.0], 150 mM glycine, and 20% methanol. The membrane was stained with 0.1% Ponceau S (Sigma) in 5% acetic acid for 5 minutes to verify equal loading of lanes, and the gel was stained with Coomassie Blue (Bio-Rad Laboratories, Inc.) to verify complete transfer of proteins.

Preparation of the membranes for visualization of the fractionated proteins was performed following the ECF Western Blot Kit (Amersham Life Science) protocol with minor modifications. Non-specific proteins were blocked by incubation of the membrane with 5% nonfat dry milk in TBS-T (20 mM Tris-HCl [pH 7.6], 137 mM NaCl, and 0.1% Tween 20) for 1 h at room temperature with agitation. Primary antibody (mouse anti-HIF-1α [Novus Biological] or mouse anti-ARNT-1 [Transduction Laboratories]) was added to the membrane at a 1:1000 dilution in TBS-T and incubated at room temperature for 1 h with agitation. The secondary antibody, fluorescein linked anti-mouse at a 1:600 dilution in TBS-T was added to the membrane and incubated at room temperature for 1 h with agitation. Anti-fluorescein alkaline phosphatase conjugate at a 1:5000 dilution in TBS-T was added to the membrane and incubated for 1 h at room temperature with agitation. ECF substrate was added at 15 μl/cm^2 membrane. Between each of the five proceeding steps (blocking, primary antibody, secondary antibody, tertiary antibody, and ECF substrate) the membrane was washed three times with TBS-T at room temperature, once for 15 minutes and twice for 5 minutes each. The membrane was immediately visualized by using a PhosphorImager after the addition of the ECF substrate. For subsequent probing, the membrane was washed with 40% methanol at room temperature for 30 minutes to remove the ECF substrate. Removal of primary and secondary antibodies was carried out at 50°C for 30 minutes in stripping buffer (0.01% 2-mercapthoethanol, 2% SDS, 62.5 mM Tris-HCl [pH 6.7]).

Electrophoretic mobility shift assay. Three synthetic oligonucleotide fragments were synthesized (Life Technologies, Inc.) as probes for electrophoretic mobility shift assays. These included: 1) AM_{wt}, an 18-mer oligonucleotide (5′-CCCGTGGCAA**ACGT**GTTC-3′) which contains the core binding sequence (bold) of the putative HIF-1 consensus site on the *AM* promoter, 2) AM_{mut} (negative control), an 18-mer oligonucleotide (5′-CCCGTGGCAA**AAAA**GTTC-3′) which contains a 3-bp mutation (underlined) at the core binding sequence of the putative HIF-1 site, and 3) $VEGF_{wt}$ (positive control), a 21-mer oligonucleotide (5′-TGCAT**ACGT**GGGCTCCAACAG-3′) which contains the HIF-1 responsive element (bold) of the vascular endothelial growth factor promoter. The sense strand of each oligonucleotide was hybridized to the antisense strand by incubating at 37°C for 30 minutes and then rapidly freezing in liquid N_2. The double-stranded oligonucleotide was end labeled with [γ^{32}P]ATP using T4 polynucleotide kinase (New England Biolab) and unincorporated γ^{32}P was removed using the Nucleotide Removal Kit (Qiagen).

The electrophoretic mobility shift assays were performed by incubating 10 μg of normoxic or hypoxic nuclear extract with 10^4 cpm (1.0 ng) of γ^{32}P-labeled, double-stranded oligonucleotide probe in buffer D (25 mM Tris-HCl [pH 7.6], 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1.2 mM sodium vanadate, and 0.3 ng poly dIdC · dIdC [Sigma]). The binding reaction was carried out on ice for 15 minutes and then resolved on a 5% nondenaturing polyacrylamide gel with 1 × TBE at 40 mA for 1 h and 45 minutes. The gel was vacuum-dried and analyzed using a PhosphorImager. Competition experiments were performed with 20 and 100 ng of unlabeled double-stranded oligonucleotides (AM_{wt}, AM_{mut}, or $VEGF_{wt}$).

RESULTS

Under hypoxic conditions AM and HIF-1α mRNA levels increase while levels of HIF-1β mRNA remain unchanged. HIF-1 is a heterodimeric protein complex, composed of a HIF-1α and a HIF-1β subunit. Northern blot analysis was used to examine whether the levels of *AM*, *HIF-1α* and *HIF-1β* mRNA are regulated by hypoxic conditions in cultured mouse HL-1 cardiac myocytes. A 2-fold increase in *AM* mRNA levels was observed at both 12-h and 24-h after exposure to

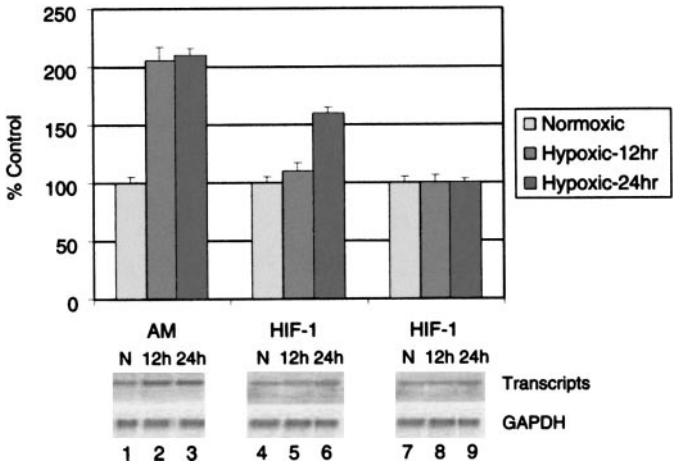

FIG. 1. Northern blot analysis of AM, HIF-1α and HIF-1β mRNA in HL-1 cardiomyocytes under normoxic and hypoxic conditions. Total RNA was isolated from HL-1 cells under normoxic (20% oxygen) and hypoxic (1% oxygen) atmospheres. After 12 and 24 h, the mRNA levels of *AM* (lanes 1, 2 & 3), *HIF-1α* (lanes 4, 5 & 6) and *HIF-1β* (lanes 7, 8 & 9) were determined by northern blot hybridization. GAPDH mRNA hybridization is shown as a control for RNA loading and for normalization (lanes 1–9). (Top) *AM, HIF-1α* and *HIF-1β* mRNA levels, normalized to GAPDH, are shown. Data are shown as mean ± SEM from three separate northern blot analyses.

hypoxia (1% O_2) (Fig. 1, lanes 1–3). The observed changes in *AM* mRNA levels were normalized to GAPDH mRNA levels, which remained constant during these studies (Fig. 1, lanes 1–9). *HIF-1α* mRNA levels also increased in HL-1 cells that were subjected to hypoxic (1% O_2) conditions, both at the 12 h and 24 h time point, as compared to normoxic controls (20% O_2) (Fig. 1, lanes 4–6). In contrast, there was no apparent change in the levels of *HIF-1β* mRNA in HL-1 cells that were exposed to hypoxic conditions (1% O_2) (Fig. 1, lanes 7–9). Previous studies have suggested that in response to hypoxic conditions, *HIF-1α* expression is up-regulated while *HIF-1β* is not induced but instead constitutively expressed (11,12,13). Therefore, this data from our northern blot analysis of *HIF-1α* and *HIF-1β* mRNA levels in HL-1 cardiomyocytes is in agreement with findings of other investigators.

HIF-1 protein is increased in cultured HL-1 mouse cardiac myocytes in response to hypoxic conditions. HL-1 cells were exposed to normoxic or hypoxic conditions for 4, 6, or 12 h. Nuclear extracts (15 μg) from these cells were fractionated by Tris/glycine SDS-PAGE and transferred to nitrocellulose membranes. The membranes were then incubated with a 1:1000 dilution of purified antibodies raised against recombinant mouse HIF-1α (Fig. 2, top) or HIF-1β (Fig. 2, bottom). Levels of HIF-1α protein, which were extremely low in HL-1 cells exposed to normoxic conditions at the 4, 6 and 12-h period (Fig. 2, top, lanes 1, 2, & 3), increased dramatically in response to hypoxic conditions (lanes 4, 5 & 6). This increase in HIF-1α protein level was substantial starting at 4-h under hypoxia (lane 4), and continued to increase at both the 6 and 12-h time period (lanes 5 & 6, respectively). Compared to HIF-1α, levels of HIF-1β protein were higher in normoxic HL-1 cells (Fig. 2, bottom, lanes 1, 2, & 3) and showed a more modest but significant induction in response to hypoxic conditions (lanes 4, 5 & 6).

Binding of HIF-1 to the adrenomedullin promoter. When an 18-bp double-stranded oligonucleotide [^{32}P]-labeled probe containing the HIF-1 binding site from the 5′-flanking sequence of the *AM* gene (Fig. 3A [AM_{wt}]) was incubated with nuclear extract from hypoxic HL-1 cells, complexes containing HIF-1 were detected (Fig. 3B, lanes 1 & 2). However, when the same probe was incubated with nuclear extract prepared from HL-1 cells cultured under normoxic conditions, no apparent complexes were observed (Fig. 3B, lanes 5 & 6). Varying amounts of unlabeled oligonucleotide AM_{wt} competed with the probe for binding of HIF-1 (Fig. 3B, lanes 3 & 4).

When a 21-bp oligonucleotide probe (Fig. 3A [$VEGF_{wt}$]) containing the HIF-1 binding site from the 5′-flanking region of the *VEGF* gene was incubated with nuclear extracts from hypoxic HL-1 cells, a complex containing HIF-1 (Fig. 3C, lane 2) was detected as previously demonstrated (14). An excess of unlabeled oligonucleotide $VEGF_{wt}$ competed with the probe for binding of HIF-1 (Fig. 3C, lane 4). Furthermore, an excess of unlabeled AM_{wt} also competed with the $VEGF_{wt}$ probe for binding of the heterodimeric HIF-1 complex (Fig. 3C, lane 3). Oligonucleotide AM_{wt} was also used as a probe and showed a pattern of binding (lane 6), which was identical to that of the $VEGF_{wt}$ probe (lane 4). The binding of HIF-1 to the AM_{wt} probe could be competed out by an excess of unlabeled AM_{wt} (lane 7) or $VEGF_{wt}$ (lane 8), but not by AM_{mut} (lane 9). In addition, when an 18-bp oligonucleotide containing a 3-bp substitution in the HIF-1 binding site (Fig. 3A [AM_{mut}]) was used as the probe in the reaction mixture, no HIF-1 complex was observed (Fig. 3C, lane 1).

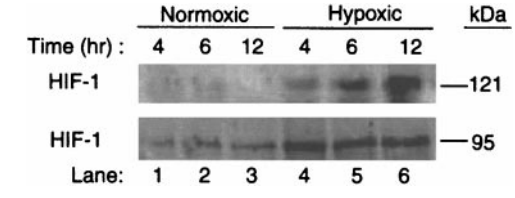

FIG. 2. Western blot analysis of HIF-1 protein levels in HL-1 cells. Nuclear extracts were prepared from cultured HL-1 cardiac myocytes that were exposed to normoxic (20% O_2) (lanes 1, 2 & 3) or hypoxic (1% O_2) (lanes 4, 5 & 6) conditions for 4, 6 or 12 h. Aliquots (15 μg) were fractionated by SDS-PAGE, transferred to nitrocellulose membranes, and incubated with a 1:1000 dilution of purified antibodies raised against recombinant mouse HIF-1α (top) or HIF-1β (bottom).

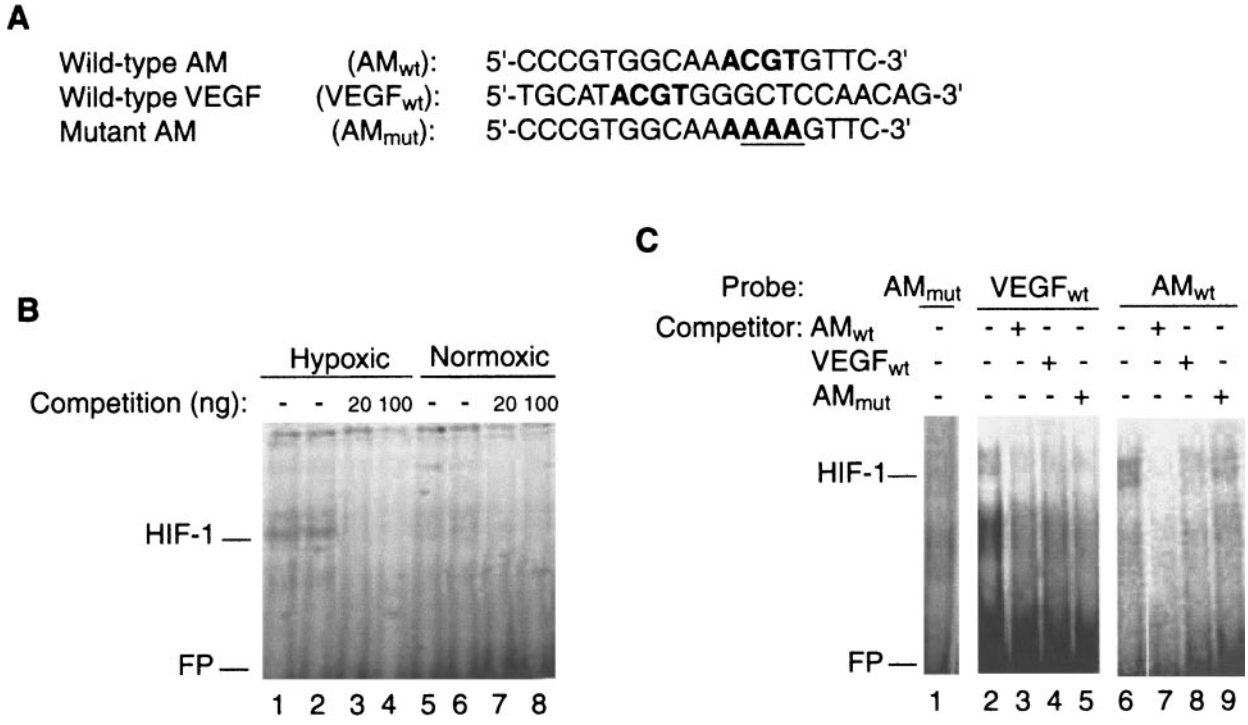

FIG. 3. Gel shift assays of HL-1 nuclear extracts for binding of HIF-1 to AM and VEGF sequences. (A) Sequences of oligonucleotides used only the coding strand sequence of each double-stranded oligonucleotide is shown. The AM_{wt} and $VEGF_{wt}$ doubled-stranded oligonucleotides contain the consensus HIF-1 binding site (bold) identified in the mouse *AM* and *VEGF* promoters. AM_{mut} oligonucleotide contains mutated changes to this core sequence (underlined bold). (B) Electrophoretic mobility shift assays. 10-μg aliquots of nuclear extract, prepared from HL-1 cells exposed to hypoxic (1% oxygen, lanes 1–4) or normoxic (20% oxygen, lanes 5–8) conditions for 12 h, were incubated with γ^{32}P-labeled AM_{wt} probe and DNA binding activities were analyzed by gel electrophoresis and autoradiography. For competition assays (lanes 3, 4, 7 & 8), the indicated amount of unlabeled AM_{wt} oligonucleotide was also incubated in the binding reaction. Complexes containing HIF-1 (HIF-1) and the location of free probe (FP) are indicated on the left. (C) 10-μg aliquots of nuclear extract, prepared from HL-1 cells exposed to hypoxic (1% oxygen) conditions for 12 h, were incubated with γ^{32}P-labeled AM_{mut}, $VEGF_{wt}$ or AM_{wt} probes. For competition assays, 50 ng of unlabeled AM_{wt} (lanes 3 & 7), $VEGF_{wt}$ (lanes 4 & 8), or AM_{mut} (lanes 5 and 9) oligonucleotides was also incubated in the binding reaction.

DISCUSSION

Previously (5), we demonstrated using the technique of differential display that the *AM* gene was differentially expressed in the developing rat heart. Exposure of cultured adult rat cardiac myocytes to hypoxia resulted in a significant, time-dependent increase in *AM* mRNA levels. We also demonstrated through transfection studies in HL-1 cells that the 5′-flanking sequence of *AM* was capable of mediating a hypoxia-inducible increase in transcription.

We now report additional evidence supporting the role of hypoxia and HIF-1 in regulating the expression of the adrenomedullin gene. We utilized northern blot analysis to show an increase in the level of *AM* mRNA in cultured HL-1 cardiac myocytes under hypoxic conditions. Using northern and western blot analyses to examine the mRNA and protein levels of HIF-1α and HIF-1β in cultured HL-1 cardiac myocytes in response to hypoxia, we observed significant similarities in the expression patterns of these two hypoxia regulated genes as was previously observed in hepatoma cells (15). We detected an increase in the mRNA levels of HIF-1α but not HIF-1β in response to hypoxia. In addition, the levels of both HIF-1α and HIF-1β proteins were observed to increase under hypoxic conditions. The heterodimeric HIF-1 protein complex has been shown to regulate the expression of many hypoxia-inducible genes including erythropoietin (10), transferrin (16) and vascular endothelial growth factor (17). Our data from electrophoretic mobility shift assays show that the HIF-1 complex binds to the HIF-1 responsive elements localized in the *AM* promoter. Collectively, we further document that the expression of the adrenomedullin, HIF-1α and β genes in cultured cardiac myocytes are regulated by hypoxia and suggest that this hypoxic regulation may have important physiological relevance in the hypoxia-compromised heart.

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