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Hypoxia inducible factor regulates the cardiac expression and secretion of apelin

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ABSTRACT **Apelin and its G-protein-coupled receptor APJ have various beneficial effects on cardiac function and blood pressure. The mechanisms that regulate apelin gene expression are not known. Because apelin gene expression has been shown to increase in cardiac ischemia, we investigated if apelin (*Apln*) gene expression was sensitive to hypoxia. Here we show that hypoxia increases the apelin expression in rat myocardium and in cultured cardiomyocytes. Pharmacological activation of hypoxia inducible factor by desferrioxamine (DFO) or expression of a constitutively active form of HIF-1α increased apelin expression in cardiomyocyte cultures. The induction of apelin by hypoxia was abolished on transient expression of the HIF inhibitory PAS protein in cardiomyocytes. Increased apelin expression induced by hypoxia or DFO was accompanied by the processing of the cellular storage form proapelin into smaller apelin peptides and increased secretion of these biologically active forms of apelin. In a rat *in vivo* model, acute myocardial infarction (24 h) led to a transient increase in ventricular apelin mRNA levels. Our results indicate that apelin gene is regulated by hypoxia in cardiac myocytes via the HIF pathway, suggesting a role for apelin as a potential marker for acute cardiac hypoxia with a possible compensatory role in myocardial tissue suffering from oxygen deprivation.—Ronkainen, V.-P., Ronkainen, J. J., Hänninen, S. L., Leskinen, H., Ruas, J. L., Pereira, T., Poellinger, L., Vuolteenaho, O., Tavi, P. Hypoxia inducible factor regulates the cardiac expression and secretion of apelin. *FASEB J.* 21, 1821–1830 (2007)**

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Apelin has been identified as an endogenous ligand of the orphan G-protein-coupled receptor APJ (1, 2). They are both widely expressed in the central nervous system (CNS) and periphery (3) with a role in the regulation of fluid and glucose homeostasis, feeding behavior, vessel formation, cell proliferation, and immunity (4, 5). Recently, it was found that insulin increases the expression of apelin in adipocytes, and, consequently, apelin expression in fat cells and apelin plasma level is increased in hyperinsulinemia-related obesity (6, 7).

However, a line of evidence indicates that the cardiovascular system is the main target of apelin. High levels of mRNA of both APJ and apelin are found in cardiac myocytes, vascular smooth muscle, and endothelial cells (8–11). Originally it was shown that apelin increases cardiac contractility in a dose-dependent manner in isolated rat hearts (12). Apelin induces acute positive inotrophy *in vivo* in normal and failing postinfarction rat hearts (13) and also long-term functional improvement of cardiac contraction without evidence of hypertrophic growth (14). In addition to the inotrophic effects of apelin, several mechanisms have been described whereby apelin regulates vascular tone and blood pressure. Intravenous injection of apelin lowers blood pressure by triggering the release of nitric oxide from endothelial cells (15) and reduces water and sodium uptake in the kidneys by inhibiting vasopressin release (16). In addition, apelin shares a degradation pathway with angiotensin II. Both are substrates for angiotensin-converting enzyme 2, an important enzyme of the renin-angiotensin system. Mice deficient in the APJ receptor have normal blood pressure but increased vasopressor response to angiotensin II (17), suggesting a primary role for apelin in counteracting angiotensin-induced vasoconstriction.

The molecular mechanisms of apelin action and especially those that regulate apelin expression are still largely unknown. Apelin is present in human plasma and myocardium, and left ventricle apelin mRNA levels increase in chronic heart failure due to coronary heart disease and dilated cardiomyopathy (18). In addition, apelin plasma levels are reported to increase especially in the early stage of left ventricular dysfunction (18, 19). Since apelin has been implicated in ischemic heart disease, we hypothesized that apelin gene expression might be controlled by oxygen levels. In this case, the

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most presumable candidate for the regulation of apelin gene expression would be hypoxia-inducible factor-1 (HIF-1). HIF-1 is activated in hypoxia due to the prevention of oxygen-dependent HIF prolyl-4-hydroxylase (HIF-P4H)-mediated proline hydroxylation, which in normoxia targets the HIF-1α subunit for ubiquitination and proteosomal degradation (20–22). HIF-1 regulates the expression of several hypoxia-inducible genes including erythropoietin, adrenomedullin, and heart-specific atrial natriuretic peptide (ANP; refs. 23–25).

In this study, we show for the first time that hypoxia regulates apelin gene expression and secretion in cardiac myocytes. We further characterize the pivotal role of HIF in the hypoxic induction of *Apln* gene expression. This finding establishes a novel regulation pathway that might improve cardiac function during compromised oxygen supply.

MATERIALS AND METHODS

Atrial superfusion model

Male Sprague-Dawley rats weighing 330–380 g from the Center for Experimental Animals at the University of Oulu (n=18) were used in the perfusion experiments with the approval of the Animal Use and Care Committee of the University of Oulu. The experimental model used in this study was the isolated perfused rat atrium, prepared as described previously (26). The pacing frequency (Grass stimulator SD9: 2 Hz, 22 V, 0.25 ms), preload (2 cm H_2O), and perfusion buffer flow (2.5 ml/min, peristaltic pump model 7553–85, Cole-Parmer Instrument, Chicago, Illinois, USA) were held constant throughout the preincubation period (30 min) and the experiments (3 h). The tissue contractility was measured with a TCB 100 pressure transducer (Millar Instruments, Houston, Texas, USA). The partial oxygen pressure of the atrium was manipulated using three different gas mixtures to bubble the Krebs-Henseleit perfusion buffer: *1)* 95% O_2 + 5% CO_2 (normoxia), *2)* 15% O_2 + 80% N_2 + 5% CO_2 (mild hypoxia), and *3)* 95% N_2 + 5% CO_2 (hypoxia). However, it is likely that the amount of oxygen used to oxygenate the buffer is not the same as that to which the tissue is exposed. In any tissue perfusion system, low oxygen percentages are hard to reach because both the perfusate and the tissue are exposed to ambient oxygen (~20%), and high oxygen percentages are equally difficult to obtain because oxygen tends to diffuse from the buffer into the air, which has a lower oxygen percentage. Actual oxygen percentages of the perfusion buffer were measured directly from the perfusion chamber using an ISO_2 dissolved oxygen meter (World Precision Instruments Inc., Sarasota, Florida, USA). The measured actual buffer O_2 percentages were 58, 18, and 12 (gases *1*, *2*, and *3*, respectively).

Infarction model

Myocardial infarction was produced by ligation of the left anterior descending coronary (LAD) artery. Male Sprague-Dawley rats weighing 250–300 g were anesthetized with medetomidine hydrochloride (Domitor 250 μg/kg ip) and ketamine hydrochloride (Ketamine 50 mg/kg ip). Rats were connected to the respirator through a tracheotomy. A left thoracotomy and pericardial incision was performed, and the LAD was ligated. After the operation, anesthesia was partially antagonized with atipamezole hydrochloride (Antisedan, 1.5 mg/kg ip) and rats were hydrated with 5 ml physiological saline solution given subcutaneously. For postoperative analgesia, buprenorphine hydrochloride (Temgesic, 0.05–0.2 mg/kg sc) was administered. The sham-operated rats underwent the same surgical procedure without the ligation of LAD. The experimental design was approved by the Animal Use and Care Committee of the University of Oulu.

Plasmids and fusion proteins

A plasmid producing constitutively active HIF-1α [pFlag-HIF-1α(1–390)-VP16] was generated by first introducing a polymerase chain reaction (PCR) product corresponding to the VP16 transactivation domain, flanked by *Nhe*I sites, into pFlag-HIF-1a previously (27) digested with the same restriction enzyme, thus generating pFlag-HIF-1α-VP16. This construct was then digested with *Sal*I and *Kpn*I (deleting the cDNA sequence corresponding to amino acids 244–822 of mouse HIF-1α) and ligated to a PCR product of the mouse HIF-1α coding sequence corresponding amino acids 244 to 390. The final product corresponds to a Flag-HIF-1α(1–390)-VP16 inframe fusion as verified by sequencing. The HIF-1α portion of a hybrid (amino acids 1–390) consists of bHLH and PAS domains being responsible for DNA binding and dimerization with HIF-1β. The VP16 portion is derived from the herpes simplex virus *trans*-activation domain and replaces the regulative domain found in endogenous HIF-1α (amino acids 391–826). The chimera protein escapes HIF-P4H-mediated hydroxylation and degradation and activates HIF-1 responses also in normoxic conditions. The pT81/hypoxia response element (HRE)-luc reporter plasmid and inhibitory PAS (Per/Arnt/Sim) domain protein (IPAS) expression vector pCMV-Flag-IPAS have been described previously (28, 29).

Cell culture and induction of hypoxia

Neonatal rat cardiomyocytes were isolated 1–2 days after birth. Ventricles were excised and cut into small pieces and incubated for 1 h in a solution containing 100 mmol/l NaCl, 10 mmol/l KCl, 1.2 mmol/l KH_2PO_4, 4.0 mmol/l $MgSO_4$, 50 mmol/l taurine, 20 mmol/l glucose, 10 mmol/l HEPES, 2 mg/ml collagenase type II (Worthington), 2 mg/ml pancreatin (Sigma P-3292), and 1% penicillin-streptomycin (PS). After incubation, the detached cells were collected in 15 ml Falcon tubes and centrifuged for 5 min at 160 *g*. The supernatant and the top layer of the pellet containing damaged cells were discarded, and the isolated cardiomyocytes were plated on 50 or 35 mm fibronectin-coated plastic dishes at a density of 2 million or 350,000 cells per dish. The cells were cultured to reach confluence in Dulbecco's modified Eagle's medium containing 10% FBS and 1% PS for 72 h before any intervention. The HL-1 cell line was a kind gift from Dr. W. C. Claycomb and was maintained as described previously (30). Pharmacological interventions and hypoxia as well as transfections were introduced to confluent HL-1 cultures, and cells were collected for measurements after 24 or 48 h. To induce hypoxia, HL-1 cells or neonatal cells were cultured in a Biospherix C-Chamber (model C-274, Biospherix, Redfield, New York, USA) inside a standard culture chamber. Oxygen concentration was set to 2%, and carbon dioxide concentration was held at 5% inside the C-Chamber by injecting N_2 and CO_2 into the chamber with a ProOx 110 oxygen controller and a ProCO2 CO_2-controller (Biospherix). To chemically mimic hypoxia responses, HL-1 cells were incubated in the presence of a HIF prolyl-4-hydroxylase

(HIF-P4H) inhibitor, desferrioxamine (DFO; 100 μmol/l, Sigma, St. Louis, MO, USA) for 24 h.

Transfection of HL-1 cells and reporter analysis

Cells were transiently transfected with Lipofectamine 2000 (10 μl) (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After a 5 h transfection period, cells were grown for 24 h at normoxia (21% O_2) and then subjected to 24 h hypoxia (2% O_2) or DFO exposure. To measure the transcriptional activity of HIF, HL-1 cells were transfected with the pT81/HRE-luc reporter plasmid (3.5 μg) (28) and the luciferase signal was measured and normalized with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions. IPAS expression vector (8.0 μg; pCMV-Flag-IPAS; ref 29) was used to suppress endogenous HIF activity. To activate HIF-1 target genes, cells were transfected with the pFlag-HIF-1α(1–390)-VP16 plasmid (8.0 μg), which produces a constitutively active, stable form of HIF-1α. In the control groups, the cells were transfected with the empty cloning vector pUC18 (8.0 μg, Fermentas International, Burlington, Ontario, Canada). Transfection efficiency was assessed using the viral vector driven GFP contruct, (pEGFP-N1, Clontech Europe, Erembodegen, Belgium). Routinely, 60–80% of the transfected cells were fluorescent after 48 h. After the incubation period, cells were lysed and total RNA was isolated.

Quantitative reverse transcriptase-PCR

Total RNA from tissue samples (auricles and left ventricles) was isolated by using the QuickPrep Total RNA Extraction Kit (Amershan Pharmacia Biotech, Piscataway, NJ, USA) and from cell cultures (HL-1 cells and neonatal cardiomyocytes) using the GenElute Mammalian Total RNA Miniprep Kit (Sigma). After cDNA synthesis (First Strand cDNA Synthesis Kit, MBI Fermentas), quantitative PCR reactions were performed with the ABI 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using the TaqMan chemistry. The forward and reverse primer sequences used were mouse apelin 5′-CATAAGGGCCCCATGCCT-3′ and 5′-GCACCGGGAGGGCACT-3′ (GenBank accession No. NM_013912), rat apelin 5′-CAAGGATCCCTTTGGCCC-3′ and 5′-AGGAGAAGCTGGGTCTCCAAG-3′ (NM_031612), mouse adrenomedullin 5′-GAGCGAAGCCCACATTC-GT-3′ and 5′-GCTGCGGGAACCCTGG-3′ (NM_009627), rat adrenomedullin 5′-CATTGAACAGTCGGGCGATG-3′ and 5′-CAGGGTGCGAAGCTCTCTG-3′ (NM_012715), mouse HIF-Prolyl-4-Hydroxylase-1 (PHD1) 5′-AGGGCG-GCTGGGCA-3′ and 5′- GATAACACGCCACCATGGC-3′ (NM_053208), mouse HIF-Prolyl-4-Hydroxylase-3 (PHD3) 5′-TGAGGCTGGATCTGGAGAAGA-3′ and 5′- AGCCGAC-CTCGTGCAGAC-3′ (NM_028133). The bifunctional fluorogenic probes for apelin were 5′-Fam-TCTAAAGCAG-GATTGAAGGGCTCGCC-Tamra-3′ for mouse and 5′-Fam-TCTTCCTGGCCACTCCTTGGACTGC-Tamra-3′ for rat. Probes for adrenomedullin were 5′-Fam-TCAAACGCTAC-CGCCAGAGCATGA-Tamra-3′ for mouse and 5′-Fam-CCCATTGGCGCCTGCGGA-Tamra-3′ for rat. Probes for PHD1 and PHD3 were 5′- Fam-ACGTCATCAATGGGCG-CACCAA-Tamra-3′ and 5′-Fam- CGCCCTGGAGTACATC-GTGCCC-Tamra-3′, respectively. The results were normalized to 18S rRNA quantified from the same samples using the forward and reverse primers 5′-TGGTTGCAAAGCT-GAAACTTAAAG-3′ and 5′-AGTCAAATTAAGCCGCAGGC-3′. The probe for the 18S amplicon was 5′-Vic-CCTGGTG-GTGCCCTTCCGTCA-Tamra-3′.

Peptide extraction

Frozen cultured neonatal ventricular cells (~500,000 cells) were homogenized in 0.5 ml ice-cold 1 M acetic acid using a UltraTurrax homogenizer. The homogenate was cleared by centrifugation at 10,000 *g* for 30 min, and the supernatant was lyophilized for use in radioimmunoassay or gel filtration HPLC. Medium samples (0.8 ml) were acidified with 160 μl 1 mol/l HCl and extracted with SepPak C_{18} cartridges using a Gilson Aspec extraction robot. Each cartridge was preconditioned with 2-propanol followed by 0.1% aqueous trifluoroacetic acid. After sample application, the cartridge was washed with 5 ml 0.1% aqueous trifluoroacetic acid followed by elution with 1 ml 80% acetonitrile, 0.1% trifluoroacetic acid. The eluates were dried in a SpeedVac centrifuge concentrator for use in radioimmunoassay or gel filtration HPLC. The SepPak extraction recovery of apelin-36 (48 fmol/ml) was found to be 84 ± 5.2% (n=8).

Apelin radioimmunoassay

Apelin was measured using a goat antiserum raised against a carbodiimide conjugate of human preproapelin$_{66-77}$ (apelin-12) and horseshoe crab hemocyanin. Preproapelin$_{62-77}$-Tyr$_{77}$ (Apelin-16 with tyrosine added to the carboxyl terminus) was radioiodinated using chloramine-T and purified by gel filtration followed by reverse-phase HPLC as described previously (31). Synthetic human preproapelin$_{42-77}$ (apelin-36, Phoenix) was used as the standard. The sensitivity of the assay was 1 fmol/tube. The intra- and interassay coefficients of variation were <10% and <15%, respectively. The antiserum cross-reacts 100% with apelin-13 (pyroglu or native), apelin-16 and apelin-36, but does not recognize preproapelin$_{68-77}$ (apelin-10), ANP, BNP, CNP, ET-1, or adrenomedullin (cross-reaction<0.1%). Synthetic peptides (apelin-10, apelin-12, apelin-13, and apelin-16) were assembled using Fmoc chemistry with an ABI 433A peptide synthesizer (Applied Biosystems) and purified by reverse-phase HPLC, as described previously (31). Apelin-36, used as the assay standard, was obtained from the human apelin-36 radioimmunoassay kit purchased from Phoenix Europe GmbH (Karlsruhe, Germany).

Gel filtration HPLC

Medium samples, extracted with SepPak C_{18} cartridges, and acid extracts of cultured rat neonatal ventricular cells, were dissolved in 500 μl 40% acetonitrile, 0.1% aqueous trifluoroacetic acid and cleared by centrifugation at 10,000 *g* for 10 min. The supernatants were passed through 0.45 μm Millipore filters, and the filtrates were subjected to GF-HPLC analysis. The samples were applied to a Protein-Pak 125 GF-HPLC column [300 × 3.9 mm (i.d.); Waters] and eluted with 40% acetonitrile in aqueous 0.1% trifluoroacetic acid. The flow rate was 1 ml/min, and 0.5 ml fractions were collected. Fractions were dried in a SpeedVac concentrator and dissolved in 0.5 ml of RIA assay buffer for use in the RIAs. The column was calibrated in separate runs with bovine serum albumin (void volume), apelin-36, apelin-16, apelin-13, apelin-12, and $^{125}I^-$ (total volume).

Immunofluorescense labeling and microscopy

HL-1 cells were first cultured for 72 h and then kept 24 h in either normoxia or hypoxia (2% O_2). Cells were rinsed with 0.1 mol/l Tris-HCl, pH 7.3, fixed with 3% paraformaldehyde for 2 min, and permeabilized for 10 min with 0.5% Triton X-100. After being washed with 0.1 mol/l Tris-HCl, pH 7.3

twice for 5 min the primary apelin (in house goat antiapelin), HIF-1α [Novus Biologicals (H1alpha67) NB 100–105], or ANP (in house rabbit anti ANP; ref 32) antibody was incubated for 1 h in 0.1 mol/l Tris-HCl (pH 6.0, 8.6, and 7.3, respectively) containing 10% FBS and 0.05% Triton X-100. Again the specimens were washed twice and the secondary antibody [Alexa Fluor 488 chicken anti-goat, Molecular Probes (Eugene, OR, USA), goat anti-mouse IgG-FITC, Santa Cruz Biotechnology (Santa Cruz, CA, USA), chicken anti-rabbit Alexa 488, Molecular Probes] was incubated (pH 7.3) for 1 h. Dilutions for primary antibodies were 1:250 (apelin), 1:2000 (HIF-1α) and 1:250 (ANP). Secondary antibody dilution was 1:750 in all groups. Actin fibers were labeled with Alexa Fluor 568 phalloidin (Molecular Probes) (1:200 dilution) in 0.1 mol/l Tris-HCl, pH 7.3 for 20 min and washed twice for 5 min. After being labeled, images were taken freshly with Olympus FV1000 confocal microscope (HIF-1α: excitation 488 nm, emission 505–525 nm, actin: excitation 543, emission 560–660 nm, apelin and ANP: excitation 488 nm, emission 505–600). The amount of stabilized and translocated HIF-1α was determined by measuring the intensity of the HIF-1α stained nuclear fluorescence. To get comparable values, background subtracted fluorescence intensities (F) were divided by the minimum cytosolic fluorescence (F_0) and normalized to the intensity in normoxia.

RESULTS

Hypoxia rapidly increases apelin expression in perfused rat atrium and in cultured neonatal rat cardiomyocytes

In the first set of experiments, we characterized how isolated rat left atrium gene expression levels respond to reduced levels of oxygen. When the O_2 content of the perfusion buffer was lowered from the normal 58–18% for 3 h, the hypoxia marker gene adrenomedullin (24) mRNA levels rose 2.7-fold ($P<0.01$; $n=6$) and the induction was 3.1-fold ($P<0.001$; $n=8$) after exposure to ~12% O_2 (**Fig. 1*A***). Apelin gene expression changes were similar to those of adrenomedullin, although 18% O_2 perfusion did not increase apelin mRNA levels significantly, but exposure to 12% O_2 increased apelin mRNA levels 2.5-fold ($P<0.05$; $n=8$) after a 3 h perfusion period (Fig. 1*B*). To further characterize the time-scale for induction of apelin expression, we exposed cultured neonatal rat cardiomyocytes to 1, 2, 8, and 24 h of hypoxia (2% O_2). Apelin gene expression increased 3.7-fold ($P<0.001$; $n=4$) after 2 h hypoxic incubation and 5.6-fold ($P<0.001$; $n=4$) after 8 h exposure. Further incubation under hypoxic conditions for up to 24 h did not result in a further increase in apelin gene expression levels (5.6-fold, $P<0.001$; $n=4$; Fig. 1*C*). Hypoxic induction of apelin was accompanied by similar augmentation of a known hypoxia marker gene adrenomedullin with 1.5-fold induction after 1 h hypoxia ($P<0.01$; $n=4$), 2.8-fold more after 2 h ($P<0.001$; $n=4$), 4.0-fold after 8 h ($P<0.001$; $n=4$), and 6.0-fold after 24 h ($P<0.001$; $n=4$) exposure (Fig. 1*C*).

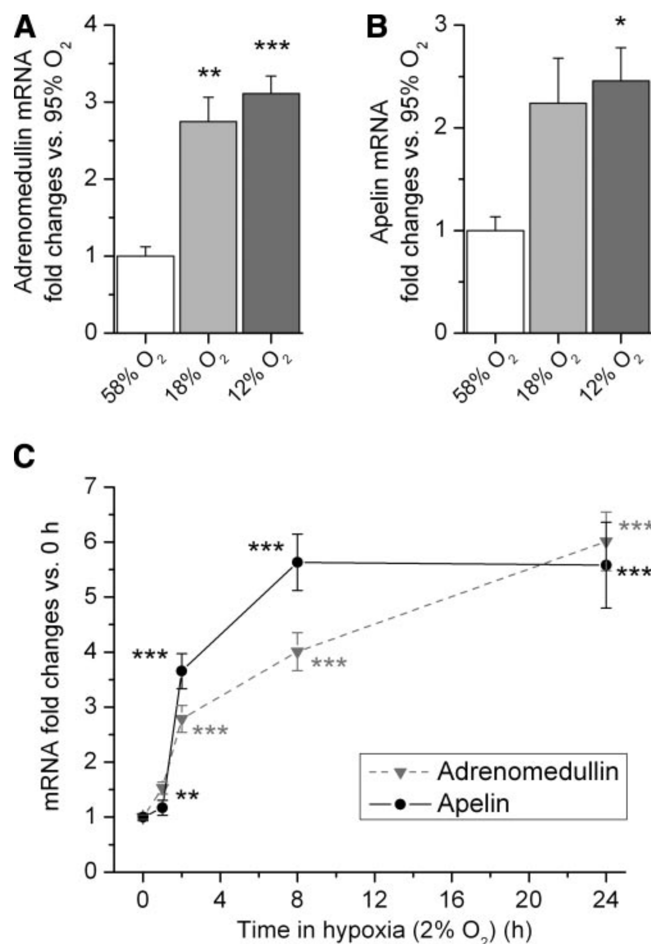


Figure 1. Reduction of the available oxygen increases apelin gene expression in perfused isolated rat left atrium and in cultured neonatal rat cardiomyocytes. Adrenomedullin (*A*) and apelin (*B*) mRNA levels in atrial tissue after 3h perfusion with buffer containing 18% O_2 ($n=6$) or 12% O_2 ($n=8$) compared to controls with 58% O_2 ($n=4$). *C*) Apelin and adrenomedullin mRNA levels in cultured neonatal rat cardiomyocytes after 1, 2, 8, and 24 h in hypoxia (2% O_2, $n=4$ in each time point). Statistical significance: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Data are mean ± SE.

Hypoxia and prolyl hydroxylase inhibition increases apelin expression in cardiac myocytes

In HL-1 cells, hypoxia (2% O_2, 24 h) or the addition of the prolyl hydroxylase inhibitor DFO resulted in HIF-1α stabilization and nuclear translocation (**Fig. 2*A***) as expected (33, 34). Nuclear HIF-1α immunofluorescence increased significantly in both DFO-treated (7.4-fold, $P<0.001$; $n=4$) and hypoxic groups (14.0-fold, $P<0.001$; $n=4$; Fig. 2*B*). Hypoxia and HIF-1 activation were accompanied by a 27-fold increase in the apelin mRNA levels ($P<0.001$; $n=6$; Fig. 2*B*). A known HIF-1 target, adrenomedullin (24, 35), responded similarly and its mRNA level was increased 3.4-fold ($P<0.001$) in hypoxia (Fig. 2*C*). Treatment of HL-1 cells with DFO (100 μmol/l) stabilized HIF-1α in normoxia (Fig. 2*A*) and increased apelin gene expression 12.8-fold ($P<0.001$) after 24 h exposure (Fig. 2*B*). Similarly DFO

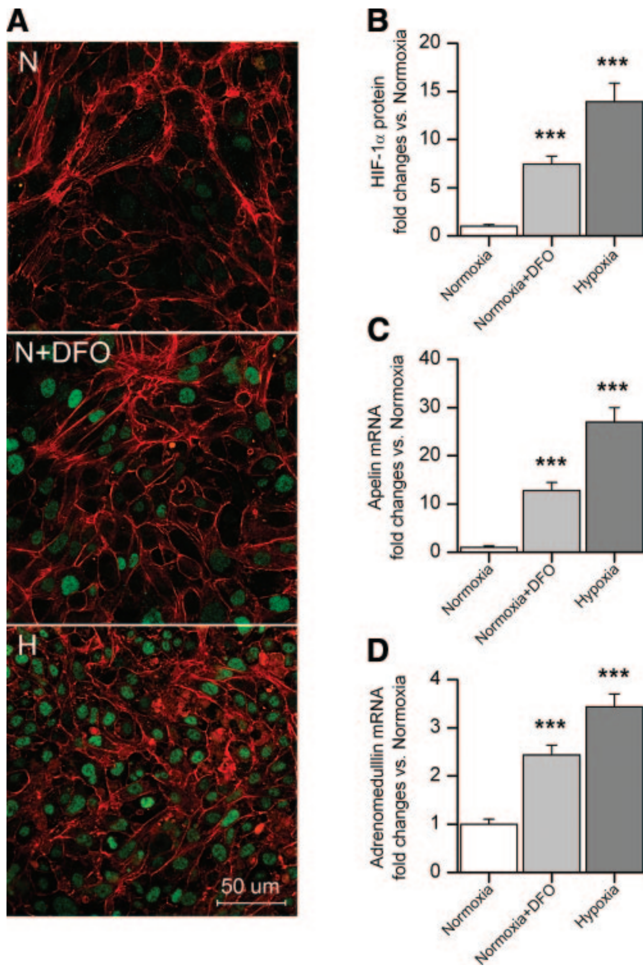


Figure 2. Hypoxia promotes HIF-1α stabilization and nuclear translocation with concomitant induction of apelin and adrenomedullin gene expression in cultured cardiomyocytes (HL-1 cells). *A)* HIF-1α immunofluorescence labeling shows a specific nuclear staining pattern (green) and increase in number of positively stained nuclei after 24 h DFO treatment (N+DFO) and more after hypoxia (2% O_2, 24 h) (H). Red color indicates phalloidin-stained actin. *B)* Amount of stabilized and nuclear translocated HIF-1α measured from HIF-1α immunolabeled cardiomyocytes (*n*=4). Apelin (*C*) and adrenomedullin (*D*) mRNA levels of cardiomyocytes exposed to hypoxia or DFO (100 μmol/l). Statistical significance compared to controls (Normoxia): $***P < 0.001$. Data are mean ± SE.

activated transcription of the adrenomedullin gene, and mRNA levels rose 2.4-fold ($P<0.001$; Fig. 2*C*)

Effect of dominant-negative and constitutively active forms of HIF-1α on HRE-driven reporter gene activity

To further characterize the role of HIF-1 in the induction of apelin expression, we performed a series of transient HL-1 cells transfections to induce the production of proteins interfering with HIF. To quantify HIF-1 transcriptional activity, HL-1 cells were transfected with a HRE-driven luciferase reporter gene construct (pT81/HRE-luc). Exposure to hypoxia or 100 μmol/l DFO for 24 h in normoxia increased the HRE activity 3.2-fold ($P<0.001$; $n=6$) and 1.9-fold ($P<0.001$; $n=6$), respectively (**Fig. 3*A***). To inhibit or activate HIF-dependent transcription, HL-1 cells were transfected with plasmids producing IPAS or HIF-1α(1–390)-VP16, respectively, together with an HRE-driven luciferase reporter construct. In IPAS-expressing HL-1 cells, the hypoxic induction of pT81/HRE-luc was significantly inhibited ($P<0.001$; $n=8$; Fig. 3*B*). Expression of the constitutively active HIF-1α(1–390)-VP16 increased luciferase expression both in normoxic ($P<0.001$; $n=8$) and hypoxic ($P<0.001$; $n=6$) groups (Fig. 3*B*).

HIF activity regulates apelin expression in HL-1 cells

Next, we characterized the effects of HIF-1α(1–390)-VP16 and IPAS on the transcriptional activity of the endogenous apelin gene. As expected, hypoxia induced the expression of apelin (89.4-fold, $P<0.001$; $n=5$) together with the known HIF target genes adrenomedullin (4.5-fold, $P<0.01$; $n=5$) and PHD3 (ref

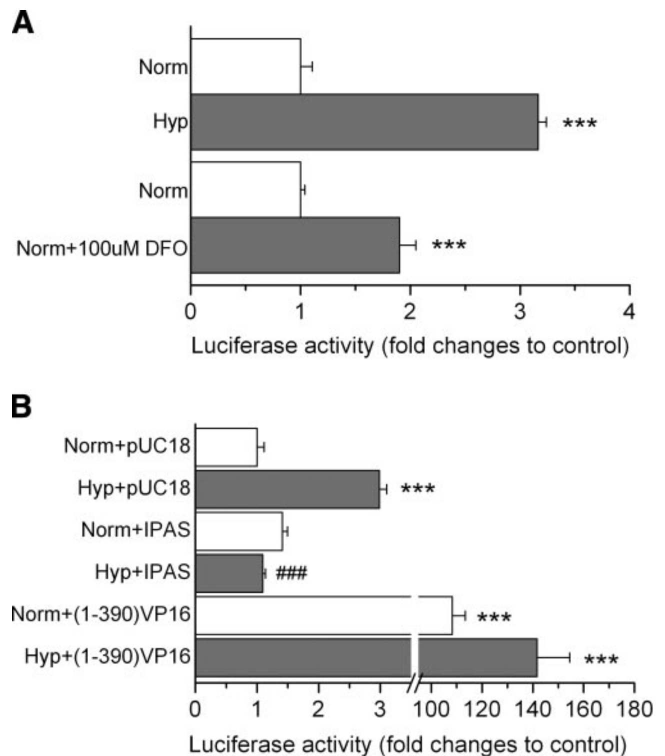


Figure 3. Hypoxia, DFO, IPAS, and HIF-1α(1–390)-VP16 modulate HIF-dependent transcription in cardiac myocytes. *A)* 24h exposure to hypoxia (2% O_2) or 100 μmol/l DFO increases HRE-driven pT81/HRE-luc luciferase production in HL-1 cell culture ($***P<0.001$, $n=8$). *B)* Expression of HIF-inhibitory IPAS (Hyp-IPAS) reduces the pT81/HRE-luc reporter activity in hypoxia compared to controls (HYP+pUC18, $###P<0.001$, $n=8$). Constitutively active HIF-1α(1–390)-VP16 increases luciferase activity in the culture after 24 h normoxia (Norm+(1–390)VP16) or hypoxia (Hyp+(1–390)VP16) compared to the control group (Norm+pUC18; ($***P<0.001$; $n=8$). Luciferase signal is normalized to renillal-luc signal and scaled to control groups. Data are mean ± SE.

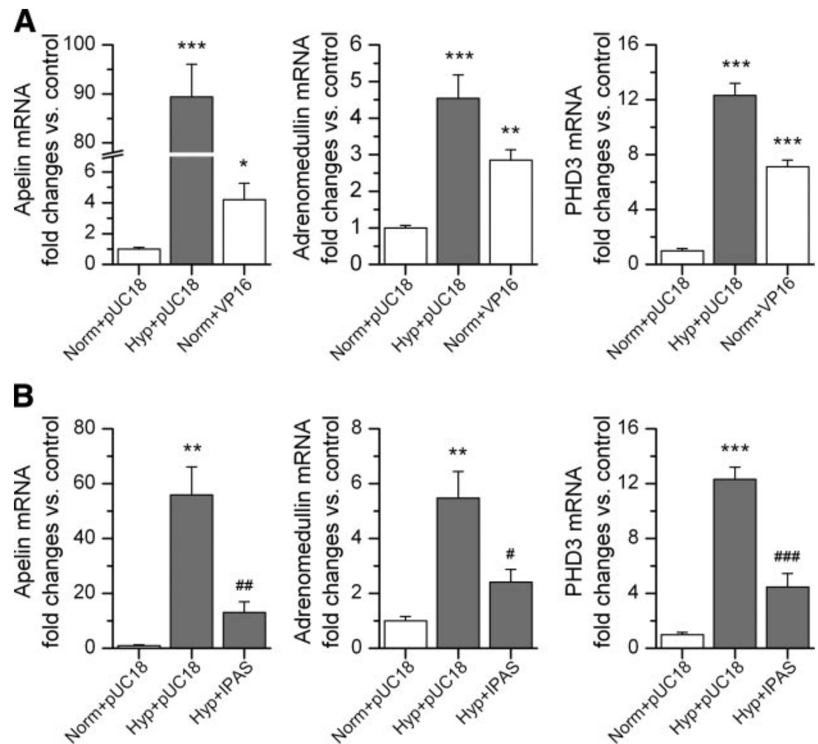


Figure 4. HIF activation in normoxia and HIF inhibition in hypoxia alter the expression of apelin and other HIF target genes in cardiac myocytes. Control groups were transfected with empty "mock" plasmid pUC18 (Norm+pUC18 and Hyp+pUC18). *A)* Constitutively active HIF-1α(1–390)-VP16 (Norm+VP16) increases apelin (left, *$P<0.05$; $n=5$), adrenomedullin (middle, **$P<0.01$; $n=5$) and PHD3 (right ***$P<0.001$; $n=6$ expression compared to control group (Norm+pUC18). *B)* HIF-inhibitory IPAS (Hyp+IPAS) decreases hypoxic apelin, adrenomedullin and PHD3 induction (##$P<0.01$, $n=5$, #$P<0.05$, $n=5$ and ###$P<0.001$, $n=6$, respectively). Stars in Hyp+pUC18 (*A*, *B*) groups indicate statistical difference compared to normoxia (Norm+pUC18). Data are mean ± SE

36; 12.3-fold, $P<0.001$; $n=6$). Control groups were transfected with the empty vector pUC18 (**Fig. 4*A, B***). Expression of HIF-1α(1–390)-VP16 increased apelin (4.2-fold, $P<0.05$; $n=5$), adrenomedullin (2.9-fold, $P<0.001$; $n=5$), and PHD3 (7.1-fold, $P<0.001$; $n=6$) mRNA levels after 48 h in normoxia, demonstrating HIF-1-dependent activation of the genes (Fig. 4*A*). Expression of the specific HIF inhibitor IPAS decreased the adrenomedullin ($P<0.05$; $n=5$) and PHD3 ($P<0.001$; $n=6$) hypoxia response and significantly blunted the increase in apelin mRNA in hypoxia ($P<0.01$; $n=5$; Fig. 4*B*), further confirming the importance of HIF in the regulation of apelin gene expression. In comparison, several HIF-1-independent genes, like PHD1 (36), were not affected by hypoxia or the expression of either HIF-1α(1–390)-VP16 or IPAS.

Hypoxia increases the secretion of biologically active forms of apelin

Increased expression of the apelin gene is likely to result in an augmented secretion of apelin from cardiac myocytes. To measure apelin protein levels, we developed a new radioimmunoassay for the detection of different forms of apelin (for details see methods). Hypoxia (2% O_2) and DFO (100 μmol/l) application in normoxia increased immunoreactive apelin ($P<0.001$ and $P<0.001$, respectively; $n=8$) in the culture medium of neonatal cardiac myocytes (**Fig. 5*A***, left). Molecular forms of secreted apelin were predominantly peptides markedly shorter than apelin-36 and surprisingly apelin-36, a common molecular form of apelin with biological activity, was not found from cardiomyocyte culture medium (Fig. 5*A*, right). The sizes of the forms secreted from cardiomyocytes appear to be between apelin-16 and apelin-12. These smaller peptides, like apelin-13, are known to be potent in mediating the cardiovascular effects of apelin (3). After exposure to hypoxia or DFO, cellular apelin immunoreactivity was not significantly altered despite the slight tendency toward cellular depletion of apelin on increased secretion (Fig. 5*B,* left). The molecular form of apelin in cellular fractions was almost exclusively a peptide that was significantly longer than apelin-36 (Fig. 5*B,* right). On the basis of apelin RIA and immunostaining, cardiomyocytes contain a rather low amount of apelin peptide suggesting that cells do not have large stores of apelin, but rather secretion relies mostly on the expression and synthesis of *de novo* apelin. Immunostained apelin localizes in the perinuclear area of the myocytes (Fig. 5*C,* top), demonstrating that synthesized proapelin is secreted via a sarco-/endoplasmic network probably by means of vesicular exocytosis. In comparison, intracellular localization of a cardiac-specific, stored, and secreted ANP (Fig. 5*C*, bottom) is similar to that of apelin, albeit ANP staining is much stronger.

Apelin gene expression is increased *in vivo* after myocardial infarction

To study the possible role of apelin in pathological developments, we measured apelin expression in infarcted rat ventricles, where lack of oxygen is likely to be one of the major stimuli affecting gene expression (37). **Figure 6*A*** shows the morphological changes that indicate dilated cardiomyopathy in the rat left ventricle 24 h after ligation of the LAD. Furthermore, echocar-

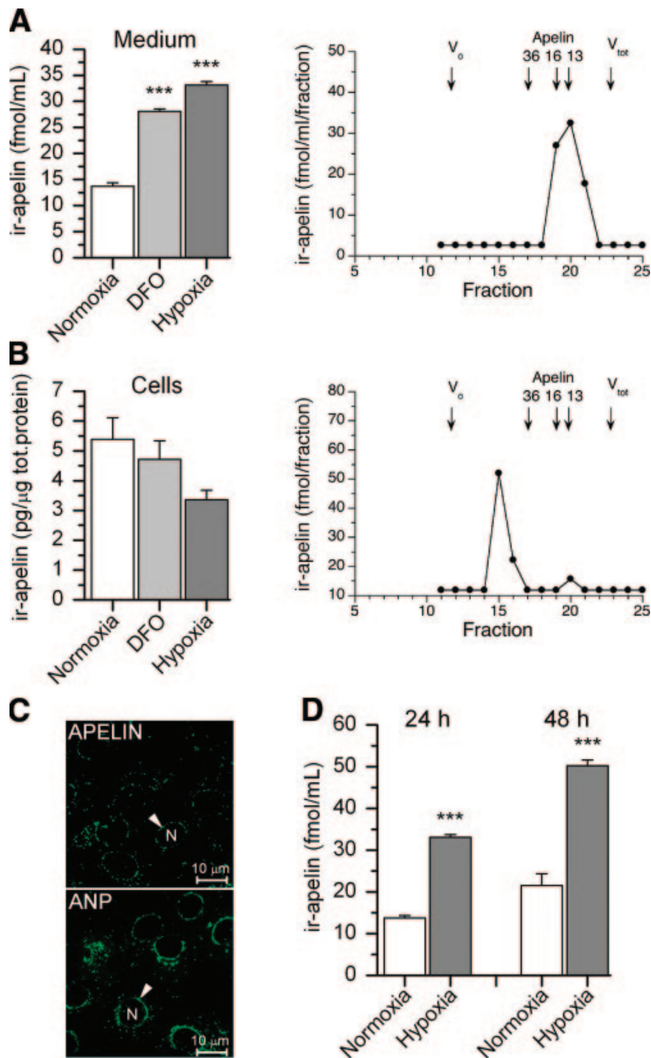


Figure 5. Hypoxia and DFO promote the production and secretion of biologically active forms of apelin from cardiac myocytes. *A)* Apelin immunoreactivity in the medium of neonatal cardiomyocyte cultures exposed to DFO (100 μmol/l) or hypoxia (2% O_2) for 24 h compared to medium from normoxic cells (left panel. Right panel) Gel filtration HPLC analysis of immunoreactive apelin in medium of hypoxia treated cells. Arrows denote positions of bovine serum albumin (V_0), apelin-36, apelin-16, apelin-13, and $^{125}I^-$ (V_{tot}). *B)* Apelin immunoreactivity in neonatal cardiomyocytes exposed to DFO (100 μmol/l) or hypoxia (2% O_2) for 24 h compared to normoxic cells (left). *B*, right panel) shows gel filtration HPLC analysis of immunoreactive apelin in hypoxia treated cells. Arrows are as in *A*, right panel. *C)* Immunostained apelin (top) and ANP (bottom) in the neonatal cardiac myocytes. N denotes nucleus and arrows indicate the perinuclear area showing intense staining of secretable peptides. *D)* Apelin immunoreactivity in neonatal cardiomyocyte culturing medium after 24 and 48 h in hypoxia (2% O_2) compared with medium from normoxic cultures. ***$P < 0.001$. Data are mean ± SE.

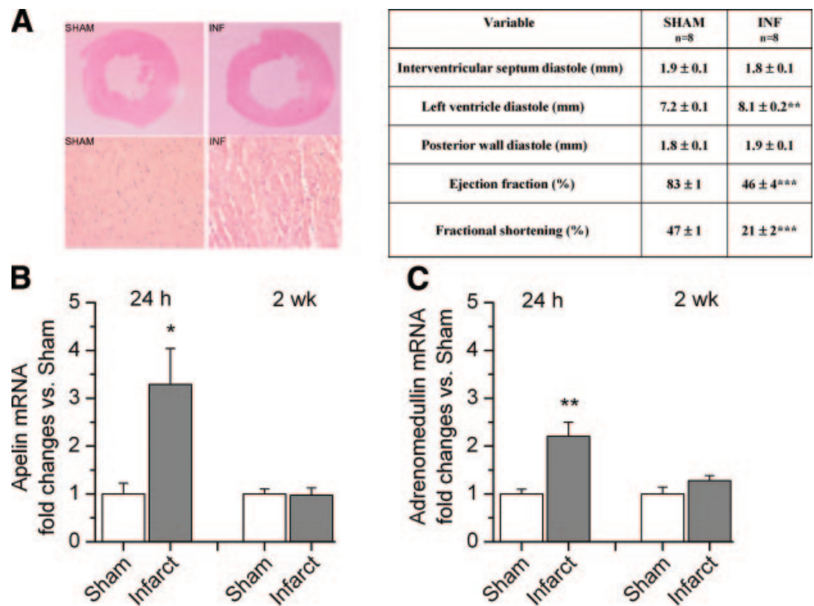
diographical findings show that 24 h after the ligation of the LAD the cardiac function was impaired; ejection fraction (P<0.001, n=8) and fractional shortening (P<0.001; n=8) were decreased with a subsequent increase in the left ventricle end diastolic volume (P<0.01; n=8). This early development of ischemic cardiac failure is accompanied by an increase in the apelin mRNA levels (P<0.05; n=5), but 2 wk after ligation of the LAD apelin expression returns to the same level with SHAM-operated hearts (Fig. 6*C*). In the acute-phase of the infarct, hypoxia is likely to be involved in the cardiac gene expression since mRNA levels of hypoxia (24) and infarction-inducible adrenomedullin (38) are changed in parallel with apelin (Fig. 6*D*).

DISCUSSION

Apelin is a peptide ligand of the APJ receptor implicated in cardiovascular diseases, obesity, and diabetes, with a wide spectrum of physiological effects in the CNS, circulation, and periphery. This study reports a novel finding that myocardial expression and secretion of apelin are activated by hypoxia via activation of HIF. Further elucidation of the processes involved in apelin secretion revealed that during hypoxia, cardiomyocytes increasingly process the storage form proapelin into smaller, biologically active, secreted forms of apelin. Results also imply that hypoxic induction of apelin is part of the acute response of heart muscle to an impaired supply of oxygen, such as in cardiac infarction.

HIF regulates apelin gene expression

Low baseline levels of apelin expression and secretion in cardiomyocytes corresponded to a low level of activated HIF in normoxia. When myocytes were exposed to hypoxia (24 h, 2% O_2) sufficient to induce HIF activation and nuclear translocation, apelin gene expression and secretion were increased. This induction was severalfold higher than the induction of adrenomedullin, a known HIF-1 target gene (24, 35). HIF-1 is a heterodimeric protein composed of HIF-1α and HIF-1β subunits (39). In hypoxia, HIF-1α escapes oxygen-dependent proline hydroxylation and degradation, accumulates, translocates to the nucleus, and heterodimerizes with HIF-1β. To activate transcription, HIF-1 interacts with the HRE of the target gene and promotes the assembly of transcriptional machinery (40, 41). Prolyl-4-hydroxylase inhibitor DFO induces HIF activation in normoxia and initiates HIF-dependent transcription. The responses of apelin and adrenomedullin to DFO treatment were similar to that induced by hypoxia, suggesting that HIF activation mediates the transcription of these genes. In the HL-1 cell culture, the overexpressed IPAS acts as a specific dominant negative regulator of HIF-1 in hypoxia. Once expressed, it combines with the HIF-1α subunit and forms a transcriptionally inactive HIF-1α-IPAS heterodimer (29). IPAS abolished the hypoxic induction of apelin, together with adrenomedullin and PHD3, further supporting the hypothesis that HIF activation is a prerequisite for hypoxic induction of these genes. In



Variable	SHAM n=8	INF n=8
Interventricular septum diastole (mm)	1.9 ± 0.1	1.8 ± 0.1
Left ventricle diastole (mm)	7.2 ± 0.1	8.1 ± 0.2**
Posterior wall diastole (mm)	1.8 ± 0.1	1.9 ± 0.1
Ejection fraction (%)	83 ± 1	46 ± 4***
Fractional shortening (%)	47 ± 1	21 ± 2***

Figure 6. Acute myocardial infarction in rats increases ventricular apelin and adrenomedullin gene expression. *A)* Hematoxylin-eosin-stained histological sections of the left ventricle (horizontal sections upper, vertical sections lower) 24 h after sham operation (SHAM) or LAD ligation (INF), showing changes in ventricular morphology and early signs of dilated cardiomyopathy. List of echocardiographical findings from Sham operated (SHAM) and infracted (INF) rats 24 h after the operation. *B)* Apelin (n=5) and *C)* adrenomedullin (n=5) mRNA levels from rat left ventricles 24 h and 2 wk after the LAD ligation compared to sham-operated ones. Statistical significance: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Data are mean ± SE

addition, expression of a nondegradable, constitutively active HIF-1α/VP16 chimera increased the pT81/HRE-luc derived luciferase activity and activated the expression of endogenous hypoxia-responsive genes. Among these, apelin mRNA levels rose significantly in HIF-1α(1–390)VP16 expressing cultures after a 48-h incubation in normoxia. Several other HIF-independent genes were not affected by the expression of the chimera. To summarize, expression of the constitutively active form of HIF and chemical activation of HIF by DFO activate apelin expression in normoxia and hypoxia induction can be inhibited by IPAS with corresponding activation and inhibition of other HIF target genes. Collectively, these data provide strong evidence for the role of HIF in the transcriptional regulation of the apelin gene. Interestingly, these data also indicate that from all of the possible cascades activated in hypoxia, HIF activation alone is sufficient to induce apelin expression.

During hypoxia and/or after HIF activation, cardiomyocytes process the apelin storage-forms into secreted forms of apelin

To date no specific stimuli for cardiomyocyte apelin production have been described; therefore, we wanted to study if hypoxia is a specific stimulus not only for apelin gene expression but also for augmented processing and secretion of biologically active apelin. The apelin gene encodes a 77 amino acid preproapelin, and it is widely expressed in various tissues and cell types (42). A 36-amino acid C-terminal fragment (apelin-36) of the prepro-apelin was the first characterized endogenous APJ receptor ligand, but several smaller C-terminal peptides were shown to be even more potent in activating APJ (1). Different apelin peptides vary in their potency to mediate different biological effects of apelin. Although apelin-36 is the most potent APJ receptor-dependent blocker of HIV infection, the smaller peptides, like apelin-13, are more potent in mediating the cardiovascular effects (3). According to our results, the storage form of apelin in cardiomyocytes is a significantly longer peptide than apelin-36, the longest biologically active apelin (3). This storage form of apelin was found in cells, localizing mostly in the sarcoplasmic network around the nucleus, just like ANP, another secreted peptide. The sizes of the forms secreted from cardiomyocytes appear to be between apelin-16 and apelin-12. The fact that cardiac myocytes secrete discrete apelin peptides raises the possibility that the different origins of the apelins in the blood could be traced on the basis of their size.

Apelin expression is increased in acute hypoxia and cardiac infarction

It has been reported that apelin tissue and expression levels are augmented in failing hearts (18, 19). The relationship between changes in cardiac apelin expression and myocardial ischemia has also been postulated (18, 19), but the underlying mechanisms have not been identified. The regulation of apelin expression by hypoxia might provide an explanation for its expression changes in ischemic hearts. Our results indicate that apelin induction is fast: in isolated atria <3 h and in cultured neonatal cardiomyocytes <2 h. In the infarcted heart, apelin gene expression is elevated *in vivo* acutely after a 24-h ischemic period but seems to be decayed again in chronic cardiac failure 2 wk after the infarction. It could be that endogenous apelin secretion is targeted to acutely compensate for the decreased contractility and oxygen supply of the myocardium suffering from hypoxic insult. Apelin has been proposed to mainly act as a paracrine modulator of cell functions, although circulating apelin levels are measurable and vary in various pathological situations (3). Accepting this view, apelin secreted from cardiomyocytes would act locally to modulate mainly the function

of coronary veins and cardiomyocytes themselves. Hypoxia causes vasodilation via various mechanisms, and apelin may well be one of the factors behind this phenomenon. It is also known that exogenous apelin has acute (12, 13) and chronic (14) positive inotropic effects in the myocardium. In cardiac myocytes, apelin was proposed to induce inotropy by activating the sarcolemmal Na^{+}-H^{+} exchanger (NHE), thus secondarily resulting in an increase in the intracellular calcium levels via reverse mode of the Na^{+}-Ca^{2+} exchanger (NCX). In this scheme, increased activity of the NHE would also lead to a rise in the intracellular pH further increasing the contraction force. Interestingly, the extent of alkalinization induced by this mechanism is likely to be greatest in the cardiac tissue suffering from acidosis (43), such as during ischemia and hypoxia. In myocardium with a limited oxygen supply, increased apelin expression could therefore serve as an adaptive mechanism to maintain the contractile function of the heart. Supporting this, apelin acutely enhances the contraction of hearts suffering from ischemic cardiomyopathy (13). Quite recently, HIF-induced gene expression was shown to protect the heart from ischemia-related damage as overexpression of HIF-1α reduced infarct size and limited the progression of infarct-induced cardiac failure in mouse (37). As a consequence of being a HIF target gene, apelin might well play a part in the early events protecting the heart against hypoxia-induced damage raising the possibility of using apelin as a therapeutic agent for ischemic heart failure patients (13). Altogether, apelin could be an important factor with numerous physiological and clinical implications in situations where the heart faces acute hypoxia. FJ

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