Original Paper

Factors Involved in Extracellular Matrix Turnover in Human Derived Cardiomyocytes

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Key Words

Matrix remodeling • Apelin • Cardiomyocyte • Hypoxia • HIF-1 • IL1 β • TNF α

Abstract

Background: The molecular mechanisms by which myocardial ischemia translates into ventricular remodeling remain unclear. **Methods:** We investigated whether hypoxia and proinflammatory cytokines are specific inducers of remodeling signals in an *in vitro* model of cultured adult human ventricular myocytes (AC16 cells). **Results:** Hypoxia modified the ratio of matrix remodeling factors by increasing the aminoterminal propeptide of type III procollagen (PIIINP) and reducing tissue inhibitor of matrix metalloproteinase type 1 (TIMP-1) secretion in AC16 cells. These effects, however, were not associated with either modifications in expression of matrix metalloproteinase type 2, collagen-I or metalloproteinase activity. Hypoxia does, actually increase the production of the cardiac antifibrogenic growth factors, Apelin and VEGF, through an Hypoxia Inducible Factor type 1-dependent mechanism. Concerning proinflammatory signaling pathways, IL1 β emerged as a powerful inducer of matrix turnover, since it significantly enhanced PIIINP, TIMP-1 and hyaluronic acid production and increased metalloproteinase activity. In contrast, TNF α did not modify matrix turnover but markedly induced the production of Apelin and VEGF. **Conclusion:** Hypoxia and increased TNF α activity likely exert cardioprotective actions by activating the cardiac antifibrogenic factors Apelin and VEGF. In contrast, IL1 β is a strong promoter of interstitial collagen remodeling that may contribute to ventricular dilation and heart failure in the ischemic myocardium.

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Introduction

Ischemic heart disease is the most important underlying condition in most cases of heart failure (HF). Myocardial infarction constitutes a significant precipitating event resulting in further deterioration of cardiac function. Although there is evidence that ischemic induction of cardiac remodeling may be an important aspect of ischemic HF [1, 2], the molecular mechanisms by which myocardial ischemia translates into ventricular remodeling and HF remain unclear.

Cardiac fibrosis is a major determinant of the remodeling process and is considered a key risk factor for the development of HF. One of the central features of cardiac fibrosis is the increased deposition of extracellular matrix (ECM), composed mainly of collagens and other proteoglycans and proteins in a dynamic equilibrium with the cellular components of the heart [3]. The dynamic turnover of collagens is controlled by several regulatory mechanisms, including *de novo* synthesis and proteolytic degradation by matrix metalloproteinases (MMPs). Under nonpathological conditions, the balance of collagen synthesis and degradation is crucial to the homeostasis of the ECM. In this regard, our comprehension of the regulation of matrix turnover has recently been enlarged recently by the identification of Apelin, a peptide that contains hypoxia response elements in their regulatory regions and displays important antifibrogenic effects [4, 5]. Since factors leading to remodeling may be the major determinants of HF prognosis rather than ventricular dilation itself [2], identification of the molecular mechanisms by which myocardial ischemia regulates ventricular remodeling and fibrosis is of relevance.

The study was conducted in cultured human ventricular myocytes (AC16 cells) that have been widely characterized and have shown to be a proliferating cell line with ultrastructural, molecular, genetic and immunocytochemical characteristics of cardiomyocytes [6]. We assessed whether hypoxia and/or proinflammatory mediators may induce ECM turnover activation or regulate the cardiac growth factors, Apelin and VEGF, in these cells. The ultimate objective was to investigate the molecular mechanisms involved in cardiac fibrogenesis.

Materials and Methods

Cell Culture

AC16 cells were generated from primary human ventricular cardiomyocytes as previously described [6]. This immortalized, stable cell line can be repeatedly frozen, thawed and propagated. Cells were seeded (1×10^6 cells/well) and grown to confluence for 36 hours in Dulbecco's Modified Eagle Medium (DMEM-F12), supplemented with 12.5% fetal calf serum, 50 U/ml penicillin and 50 µg/ml streptomycin. Thereafter, cells were switched to serum-free DMEM-F12 for 10 hours and subsequently incubated (37°C) at variable time intervals in DMEM-F12 under normoxic (21% O₂, 5% CO₂, 74% N₂) or hypoxic (5% O₂, 5% CO₂, 90% N₂) conditions in a controlled-O₂ water jacketed CO₂ incubator (Forma Scientific Series II, 3131, Marietta, OH). When indicated, the cell culture medium also contained the HIF-1α stabilizer CoCl₂ (150 µM), the HIF-1 inhibitor rotenone (2.5 µM), TNFα (20 ng/ml), IL1β (20 ng/ml) or vehicle (0.001% DMSO in cell culture medium). Cell bioavailability was assessed in AC16 cells for up to 24 hours by adding 0.4 mg of MTT (Thiazolyl Blue Tetrazolium Bromide, Sigma, St Louis, MO) and incubated at 37°C during 90 minutes; the media were discarded and 200 µl of N-propanol was added to each well. After 5-10 minutes of reaction, 50 µl of the solution was taken from each well and transferred into a 96-well plate and the O.D. was measured at 570 nm (SpectraMax 340, Molecular Devices, Sunnyvale, Ca).

Effect of hypoxia and proinflammatory cytokines on the production of matrix turnover components and growth factors in AC16 cells

Following cell culture, medium samples were obtained on completing the incubation periods to measure the aminoterminal propeptide of type III procollagen (PIIINP), the tissue inhibitor of matrix metalloproteinase type 1 (TIMP-1), hyaluronic acid (HA), Apelin and Vascular Endothelial Growth Factor (VEGF). Samples were collected, centrifuged at 2000 rpm for 10 min to remove cellular debris, decanted into

clean tubes and stored at -80 °C until analysis. Media samples for Apelin measurements were concentrated using 3000MW Amicon Ultra centrifugal filters (Millipore, Carrigtwohill, Ireland). The protein concentration in the media was determined using the Bradford assay [7].

Messenger expression of matrix turnover components and growth factors

Total RNA was extracted from AC16 cells using a commercially available kit (RNeasy, Qiagen, Germany). All samples were DNase treated before transcription using the TURBO DNA-free kit (Ambion, Austin, TX) and RNA concentrations were determined by spectrophotometric analysis (ND-100 Spectrophotometer, Nanodrop Technology, USA). One µg of DNase-treated total RNA was reverse transcribed (RT) using a complementary DNA synthesis kit (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Foster City, CA). Primers for Apelin (left: 5'-GCT CTG GCT CTC CTT GAC C-3' right: 5'-CCA TTC CTT GAC CCT CTG G-3'), VEGF-A (left: 5'-GCA GCT TGA GTT AAA CGA ACG-3' right: 5'-GGT TCC CGA AAC CCT GAG-3'), Col1α1 (left: 5'-CCC TGG AAA GAA TGG AGA T-3', right: 5'-AAT CCT CGA GCA CCC TGA-3'), Col1α2 (left: 5'-CCA GCT GGT CCA AAT GGT -3', right: 5'-CAC CAG GGA AAC CAG TCA TAC-3'), PDGFRβ (left: 5'-TTC CAT GAG GAT GCT GAG GT-3', right: 5'-CAG GGT GGC TCT CAC TTA GC-3'), TGFβR-1 (left: 5'-AGG CCA AAT ATC CCA AAC A-3', right: 5'-CCA ACA TTC TCT CAT AAT TTT AGC C-3'), MMP2 (left: 5'-CCC AAA ACG GAC AAA GAG TT-3', right: 5'-CTT CAG CAC AAA CAG GTT GC-3'), TIMP-1 (left: 5'-GGG CTT CAC CAA GAC CTA CA-3', right: 5'-TGC AGG GGA TGG ATA AAC AG-3'), TIMP-2 (left: 5'-GAA GAG CCT GAA CCA CAG GT-3', right: 5'-CGG GGA GGA GAT GTA GCA C-3'), and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), used as an endogenous standard (left: 5'-GCC ACA TCG CTC AGA CAC-3' right: 5'-CGC CCA ATA CGA CCA AAT-3') were designed according to human Apelin, VEGF-A, Col1A1, Col1A2, PDGFRβ, TGFβR-1, MMP2, TIMP-1, TIMP-2 and GAPDH sequences (GenBank NM_017413.3, NM_001025366, NM_000088.3, NM_000089.3, NM_002609.3, NM_004612.2, NM_004530.2, NM_003254.2, NM_003255.4 and NM_002046, respectively). The primers and probes used for the genes studied were designed to include intron spanning using the Universal Probe Library Assay Design Center (Roche Applied Science, <http://www.roche-applied-science.com/sis/rtpcr/upl/adc.jsp>). Real time quantitative PCR was performed in duplicate and analyzed with Light Cyclers 480 (Roche Diagnostics, Indianapolis, IN). Twenty µl total volume reaction of diluted 1:16 cDNA, 200 nM primer dilution, 100 nM pre-validated 9-mer probe (Universal ProbeLibrary, Roche Diagnostics) and FastStart TaqMan Probe Master (Roche Diagnostics) was used in each PCR reaction. Fluorescence signal was captured during each of the 45 cycles (denaturing 10 sec at 95°C, annealing 30 sec at 60°C and extension 30 sec at 72°C). GAPDH was used as the reference gene for normalization and water was used as the negative control. Relative quantification was calculated using the comparative threshold cycle (CT), which is inversely related to the abundance of mRNA in the initial sample. The mean CT of duplicate measurements was used to calculate ΔCT as the difference in CT for target and reference. The relative quantity of product was expressed as fold-induction of the target gene compared with the reference gene according to the formula $2^{-\Delta\Delta CT}$, where ΔΔCT represents ΔCT values normalized with the mean ΔCT of basal samples.

Gelatinase activity assay

MMPs activity was examined by gelatin zymography. Cell cultures obtained from hypoxia or IL1β treated cells were concentrated using 3000MW Amicon Ultra centrifugal filters and proteins in the resulting pellet measured as previously described. One hundred µg of protein per lane were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 0.2% gelatin-containing gels. After electrophoresis, gels were washed in 2.5% Triton X-100 to remove the SDS for 30 minutes and then incubated for 23 hours at 37°C in 50 mM Tris-HCl 1M pH 8 containing 5 mM CaCl₂, 2 µM ZnCl₂ and 0.02% NaN₃. Finally, gels were stained with 0.4% Coomassie brilliant blue R250 and destained in 10% acetic acid/40% methanol. Proteolytic bands of 62 kDa, which correspond to the active form of MMP-2, were scanned and the intensity of the bands analyzed.

Effect of hypoxia on HIF-1 activation in AC16 cells

The intranuclear activity of HIF-1 was determined in nuclear extracts of AC16 cells obtained following hypoxic or normoxic exposure using a nuclear extract kit (Active Motif, Carlsbad, CA) according to the manufacturer's instructions. The cells were collected and lysed, and the cytosolic fraction was removed. The nuclear pellets were homogenized, and the nuclear extracts were collected. Protein concentrations in the nuclear extracts were measured. The activation of HIF-1 was quantified using a TransAM kit (Active

Motif), which measures binding to a 26-bp hypoxia response element oligonucleotide [8]. Nuclear extract (6 µg) were used per sample. Nuclear extracts from COS-7 cells treated with the HIF-1α-stabilizer CoCl₂, which were provided with the kit, were used as a positive control. Nuclear extracts incubated with an HIF-1-specific competitor oligonucleotide served as a negative control. To assess the effect of inhibiting HIF-1 activation, the AC16 cells were exposed to rotenone (2.5 µM), an inhibitor of hypoxic HIF-1α stabilization [9] for 40 min followed by exposure to hypoxia (5% O₂) or normoxia (21% O₂) for 24 h. Thereafter, HIF-1 activity was determined and the medium was collected for further determination of PIIINP, TIMP-1, HA, Apelin and VEGF concentrations.

Measurements and statistical analysis

HA, TIMP-1 and PIIINP were measured using chemiluminometric immunoassays run on the ADVIA Centaur XP Immunochemistry analyzer (Siemens Healthcare Diagnostics, Tarrytown, NY, USA). Apelin was measured using the Apelin-12 microplate ELISA assay kit (Phoenix Pharmaceuticals, Burlingame, CA) according to the manufacturer's instructions. The antibody cross-reacts 100% with human Apelin-12, -13 and -36. The minimum detectable concentration was 70 pg/ml and the intraassay and interassay coefficient of variation were <5% and <14%, respectively. VEGF was measured by enzyme-linked immunosorbent assay (Quantikine, Immunoassay, R&D Systems, Minneapolis, MN).

The experimental data are presented as means ± SE from independent experiments. Comparisons between normoxic and hypoxic conditions and between vehicle and treated cells were performed with the unpaired Student's t-test. This test was also used to compare the effect of rotenone on Apelin and VEGF production under hypoxic conditions in AC16 cells. Differences were considered significant at a value of $p < 0.05$.

Results

Hypoxia activates PIIINP, Apelin and VEGF production but represses TIMP-1 expression in human cardiomyocytes

To investigate whether reduced oxygen tension could be a specific stimulus for the secretion of matrix turnover components and growth factors in human cardiomyocytes, we incubated AC16 cells under hypoxic conditions for increasing time periods and PIIINP, TIMP-1, HA, Apelin and VEGF concentrations in the cell culture medium were determined. No differences in cell viability, as assessed by the MTT assay, were observed between normoxic and hypoxic cells (data not shown). AC16 cells time-dependently released PIIINP, TIMP-1 and HA levels within the 48-hour incubation period. Moreover, the secretion of PIIINP was significantly higher at 24 and 48 hours of hypoxic exposure than under normoxic conditions (Fig. 1A). In fact, PIIINP levels in the media of hypoxic cells were two- to threefold higher than those in normoxic cells. By contrast, the secretion of TIMP-1 was significantly lower at 48 hours of hypoxic exposure than under normoxic conditions (Fig. 1B). Secretion of HA did not experience any modification due to hypoxic exposure of up to 48 hours. Secretion of Apelin was significantly higher at 24 and 48 hours of hypoxic exposure than under normoxic conditions (Fig. 1C). The hypoxic induction of Apelin was accompanied by a parallel augmentation of the hypoxia-sensitive factor VEGF (Fig. 1D).

Hypoxia inhibits TIMPs and activates Apelin and VEGF mRNA levels in AC16 cells

The effect of hypoxia on cardiac remodeling and growth factors was also evaluated at the mRNA level. We harvested cells for the measurement of TIMP-1, TIMP-2, Apelin, VEGF, PDGFRβ, TGFβR1, MMP2, Col1α1 and Col1α2 gene expression. GAPDH was used as an endogenous control. No significant differences were found in GAPDH mRNA expression over the experimental period in either normoxia- and hypoxia-exposed AC16 cells. Real-time PCR analysis indicated that hypoxic exposure significantly decreased TIMP-1 and TIMP-2 messengers and enhanced Apelin and VEGF mRNA levels (Table 1). There was a trend toward lower concentrations of PDGFRβ, and TGFβR1 in AC16 cells exposed to 5% O₂, although the differences did not reach statistical significance. Neither were there significant differences

Fig. 1. Production of the aminoterminal propeptide of type III procollagen (PIINP) (A), tissue inhibitor of matrix metalloproteinase type 1 (TIMP-1) (B), Apelin (C) and vascular endothelial growth factor (VEGF) (D) following 24 and 48 hours of incubation, under normoxic (21% O₂) or hypoxic (5% O₂) conditions. p<0.05 or p<0.01 vs. normoxic conditions (unpaired Student's t-test); n=6 independent experiments.

Table 1. Gene expression fold change of tissue inhibitor of matrix metalloproteinase type 1 (TIMP-1) and TIMP-2, Apelin, vascular endothelial growth factor (VEGF), platelet-derived growth factor receptor β (PDGFR β), transforming growth factor β receptor 1 (TGF β R1), matrix metalloproteinase 2 (MMP2), Collagen-I α 1 (Col1 α 1) and Collagen-I α 2 (Col1 α 2) measured in AC16 cells under normoxic (21% O₂) or hypoxic (5% O₂) conditions. ^aResults are given as means $\pm$ SE (unpaired Student's t-test)

Genes	Normoxia (21% O ₂)	Hypoxia (5% O ₂)	Significance ^a
TIMP-1	1.04 $\pm$ 0.19	0.48 $\pm$ 0.06	p<0.05
TIMP-2	1.01 $\pm$ 0.11	0.51 $\pm$ 0.13	p<0.05
Apelin	1.01 $\pm$ 0.29	4.36 $\pm$ 0.69	p<0.005
VEGF	1.17 $\pm$ 0.19	4.66 $\pm$ 0.73	p<0.01
PDGFR β	1.06 $\pm$ 0.26	0.66 $\pm$ 0.24	0.32
TGF β R1	1.05 $\pm$ 0.23	0.65 $\pm$ 0.21	0.27
MMP2	1.01 $\pm$ 0.12	0.96 $\pm$ 0.12	0.76
Col1 α 1	0.93 $\pm$ 0.24	1.00 $\pm$ 0.14	0.83
Col1 α 2	1.17 $\pm$ 0.46	0.92 $\pm$ 0.48	0.72

in MMP2, Col1 α 1 and Col1 α 2 gene expression between cells cultured under normoxic or hypoxic conditions (Table 1).

HIF-1 regulates Apelin and VEGF secretion but not matrix turnover components in AC16 cells

Next we sought to determine whether HIF-1 mediates the changes in matrix turnover components during hypoxic exposure. PIINP and TIMP-1 concentrations in the cell culture medium did not experience any modification regardless of whether the experiments were

Fig. 2. Apelin and VEGF mRNA expression in AC16 cells. Cells were exposed to vehicle or CoCl₂ for 6 hours. Total RNA was obtained and mRNA expression assessed by real-time PCR. Results are expressed as means $\pm$ SE. $p < 0.01$ vs. vehicle (unpaired Student's t-test); $n = 4$ independent experiments.

Fig. 3. Production of the aminoterminal propeptide of type III procollagen (PIIINP), tissue inhibitor of matrix metalloproteinase type 1 (TIMP-1) and hyaluronic acid (HA) in AC16 cells exposed to IL1 β (20 ng/ml) or vehicle for 24 hours. Results are expressed as means $\pm$ SE. $p < 0.05$ or $p < 0.01$ vs. vehicle (unpaired Student's t-test); $n = 6$ independent experiments.

performed in the presence or in the absence of CoCl₂. Moreover, rotenone did not prevent the effect of hypoxia on PIIINP and TIMP-1 secretion in the cardiomyocyte cell line (data not shown).

Contrary to what occurred on assessing the involvement of HIF-1 in the secretion of matrix turnover components, this transcription factor was related to the hypoxia-induced secretion of Apelin and VEGF observed in AC16 cells. Real-time PCR analysis indicated that within six hours CoCl₂ mimics the hypoxia-induced overexpression of Apelin and VEGF in human cardiomyocytes (Fig. 2). A direct regulatory effect of HIF-1 was further confirmed by assessing whether rotenone may prevent the hypoxic-induced secretion of growth factors in AC16 cells. Hypoxic cells pretreated with rotenone did not increase HIF-1 DNA binding in comparison to cells under normoxic conditions, indicating that rotenone is able to inhibit HIF-1 DNA binding in hypoxic cells (data not shown). Moreover, rotenone was able to prevent the hypoxia-induced increase of Apelin (777 ± 146 vs 371 ± 59 pg/mg protein; $p < 0.01$) and VEGF (1922 ± 129 vs 223 ± 22 pg/ml; $p < 0.01$) secretion in the cultured medium of human-derived cardiomyocytes, indicating that the mechanism of hypoxia-stimulated Apelin and VEGF secretion is mediated by increased HIF-1 DNA binding.

IL1 β , but not TNF- α , markedly activates extracellular matrix turnover components in human cardiomyocytes

To investigate whether proinflammatory cytokines could be a specific stimulus for the secretion of matrix turnover factors, we incubated AC16 cells with TNF α (20 ng/ml) and IL1 β (20 ng/ml) or vehicle for 24 hours, and PIIINP, TIMP-1 and HA concentrations

Fig. 4. Effect of TNFα (20 ng/ml) on Apelin and Vascular Endothelial Growth Factor (VEGF) expression in AC16 cells. Cells were stimulated with TNFα for 24 or 3 hours for protein or messenger assessment, respectively. Culture medium or cellular extracts were collected to measure protein concentration and mRNA abundance of Apelin and VEGF. Results are expressed as means ± SE. $p < 0.01$ vs. vehicle (unpaired Student's t-test); $n = 4$ independent experiments.

were measured. IL1β stimulation produced a significant increase of PIIINP, TIMP-1 and HA (Fig. 3). In fact, PIIINP and HA levels in the media of cells stimulated with IL1β were more than twofold and fourfold higher than in the media of vehicle-treated cells, respectively. In contrast, TNFα did not modify the secretion pattern of matrix turnover components in human cardiomyocytes.

TNFα, but not IL1β, increases the synthesis and secretion of Apelin in AC16 cells

To investigate whether proinflammatory cytokines could be a specific stimulus for the secretion of growth factors, we incubated AC16 cells with TNFα (20 ng/ml), IL1β (20 ng/ml) or vehicle for 24 hours and Apelin and VEGF concentrations were measured. Stimulation of AC16 cells with TNFα resulted in a significant twofold increase in Apelin and VEGF secretion (Fig. 4). AC16 cells incubated with IL1β importantly enhanced VEGF secretion in comparison with cells incubated with vehicle (436 ± 45 vs. 2728 ± 75 pg/ml; $p < 0.01$). However, IL1β did not produce noticeable changes in Apelin secretion (307 ± 43 vs. 297 ± 38 pg/mg protein; not significant).

The increase in Apelin and VEGF secretion induced by these cytokines was paralleled by a similar induction pattern in mRNA expression. Actually, following a 3-hour incubation, TNFα induced a 1.5-fold increase in both Apelin and VEGF mRNA expression in cultured human derived cardiomyocytes (Fig. 4). Moreover, IL1β induced a 1.6-fold increase in VEGF mRNA expression but did not modify Apelin mRNA expression.

IL1β increases gelatinolytic activity in human cardiomyocytes

Zymography of AC16 cells media demonstrated the presence of MMP activity. As seen in the zymogram (Fig. 5), gelatinolytic activity was concentrated in the area corresponding to the molecular weight range of 62 kDa, which was suggestive of active MMP-2. Media of AC16 cells incubated with IL1β displayed a significantly higher proteolytic activity than that of vehicle cells (1.07 ± 0.03 vs. 1.98 ± 0.06 densitometric arbitrary units; $p < 0.05$). By contrast, proteolytic activity of media of AC16 cells incubated under hypoxic conditions

Fig. 5. Effect of hypoxia and IL1 β on MMP activity in media of cultured AC16 cells (100 μ g of protein per lane). Gelatin zymography depicting the effect on MMP activity in the media induced by hypoxic exposure (5% O₂) or IL1 β (20 ng/ml) treatment of AC 16 cells. MW: Molecular Weight. Results are expressed as means $\pm$ SE. $p < 0.01$ vs. vehicle (unpaired Student's t-test); $n = 5$ independent experiments.

was not significantly higher than that in the media of AC16 cells incubated under normoxic conditions.

Discussion

The current study was aimed to investigate whether hypoxia and proinflammatory cytokines could be specific inducers of remodeling signals in human cardiac myocytes. Our findings may help to define the underlying molecular mechanisms by which myocardial ischemia translates into ventricular remodeling.

Changes in myocardial ECM can lead to altered myocardial function. In this regard, modifications in the balance between matrix deposition and degradation play a key role in adverse myocardial remodeling. It is known that the increased ECM turnover observed during the progression to HF occurs as a result of increased MMP proteolytic activity and/or decreased levels of TIMPs [10]. TIMP-1 is a key factor in regulating MMP activity after myocardial infarction, both in ECM structure and in myocyte growth and myocardial function [11]. Collagen types I and III are major components of ECM with a central role in the preservation of cardiac structure and function [10]. Cardiac myocytes are important sources of collagen, TIMPs and MMPs [12-14]. It has recently been demonstrated that hypoxia-reoxygenation upregulates procollagen I and of MMP activity [15]. This is coincident with our results showing that hypoxia induces an increase in PIIINP and a decrease in TIMP-1 synthesis and production. It should be noted, however, that analysis of the *in vitro* collagenolytic activity of culture medium obtained from the hypoxia-exposed AC16 cells was not significantly different from that of AC16 cells incubated under normoxic conditions. This, in turn, suggests that the hypoxia-induced ECM-related products in human cardiomyocytes are not of major relevance in terms of biological activity.

In the current study, the effects of reduced oxygen availability in AC16 cells were assessed by comparing cultures carried out at standard cell culture conditions (ambient oxygen concentration) with cells cultured at 5% O₂ (moderate hypoxia). Hypoxic cell cultures of the human myocytes depicted enhanced Apelin synthesis and release. Overexpression of Apelin in response to hypoxia has also been previously observed in several cell types including endothelial cells, adipocytes and enteric cells [16-20]. Moreover, it has been

observed that Apelin mRNA is upregulated in response to ischemia and returns to baseline after reperfusion of the ischemic myocardium in isolated rat hearts [21]. Apelin is a peptide with significant cardioprotective properties [21-24], including protection against cardiac fibrosis and vascular remodeling [4, 5, 25, 26]. Our results indicate that the early *in vivo* ischemic induction of myocardial Apelin expression is related to reduced oxygen tension in the myocardium and constitutes a homeostatic response to prevent the progression of cardiac fibrosis.

VEGF is one of the most important protective cellular factors induced by hypoxia. Myocyte-derived VEGF is of critical importance in cardiac morphogenesis and the determination of heart function. Like Apelin, VEGF exerts cardioprotective effects on the ischemic heart and cardiomyocytes [27], including a reduction of both myocardial infarct size and fibrosis [28]. In the current investigation, hypoxic cell cultures depicted enhanced VEGF synthesis which was of similar intensity to that of Apelin. The parallel behavior of Apelin and VEGF in response to decreased O₂ tension further emphasizes the cardioprotective role of these substances in the ischemic heart.

HIF-1 is the key transcription factor in mediating cellular responses under conditions of diminished oxygen availability. Therefore, we next investigated whether this transcription factor regulates the changes observed in ECM remodeling and cell growth, promoting substances in hypoxic cardiomyocytes. Whereas no relation was observed between HIF-1 and matrix turnover substances, a direct involvement of HIF-1 in the hypoxic induction of Apelin and VEGF was demonstrated. Hypoxic cell cultures depicted enhanced Apelin synthesis and release, a phenomenon that was not seen when the cells were incubated with the HIF-1 inhibitor rotenone. Moreover, Apelin synthesis was upregulated when AC16 cells were incubated with CoCl₂, a chemical inducer of HIF-1 that prevents HIF-1 α ubiquitination and degradation [29]. The inhibitory action of rotenone and the stimulatory effect of CoCl₂ on Apelin was paralleled by a similar effect on VEGF production, indicating that the mechanisms of hypoxia-induced increase in Apelin secretion in AC16 cells is closely related to HIF-1 stabilization, further emphasizing the existence of a transcriptionally regulated mechanism for Apelin secretion in response to decreased oxygen tension in human ventricular cardiomyocytes. These results are in agreement with previous observations of putative HIF-1 binding sites in the promoter of the Apelin gene [30] and of a similar pattern of activation of the Apelin and known HIF-1 target genes such as VEGF in the ischemic rat heart [31]. HIF-1 target genes include those with protein products which are involved in angiogenesis, energy metabolism, erythropoiesis, cell proliferation and viability, vascular remodeling, and vasomotor responses [32], conferring adaptive responses against hypoxia. Therefore, activation of Apelin and VEGF in response to hypoxia represent endogenous protective mechanisms against myocardial ischemic injury.

Pronounced inflammatory response is another characteristic feature of the ischemic myocardium. Patients with ischemic HF present raised circulatory and intracardiac levels of pro-inflammatory cytokines such as TNF α or IL1 β [33]. Recently, it has been observed that IL18 is an inducer of various MMPs in cardiomyocytes [34]. In our study, IL1 β (but not TNF α) exerted a potent effect on PIIINP and TIMP-1 release in human cardiomyocytes, indicating that this proinflammatory cytokine may directly activate ECM turnover. Zymography experiments confirmed that IL1 β produced a strong increase of MMP activity, demonstrating a functional effect of IL1 β on ECM degradation. In addition to increasing PIIINP production and MMP activity, IL1 β produced a strong enhancement of the secretion of the extracellular matrix component HA in AC16 cells. It has previously been observed that HA synthesis is upregulated in experimental myocardial infarction and myocarditis [35]. Our results suggest that IL1 β , but not hypoxia or TNF α , is a major factor in the induction of HA synthesis in ischemic myocardium. This finding also points to human cardiomyocytes as major producers of HA under stress conditions, extending the previous findings of Hellman et al. [36] in mouse cardiomyocytes. Overall, our results suggest that IL1 β is one of the main factors involved in the induction of matrix turnover remodeling in the ischemic myocardium.

Stimulation of cardiac AC16 cells with TNF α produced an increase in Apelin release, which was secondary to an early elevation of Apelin gene transcription. TNF α is increased in patients with acute myocardial infarction and HF and is closely associated with the myocardial inflammatory response present in cardiac ischemia [37-43]. A close correlation has previously been observed between Apelin and TNF α expression in human adipose tissue [44]. Recently, it has been described that Apelin significantly reduces aneurysm formation by blocking macrophage burden, as well as inflammatory chemokine and cytokine production in the aneurysmal aorta [45]. In addition, evidence for a direct anti-inflammatory effect of Apelin (including TNF α mRNA downregulation) has been obtained from studies in cultured macrophages [45]. In our study, TNF α also induced Apelin and VEGF secretion in AC16 cells. This differs from the effect of IL1 β which only affected the pattern of secretion of VEGF. Activation of VEGF production by this proinflammatory cytokine has also been described in rat cardiomyocytes via activated MAP kinases [46, 47]. Accordingly, the hypothesis has been raised that this could be a beneficial response to the ischemic myocardium mediating collateral vessel neof ormation.

In summary, these findings further contribute to understanding the role of hypoxia and inflammation in the cardiac remodeling process of the ischemic myocardium. IL1 β is a specific significant inducer of the remodeling process in ventricular myocytes whereas hypoxia and TNF α upregulate the cardiac antifibrogenic growth factors, Apelin and VEGF.

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