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Human cardiac fibroblasts express B-type natriuretic peptide: fluvastatin ameliorates its up-regulation by interleukin-1α, tumour necrosis factor-α and transforming growth factor-β

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Abstract

B-type natriuretic peptide (BNP) is a cardiac hormone, which plays a major role in body fluid and cardiovascular homeostasis. Produced by cardiac ventricles, its expression is highly regulated by various mediators. Canine cardiac fibroblasts have been identified as a source of BNP. Cardiac fibroblasts are key regulators of myocardial structure and function. We treated cultured human adult cardiac fibroblasts (HACF) with 2000 U/ml tumour necrosis factor-α (TNF-α), 200 U/ml interleukin-1α (IL-1α) or 50 ng/ml transforming growth factor-β (TGF-β) in the presence or absence of 500 nM fluvastatin. N-terminal pro-BNP (Nt-proBNP) concentration was determined by a competitive enzyme immunoassay. RealTime polymerase chain reaction (real-time PCR) was performed to investigate changes in BNP mRNA expression. Nt-proBNP peptide was present in the conditioned media of HACF and incubation with fluvastatin significantly reduced Nt-proBNP peptide levels. Treatment of HACF with TNF-α, IL-1α or TGF-β significantly increased Nt-proBNP levels compared with untreated cells. This effect was completely abolished in the presence of fluvastatin. Real-time PCR analysis confirmed these changes at the level of mRNA expression. Our data suggest that cardiac fibroblasts are a potential source of BNP in the human heart. Pro-inflammatory cytokines, associated with ventricular dysfunction and cardiac fibrosis, seem to be major inducers of BNP production in cardiac fibroblasts. This effect can be reverted by a statin. Based on our data, we speculate that elevated plasma BNP levels might not only reflect increased myocardial stretch but also inflammatory and remodelling processes. A possible benefit of statin-induced reduction in BNP production requires further studies.

Keywords: B-type natriuretic peptides • human adult cardiac fibroblasts • statins • pro-inflammatory cytokines

Introduction

B-type natriuretic peptide (BNP) is a cardiac hormone with potent diuretic, natriuretic and vasodilatory effects that plays a major role in body fluid and cardiovascular homeostasis [1]. It is predominantly produced in cardiac ventricles and is secreted after myocardial stretch mainly from the left ventricular wall [1]. Various biomolecules such as cardiotrophin-1, endothelin-I, angiotensin-II, lipopolysaccharide, interleukin-1 (IL-1), triiodothyronine and α-adrenergic agonists have been shown to increase BNP expression in cardiac myocytes [1, 2]. Recently, canine cardiac fibroblasts have been identified as a source for BNP [3]. Cardiac fibroblasts are activated in pathological conditions and play an important role in the inflammatory response and in the regulation of matrix remodelling in response to myocardial injury [4].

In this paper, we show that human cardiac fibroblasts express BNP and that its expression is up-regulated by cytokines such as tumour necrosis factor-α (TNF-α), IL-1α and transforming growth factor (TGF)-β that have been implicated in the pathogenesis of ventricular remodelling and progression of cardiac dysfunction and whose plasma levels are elevated in patients with heart failure or myocardial infarction and correlate with disease severity and

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Table 1 Primers used for real-time PCR

Primer	fwd-Primer (corresponding position)	Rev-primer (corresponding position)	UPL probe (amplicon size [bp])
proBNP	5′-CAG CCT CGG ACT TGG AAA-3′ (200–217)	5′-ATC TTG GGG CTT CGT GGT-3′ (416–398)	(199)
GAPDH	5′-GAGGTGTGAGTGGGATGGTGG-3′ (681–702)	5′-GCCTGCTTCACCACCTTCTTG-3′ (890–869)	(189)
IL1R1	5′-GTT CAT TTA TGG AAG GGA TGA-3′ (1350–1371)	5′-TCT GCT TTT CTT TAC GTT TTC ATT-3′ (1403–1427)	#60 (78)
IL1R2	5′-TAC GCA CCA CAG TCA AGG AA-3′ (1239–1258)	5′-AAG AAG GCC AGT GAA AGT GG-3′ (1295–1314)	#2 (76)
TGFBR1	5′-AAA TTG CTC GAC GAT GTT CC-3′ (1305–1324)	5′-CAT AAT AAG GCA GTT GGT AAT CTT CA-3′ (1339–1364)	#31 (60)
TGFBR2	5′-GGG AAA TGA CAT CTC GCT GTA-3′ (1821–1841)	5′-CAC CTT GGA ACC AAA TGG AG-3′ (1872–1891)	#7 (71)
TNFRSF1A	5′-GAG AGG CCA TAG CTG TCT GG-3′ (261–280)	5′-GAG GGG TAT ATT CCC ACC AAC-3′ (335–355)	#59 (95)
TNFRSF1B	5′-GCA GTG CGT TGG ACA GAA G-3′ (1093–1111)	5′-CCA CCA GGG GAA GAA TCT G-3′ (1192–1210)	#5 (118)

progression [5–8]. Furthermore, we provide evidence that this up-regulation of BNP by inflammatory mediators is counteracted by fluvastatin, a 3-hydroxy-3-methylglutaryl-coenzyme-A-reductase inhibitor.

Materials and methods

Materials

Fluvastatin (kindly provided by Novartis, Basel, Switzerland) was handled as described previously [9]. Recombinant human (rh) TNF-α, rh IL-1α, rh TGF-β were purchased from Roche (Basel, Switzerland) and rh IL-6, rh IL-11, rh oncostatin M (OSM) and rh leukaemia inhibitory factor (LIF) were purchased from R&D Systems (Minneapolis, MN).

Methods

Human adult cardiac fibroblasts (HACF) and human adult cardiac myocytes (HACMs) were isolated, characterized and cultured as previously published [10]. More than 95% of HACF stained positive for fibroblast-specific antigen. HACF neither stained for the cardiac myocyte markers troponin I, tropomyosin, cardiotin and myocardial muscle actin, nor for the endothelial marker vWF or for smooth muscle actin, ruling out contamination with myocytes, smooth muscle cells and endothelial cells, respectively. More than 95% of HACM stained positive for the cardiac myocyte markers troponin I, tropomyosin, cardiotin and myocardial muscle actin. HACM neither stained for fibroblast-specific antigen nor for the endothelial marker vWF or for smooth muscle actin, ruling out contamination with fibrobasts, smooth muscle cells and endothelial cells, respectively. All human material was obtained and processed according to the recommendations of the hospital's Ethics Committee, including informed consent.

Twenty-four hours prior to the experiments, cells were starved with M199 containing 0.1% BSA (both Sigma, St. Louis, MO). For stimulation experiments with cytokines, IL-1α, TNF-α or TGF-β with and without fluvastatin were added to the cells at the concentrations indicated. To characterize a possible effect of fluvastatin in additional experiments, HACF were simultaneously treated with the respective cytokine and 500 nM fluvastatin alone or together with 100 μM mevalonate (Sigma) or 10 μM geranyl-geranyl pyrophosphate (GGPP; Sigma) or pre-treated with 5 μM Y26372 [(+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl)cyclohexanecarboxamide dihydrochloride] (Sigma), a specific inhibitor of Rho-associated kinases (ROCKs) for 1 hr before addition of the respective cytokine. Nt-proBNP levels in the conditioned media of HACF were measured by a competitive enzyme immunoassay using a sheep antibody specific for proBNP 8-29 according to the manufacturer's instruction (Biomedica, Vienna, Austria). The detection limit of this assay is 5 fmol/ml.

For specific proBNP mRNA quantification cells were treated with cytokines as described earlier and mRNA was isolated using QuickPrep™ Micro mRNA Purification Kit (Amersham Biosciences, Buckinghamshire, UK) according to the manufacturer's instructions. Real-time PCR was performed using LightCycler-RNA Master SYBR Green I (Roche) according to the manufacturer's instructions. Primers are shown in Table 1 and were designed using the LightCycler Probe Design Software Version 1.0 (Roche) and the Primer3 Software (http://frodo.wi.mit.edu/). The amplification conditions consisted of an initial incubation at 61°C for 20 min., followed by incubation at 95°C for 30 sec., 50 cycles of 95°C for 1 sec., 65°C for 10 sec. and 72°C for 10 sec., a melting step from 45°C to 95°C increasing 0.1°C per second and a final cooling to 40°C. Specific mRNA for the receptors gp130, IL-6 receptor (IL-6R), IL-11R, OSMR and LIFR was determined by Real-time PCR using conditions as described recently [11]. Real-time PCR

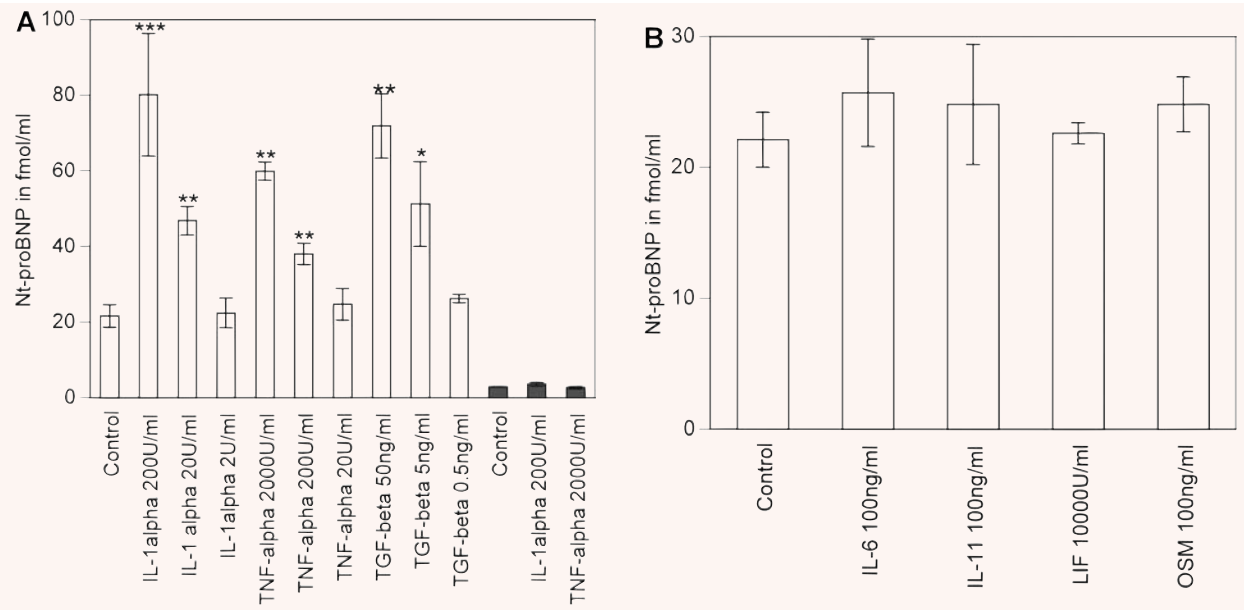


Fig. 1 Effect of IL-1α, TNF-α, TGF-β, IL-6, IL-11, OSM and LIF on proBNP production in HACF and HACM. Confluent monolayers of HACF (open bars) were incubated for 24 hrs with or without 2, 20 and 200 U/ml IL-1α; 20, 200 and 2000 U/ml TNF-α; or 0.5, 5 and 50 ng/ml TGF-β, respectively (panel **A**). Confluent monolayers of HACM (hatched bars) were incubated for 24 hrs with or without 200 U/ml IL-1α; or 2000 U/ml TNF-α (panel **A**). Confluent monolayers of HACF were incubated for 24 hrs with or without 100 ng/ml IL-6; 100 ng/ml IL-11; 10,000 U/ml LIF; or 100 ng/ml OSM, respectively (panel **B**). Nt-proBNP was determined in conditioned media using a specific ELISA and values are given in fmol/ml and represent mean values ± S.D. of three independent determinations. Experiments were performed three times with cells isolated from three different donors. A representative experiment is shown. $***P < 0.001$, $**P < 0.005$, $*P < 0.05$ as compared with untreated control cells.

for IL-1 receptor-1 (IL-1R1), IL-1R2, TGF-βR1, TGF-β2, TNFR superfamily 1A (TNFRSF1A) and TNFRSF2A was performed using LightCycler® TaqMan® Master (Roche) according to the manufacturer's instructions. Primers, shown in Table 1, were designed using the Roche Universal ProbeLibrary Assay Design Center (http://www.universalprobelibrary.com/). The amplification conditions consisted of an initial incubation at 95°C for 10 min., followed by 45 cycles of 95°C for 10 sec., 63°C for 20 sec. and 72°C for 6 sec. and a final cooling to 40°C. Data were analysed using LightCycler Software Version 3.5 (Roche).

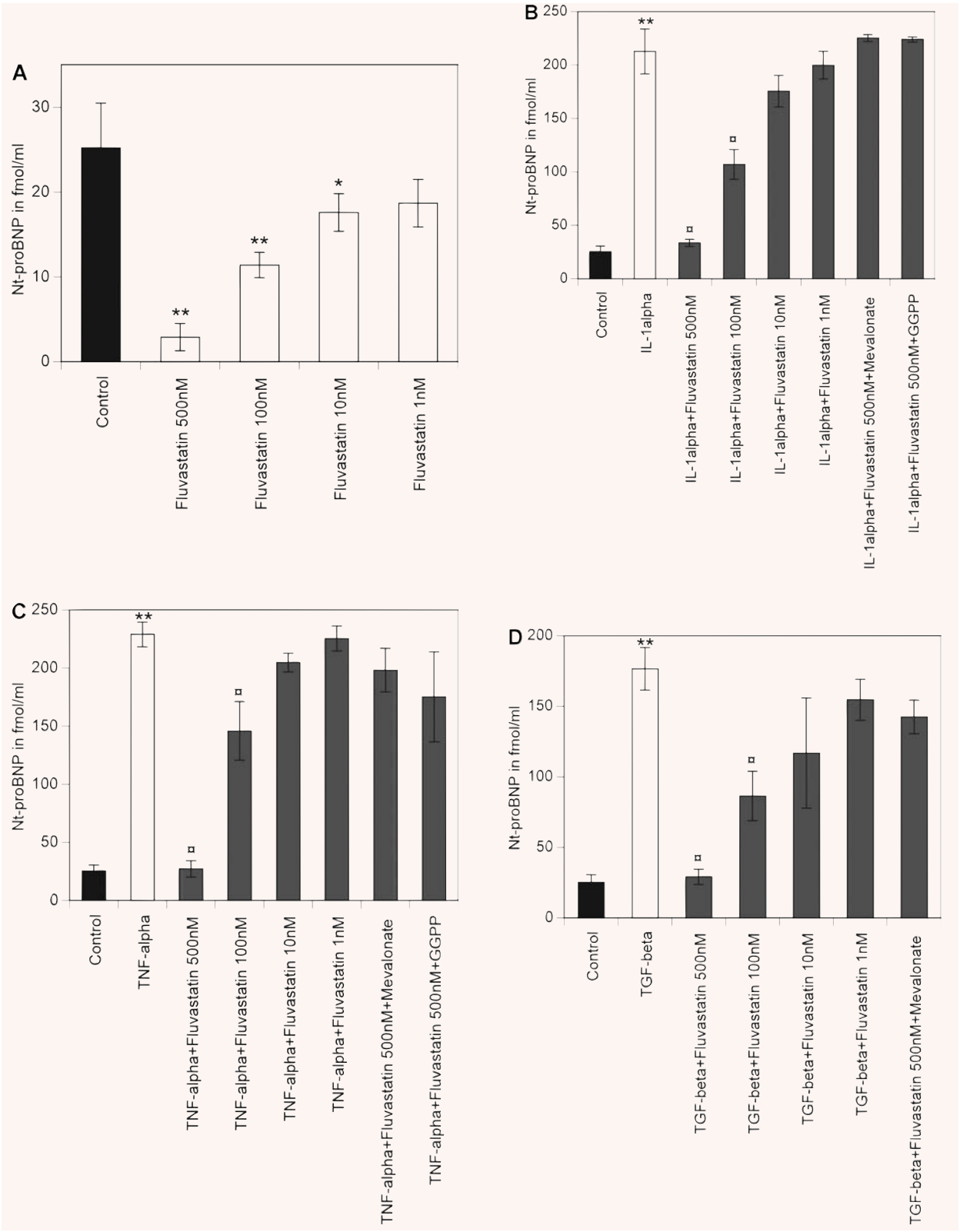
To determine possible cytotoxic effects of fluvastatin, lactate dehydrogenase (LDH) leakage was measured in cultures treated with the statin using a commercially available assay for photometric determination of LDH activity (Sigma). In addition, cells were counted at the end of the respective experiments using a haemocytometer.

We used t-test for independent variables to compare Nt-proBNP levels in the conditioned media of treated cells with those of control cells as well as to detect significant effects of fluvastatin on BNP secretion. Statistical significance was present if $P < 0.05$.

Results

Nt-proBNP concentration increased dose dependently in condition media of HACF treated with IL-1α, TNF-α or TGF-β, whereas IL-6, IL-11, OSM or LIF had no effect (Fig. 1A and B). In further analyses, we used the most effective concentrations of IL-1α (200 U/ml), TNF-α (2000 U/ml) or TGF-β (50 ng/ml). In addition, specific mRNA IL-1R1, IL-1R2, TGF-βR1, TGF-β2, TNFRSF1A, TNFRSF2A, gp130, IL-6R, IL-11R, OSMR and LIFR was detected in these cells by real-time PCR (data not shown). As can be also seen from Fig. 1A, HACMs produced approximately 1/10 of the amount of Nt-proBNP secreted by HACF under the same culture conditions. Furthermore, Nt-proBNP production was not affected by IL-1α or TNF-α in HACM.

Fluvastatin dose dependently reduced baseline Nt-proBNP secretion of untreated HACF. A significant reduction of Nt-proBNP was seen with concentrations of fluvastatin between 10 nM and 500 nM (Fig. 2A). Furthermore, the increase in Nt-proBNP secretion by HACF induced by IL-1α, TNF-α and TGF-β, respectively, was reduced to Nt-proBNP levels seen in untreated control cells by the presence of 500 nM fluvastatin. A significant reduction of Nt-proBNP was also seen with 100 nM fluvastatin (Fig. 2B–D). To determine whether this effect of fluvastatin on BNP secretion depends on its capacity to inhibit mevalonate synthesis by HMG-CoA reductase, HACF were simultaneously treated with the respective cytokine and 500 nM fluvastatin alone or together with 100 μM mevalonate or 10 μM GGPP. In these experiments, the inhibiting effect of fluvastatin on IL-1α-, TNF-α- and TGF-β-induced Nt-proBNP secretion was completely reversed by mevalonate or GGPP. Mevalonate or GGPP alone had no effect on Nt-proBNP production



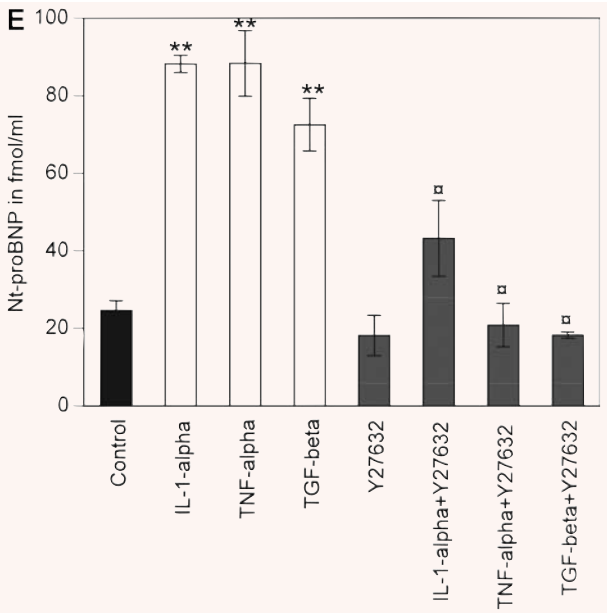


Fig. 2 Effect of TNF-α, IL-1α, TGF-β and fluvastatin on proBNP production in HACF. Confluent monolayers of HACF were incubated for 24 hrs without (panel **A**) or with 200 U/ml IL-1α (panel **B**), 2000 U/ml TNF-α (panel **C**) or 50 ng/ml TGF-β (panel **D**), respectively, in the absence (open bars) or presence of 500 nM, 100 nM, 10 nM or 1 nM fluvastatin without or with 100 μM mevalonate or 10 μM GGPP (hatched bars). In panel **E**, confluent monolayers of HACF were pre-treated with medium (open bars) or 5 μM Y27632 (hatched bars) for 1 hr and then incubated for 24 hrs without or with 200 U/ml IL-1α, 2000 U/ml TNF-α or 50 ng/ml TGF-β, respectively. Nt-proBNP was determined in conditioned media using a specific ELISA and values are given in fmol/ml and represent mean values ± S.D. of three independent determinations. Experiments were performed 3 times with cells isolated from three different donors. A representative experiment is shown. $^{**}P < 0.001$, $^{*}P < 0.05$ as compared with untreated control cells; ¤ $P < 0.001$ as compared with cells incubated without fluvastatin or Y27632.

by HACF (data not shown). When cells were incubated with fluvastatin for 48 hrs at a concentration of 500 nM, cell viability was not significantly affected by this treatment as LDH leakage did not exceed 105% of control. In addition, no changes in cell numbers were observed. A cytotoxic effect was only observed at concentrations greater than 5 μM of the statin (data not shown). As can be seen from Fig. 2E, the ROCKs inhibitor Y26372 mimicked the effect of fluvastatin as it inhibited IL-1α-, TNF-α- and TGF-β-stimulated Nt-proBNP secretion. Y26372 at the concentration of 5 μM did neither affect cell viability as judged by LDH leakage nor change cell numbers.

Incubation of HACF for 24 hrs with IL-1α (200 U/ml), TNF-α (2000 U/ml) or TGF-β (50 ng/ml), respectively, significantly increased BNP mRNA levels, whereas fluvastatin (500 nM) blunted the effects of these cytokines (Table 2). It should be noted that fluvastatin at a concentration of 500 nM did not systematically decrease mRNA levels in cardiac fibroblasts as it had no down-regulating effect on mRNA levels specific for IL-6, IL-8, plasminogen activator inhibitor type-1 (PAI-1) and vascular endothelial growth factor (VEGF) in these cells. When HACF were incubated for 24 hrs with 500 nM fluvastatin the respective mRNA levels increased slightly for IL-6, IL-8 and PAI-1 (1.3-fold, 1.5-fold and 1.3-fold, respectively) and 2.3-fold for VEGF. Furthermore, mRNA specific for GAPDH did not change significantly (1.1-fold over control) when the cells were treated with 500 nM fluvastatin for 24 hrs.

Table 2 Effect of TNF-α, IL-1α, TGF-β and fluvastatin on proBNP mRNA expression in HACF

	Without fluvastatin	With fluvastatin 500 nM
Control	1.0 ± 0.1	0.6 ± 0.1
IL-1α 2000 U/ml	2.9 ± 0.5*	0.8 ± 0.1†
TNF-α 200 U/ml	2.9 ± 0.7*	0.9 ± 0.2†
TGF-β 50 ng/ml	2.6 ± 0.5*	0.9 ± 0.2†

Confluent monolayers of HACF were incubated for 24 hrs without or with 200 U/ml IL-1·, 2000 U/ml TNF-α or 50 ng/ml TGF-β, respectively, in the absence or presence of 500 nM fluvastatin. Real-time PCR with primers specific for BNP and GAPDH was performed. BNP mRNA levels were normalized according to the respective GAPDH levels. Values are given as fold of control and represent mean values ± S.D. of three independent determinations. Experiments were performed 2 times with HACF isolated from two different donors. A representative experiment is shown. $^{*}P < 0.001$ as compared with untreated control cells, †$P <$ 0.001 as compared with cells incubated without fluvastatin.

Discussion

Initial results on BNP production originate from *in vitro* data of cultivated neonatal rat cardiac myocytes. Limited data are, however, available on human myocardial cells [12]. In this study, we provide evidence for the first time that similar to canine cardiac fibroblast [3], cultured human adult cardiac fibroblasts also express BNP-mRNA and secrete BNP and that inflammatory mediators such as TNF-α, IL-1-α and TGF-β are potent inducers of BNP mRNA expression and protein secretion by these cells. HACF also express the respective receptors for TNF-α, IL-1-α and TGF-β. Other inflammatory cytokines such as IL-6, IL-11, OSM or LIF did not affect BNP production in HACF. The respective receptors, however, were expressed by these cells. In contrast, human adult cardiac myocytes produced only 1/10 of the amount of BNP secreted by cardiac fibroblasts and BNP production in human cardiac myocytes was not affected by TNF-α or IL-1-α. It should be emphasized that cultured rat cardiac myocytes produced only minute amounts of BNP as compared with mixed cultures of cardiac myocytes and non-myocytes. In addition, only these mixed cultures responded to IL-1 with a significant increase in BNP production [13].

TNF-α, IL-1-α and TGF-β, shown to up-regulate BNP in cardiac fibroblasts in our study, are known to be associated with cardiac dysfunction and remodelling of the myocardium and are elevated in the plasma of patients with heart failure or myocardial infarction [5–8]. Thus, one could speculate that BNP might not only reflect reduced ventricular function but also inflammatory and/or remodelling processes within the myocardium.

Statin therapy in patients with heart failure improves ventricular function and symptoms of heart failure and reduces ventricular dimensions as well as plasma concentrations of BNP, TNF-α and IL-6 independent of plasma cholesterol levels [14, 15]. In the present study, fluvastatin between 10 nM and 500 nM significantly and dose dependently decreased baseline levels of BNP. Fluvastatin at concentrations of 100 nM and 500 nM also reduced the cytokine-induced expression of BNP whereby fluvastatin at the latter concentration reduced the cytokine-induced expression of BNP to control levels. In this respect, it should be noted that in patients undergoing 6 weeks of statin therapy with 40 mg of fluvastatin daily, plasma concentrations of approximately 250 nM were measured [16]. In our study, the inhibiting effect of fluvastatin on the IL-1α-, TNF-α- and TGF-β-induced up-regulation of Nt-proBNP production by cardiac fibroblasts was completely reversed by mevalonate and GGPP, suggesting that this effect was brought about by inhibition of the mevalonic acid pathway and protein prenylation. In particular, GGPP is essential for the isoprenylation of the GTPases Rap, Rab and Rho. Blocking isoprenylation of these GTPases causes their inactivation and thus prevents ROCK signalling [17]. Indeed, statins have been shown to inhibit the prenylation of ROCK [18–20]. Of note, Rho is crucial for the activation of rac, which is involved in IL-1-dependent regulation of the human BNP promoter [21]. We show here that the ROCK inhibitor Y27632 inhibits the IL-1α-, TNF-α- and TGF-β-induced up-regulation of Nt-proBNP similarly to fluvastatin. These results suggest that fluvastatin exerts its effect on IL-1α-, TNF-α- and TGF-β-induced up-regulation of Nt-proBNP by inhibiting the ROCK signalling pathway. The stimulating effect of IL-1α, TNF-α and TGF-β on BNP expression and its inhibition by fluvastatin was also seen at the level of specific BNP-mRNA expression in human cardiac fibroblasts. It should be emphasized, however, that we could show here that fluvastatin does not systematically decrease mRNA levels in cardiac fibroblasts as in these cells it slightly increased mRNA levels specific for the IL-6, IL-8 and PAI-1, whereas mRNA levels specific for VEGF doubled after incubation with fluvastatin in cardiac fibroblasts. Furthermore, mRNA specific for the housekeeping gene GAPDH was not affected significantly by fluvastatin. However, at this point further studies are needed to determine if the down-regulatory effect of fluvastatin on basal and cytokine-induced BNP expression in human cardiac fibroblasts is brought about by modulating directly gene transcription, stability of mRNA or post-translational modification. In that respect, it is of interest that it was shown recently that atorvastatin decreases the stability of mRNA for monocyte chemoattractant protein-1 (MCP-1) [22].

Our finding that human cardiac fibroblasts secrete BNP in considerable amount reveals a previously unknown function of these cells. Cardioprotective effects of BNP, like natriuresis, diuresis and inhibition of the renin–angiotensin–aldosterone system have been shown in healthy individuals and in patients with heart failure suggesting that elevated BNP expression is an adaptive mechanism of the heart in order to maintain cardiac performance during ventricular overload [2]. On the other hand, BNP has been shown to enhance hypoxia-induced apoptosis of myocardial cells [23], to have negative inotropic effects within the myocardium and to adversely affect cardiac contractility through inhibition of the expression of sarcoplasmic reticulum Ca^{2+} ATPase [24, 25]. BNP also inhibits expression of several pro-inflammatory cytokines, which are essential to protect the heart against reperfusion injury and ischaemic myocardial damage [26, 27], increases the expression of matrix metalloproteinases involved in remodelling [3], promotes the accumulation of inflammatory cells in the infarcted myocardium [28], suppresses the mitochondrial respiration in the myocardium, reduces the effects of catecholamines, induces systemic vasodilation and potentiates the generation of nitric oxide, which in turn is a known inhibitor of myocardial contractility [25, 29–32]. Thus, inhibiting the excessive expression of BNP, as shown by our study, by administration of statins might at least in theory result in prevention of ventricular dilation, and could improve myocardial performance and outcome of patients. Certainly, this possibility requires further intensive investigations, in particular in the light of a recent study published by the GISSI-group, which did not find any benefit for patients suffering from chronic heart failure who received rosuvastatin [33].

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