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Mechanisms of β_3-adrenoceptor-induced eNOS activation in right atrial and left ventricular human myocardium

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1 β-adrenoceptors are important modulators of cardiac function. The present study investigated β_3-adrenergic eNOS activation in human myocardium.

2 We measured nitric oxide (NO) liberation (diaminofluorescence) and signal transduction (immunohistochemistry, phosphorylation of eNOS^{Ser1177}, eNOS^{Thr495}, eNOS^{Ser114}, Akt/protein kinase B (Akt/PKB), and eNOS translocation) in human right atrial (RA, aortocoronary-bypass OP) and left ventricular nonfailing (LV, rejected donor hearts) myocardium after application of BRL 37344 (BRL), a preferential β_3-adrenoceptor agonist.

3 In both RA and LV, BRL (10 μl) induced a liberation of NO. An eNOS activation *via* translocation was only observed in RA after application of BRL (10 μM). Yet, the NO liberation in both LV and RA was accompanied by phosphorylation of eNOS^{Ser1177} and Akt/PKB. BRL-induced eNOS phosphorylation was abolished by LY292004, a blocker of PI-3 kinase. eNOS-Ser^{114} phosphorylation was unchanged in RA, but decreased in LV after β_3-adrenergic stimulation. BRL did not alter phosphorylation of eNOS^{Thr495}.

4 In conclusion, receptor-dependent eNOS activation is differentially regulated in the human heart. In the left ventricle, eNOS activation *via* phosphorylation seems to be of major importance, whereas in human atrial myocardium eNOS translocation is the predominant mechanism induced by β_3-adrenergic activation.
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Abbreviations: BRL, BRL 37344, BRL, ((*RR* + *SS*)-(±)-4-[2-(2-(3-chlorophenyl)-2-hydroxyethyl)amino)propyl] phenoxy-acetate; DAF-FM DA, diaminofluorescein; eNOS, endothelial nitric oxide synthase; L-NAME, L-nitromethylarginine methylester; NO, nitric oxide

Introduction

Different mechanisms have been recognized modulating the activity of the endothelial nitric oxide (NO)-synthase (eNOS) in endothelial cells. Activation of eNOS by translocation, dissociation from caveolin-1 and subsequent binding of calcium-calmodulin is the main mechanism leading to increased eNOS activity (Wu, 2002). In addition, phosphorylation at eNOS^{Ser1177}, eNOS^{Thr495} and eNOS^{Ser114} could be identified as additional mechanisms modulating eNOS activity (Bauer *et al*., 2003; for reviews, see Goligorsky *et al*., 2002; Flemming & Busse, 2003). Whereas eNOS^{Ser1177} phosphorylation has been implicated with an activation of the enzyme, phosphorylation of eNOS^{Ser114} and eNOS^{Thr495} has been demonstrated to decrease enzyme activity (Dimmeler *et al*., 1999; Bauer *et al*., 2003; Flemming & Busse, 2003). Very recently, the phosphorylation/dephosphorylation of eNOS^{Thr495} (Lin *et al*., 2003), as well as of eNOS^{Ser114} (Flemming & Busse, 2003), has been speculated to be an intrinsic switch mechanism that determines whether eNOS generates NO *versus* superoxide anions. Furthermore, evidence is provided that a kind of cross-talk or interrelationship may exist between eNOS^{Ser1177}, eNOS^{Ser114} and eNOS^{Thr495} phosphorylation, at least in endothelial cells (Dimmeler *et al*., 1999; Bauer *et al*., 2003; Flemming & Busse, 2003).

β-Adrenoceptors are important modulators of cardiac function. β_3-Adrenoceptors stimulated by the preferential β_3-adrenoceptor agonist BRL 37344 (BRL, ((*RR* + *SS*)-(±)-4-[2-(2-(3-chlorophenyl)-2-hydroxyethyl)amino)propyl] phenoxy-acetate)) induced a $G_{i/o}$-protein dependent eNOS activation in the presence of nadolol (β_1-/β_2-adrenoceptor blockade) (Gauthier *et al*., 1998; Moniotte *et al*., 2001). Yet, the knowledge about the mechanisms resulting in an activation of eNOS in cardiomyocytes is limited (for a review, see Bloch *et al*., 2001a). Recently, a stretch-dependent eNOS activation

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by phosphorylation at eNOS^{Ser1177} could be shown as a further eNOS regulatory mechanism in rat cardiomyocytes (Petroff *et al*., 2001).

To get a closer understanding of the mechanisms involved in β_3-adrenergic modulation of eNOS activity, the present study investigated the influence of the preferential β_3-adrenoceptor agonist BRL 37344 in isolated, electrically stimulated trabeculae obtained from human myocardium. Since regional differences have been described for β-adrenergic stimulation, for example, spare receptors in the right atrium (Schwinger *et al*., 1990), human right atrial and left ventricular nonfailing myocardium was studied in comparison for NO liberation and eNOS activation *via* translocation or phosphorylation.

Methods

Patients

Human ventricular trabeculae were obtained from 12 nonfailing donor hearts that could not be transplanted for technical reasons. The mean age of the donor group was 49 ± 4.7 years (three women, nine men). No cardiac catheterization had been performed in the organ donor group, but none of the donors had a history of heart disease and all had normal left ventricular function as measured by echocardiography. Right atrial myocardium was obtained from patients who underwent aortocoronary bypass operations without clinically signs of heart failure (33 male, 12 female; age 54.9 ± 4.2 years) with either coronary heart disease ($n = 32$) or valvular disease ($n = 13$)). Immediately after explantation, the myocardial tissues were placed in ice-cold aerated modified Tyrode's solution (see below) and delivered to the laboratory within 10 min. The study was approved by the local ethics committee and conforms with the Declaration of Helsinki.

Single-cell NO imaging

Diaminofluorescein (DAF-FM DA) is converted *via* an NO-specific mechanism to an intensely fluorescent triazole derivative (Kojima *et al*., 1998). We have used in the present study DAF-FM DA to detect changes of the NO level induced by BRL.

For this purpose, shock-frozen right atrial and left ventricular myocardial tissue stored at -80°C was used. Prior to the experiments, the tissue was equilibrated at -20°C for at least 1 h and sliced to 25 μm thickness. Tyrode's solution was added immediately, containing (in mM): $CaCl_2$ 1.8, $MgCl_2$ 1.1, KCl 5.4, NaCl 140, NaH_2PO_4 4.2, glucose 10, $NaHCO_3$ 23 and L-arginine 1. Slices were loaded with the NO-sensitive dye DAF-FM DA (10.0 μM, Molecular Probes, U.S.A.) for 30 min at room temperature. BRL 37344 (10 μM, Tocris, U.K.), Spermine NONOate (10 μM, Alexis, U.S.A.) and L-NAME (100 μM Alexis, FRG) were employed in individual experiments.

Changes of fluorescence were monitored as reported earlier (Pott *et al*., 2003). Briefly, excitation light (475 nm) was generated by a monochromator (TIL Photonics, Germany) and the emitted fluorescence imaged through a 470 nm long pass filter using an intensified charge-coupled device camera (Thetha, Germany). Fluorescence images (25 ms exposure time) were acquired at a frequency of 0.1 Hz using the Fucal fluorescence software package (TIL Photonics, Germany). Cardiomyocytes could be easily identified under the transmitted light and regions of interests selected accordingly.

Data analysis was done off-line using the Fucal software package. First, the data were normalized (F/F_0 where F_0 is the initial fluorescence), then changes of fluorescence were quantified. Responses are expressed in percent by defining the fluorescence intensity prior to the application of agonists 100%, and % of increase by measuring the maximal fluorescence intensity after addition of the agonist. Experiments with a prominent steady-state drift were not included in the analysis. For quantitative estimation of NO generation after application of BRL, only responding cells were included, whereas when using preincubation with L-NAME (100 μM for 90 min) all cells were taken.

Immunohistochemistry

Investigation of activated and/or phosphorylated molecules made it necessary to conduct preincubation procedures with freshly obtained tissue. All experiments were performed in the presence of propranolol (right atrium: 1 μM, left ventricle: 0.3 μM). Experiments were performed as described previously (Pott *et al*., 2003). Immunohistochemical measurements were performed at $t = 0$ min (control) and 5 min after application of BRL. Previous experiments have shown that eNOS activation is maximal 5 min after application of BRL (Pott *et al*., 2003). For intensity analysis of immunostaining in cardiomyocytes, we measured the gray values of 30 cardiomyocytes from three randomly selected areas of each slice. The intensity of immunostaining was reported as the mean of measured cardiomyocyte gray value minus background gray value. The background gray value was measured at a cell-free area of the slice. For staining intensity detection, a Zeiss Axiophot microscope coupled to a three-chip CCD camera was used and the analysis was performed using the Optimas 6.01 image analysis program.

Materials

The preferential β_3-AR agonist BRL 37344 was obtained by Tocris (Bristol, U.K.). The following primary antibodies were used for immunohistochemistry: (1) rabbit anti-eNOS antibody against the bovine eNOS peptide (599–613) plus additional C-terminal Cys conjugated to KLH (PYNSSPREQHKSYKC) (Biomol, Hamburg, Germany); this antibody has been previously shown to be specific in detecting the eNOS protein after dissociation from caveolin, that is, after translocation (Bloch *et al*., 2001b), anti-phospho-Akt/PKB (Upstate, Lake Placid, U.S.A.), corresponding to amino acids 301–312 of mouse pAkt/PKB, anti-phospho-eNOS^{Ser1177} (Upstate, Lake Placid, U.S.A.), corresponding to amino acids 1172–1183 of human eNOS and anti-phospho-eNOS^{Thr495} (Upstate, Lake Placid, U.S.A.), corresponding to amino acids 489–501 of human eNOS as well as anti-phospho-eNOS^{Ser114} corresponding to amino acids 106–118 of bovine eNOS (Upstate, Lake Placid, U.S.A.). As secondary antibodies, a biotinylated goat anti-rabbit, or biotinylated goat anti-mouse antibody (Dako, Hamburg, Germany) was used for accentuation.

All other chemicals were of analytical grade or the best grade, commercially available. For studies with isolated myocardium and trabeculae, stock solutions were prepared

and added to the organ bath. All compounds were dissolved in twice distilled water and did not change the pH of the medium.

Statistical analysis

All data are presented as mean ± s.e.m. Data analysis was performed using Student's *t*-test for paired and unpaired data, where appropriate. Significance was considered at a *P*-value <0.05.

Results

NO imaging

To investigate the signalling mechanism of BRL, we have investigated intracellular NO concentration by imaging human atrial and ventricular cardiomyocytes employing the NO-sensitive dye DAF-FM DA. As can be seen in Figure 1, application of BRL (10 μM) led to a transient rise of NO (Figure 1a) in the majority (95.4%, Figure 1e) of ventricular

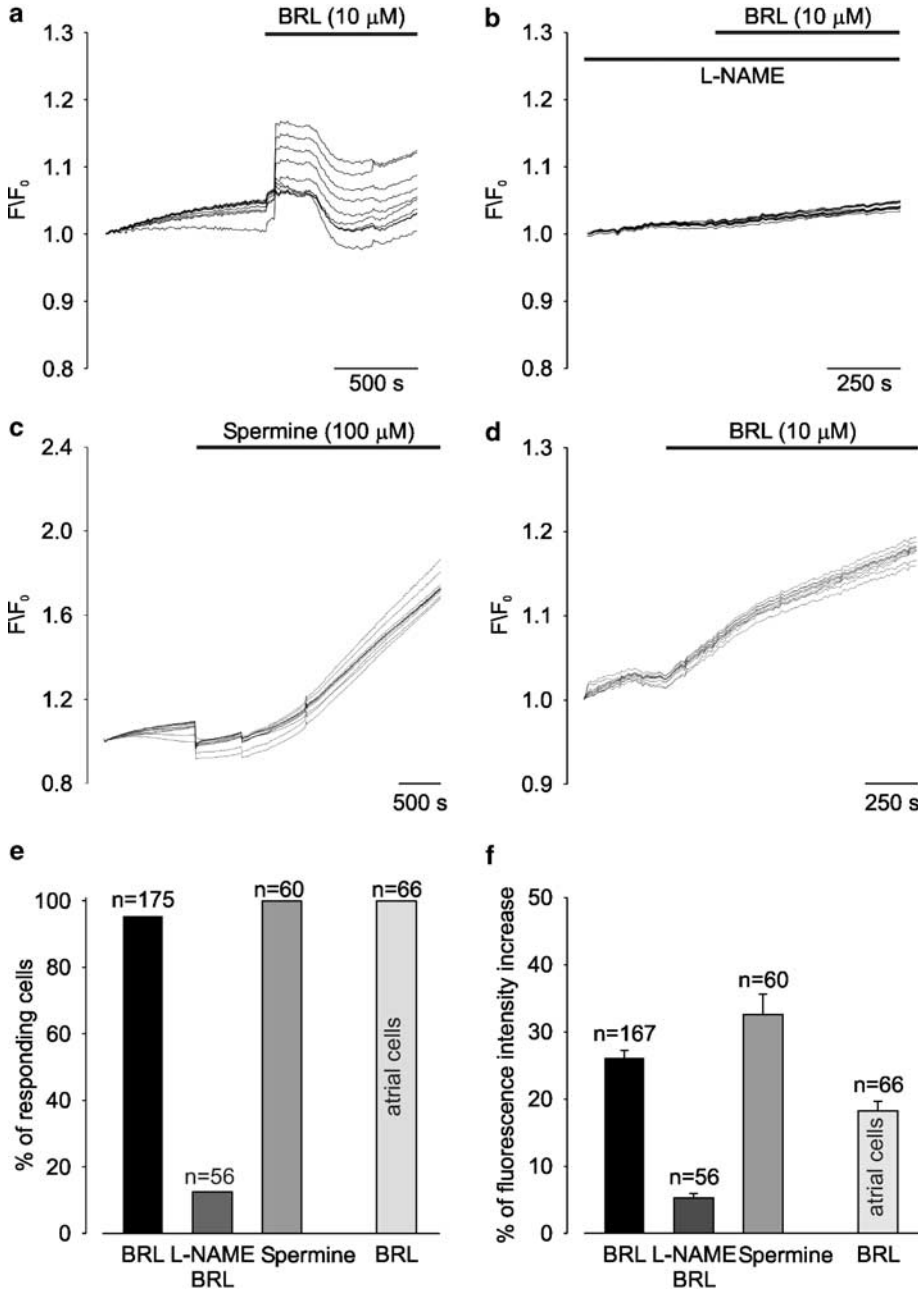

Figure 1 NO imaging: (a) DAF fluorescence increases in ventricular cardiomyocytes after application of BRL. (b) After preincubation with the NO inhibitor L-NAME for 90 min, no change in fluorescence was observed upon application of BRL. (c) The NO donor spermine NONOate led to pronounced increase of DAF fluorescence in ventricular cardiomyocytes. (d) Similar to the ventricular cardiomyocytes, NO increase was also observed upon BRL application in atrial cardiomyocytes. (e) The percentage of cells responding with an increase in fluorescence is displayed. (f) Quantitation of changes in the fluorescence intensity. Note that, for quantitative comparison, nonresponding and responding cells are included in the L-NAME group.

cardiomyocytes ($n=175$, 10 experiments). This was corroborated by preincubation (90 min) with the NO inhibitor L-NAME (100 μM, Figure 1b), where the majority (87.6%, Figure 1e) of ventricular cells ($n=56$, four experiments) did not show any more responses to BRL. This is also demonstrated quantitatively where BRL responders lead to a $26.1\pm1.1\%$ increase of fluorescence, whereas in cells after L-NAME preincubation only a significantly lower augmentation of the fluorescence signal by $5.2\pm0.7\%$ was noted (Figure 1f). The very low frequency of BRL responses in the presence of L-NAME (six out of 56 cells) may be related to incomplete pharmacological blockade of the different NOS isoforms. As expected, the most prominent generation of NO was detected in ventricular cardiomyocytes after application of the potent NO donor spermine NONOate (Figure 1c). This led to an augmentation of the DAF fluorescence by $32.5\pm3.0\%$ ($n=60$, four experiments, Figure 1f); however, when comparing BRL- and spermine-induced NO increase in ventricular cardiomyocytes, BRL appears to lead also to robust NO generation (Figure 1f). Besides ventricular

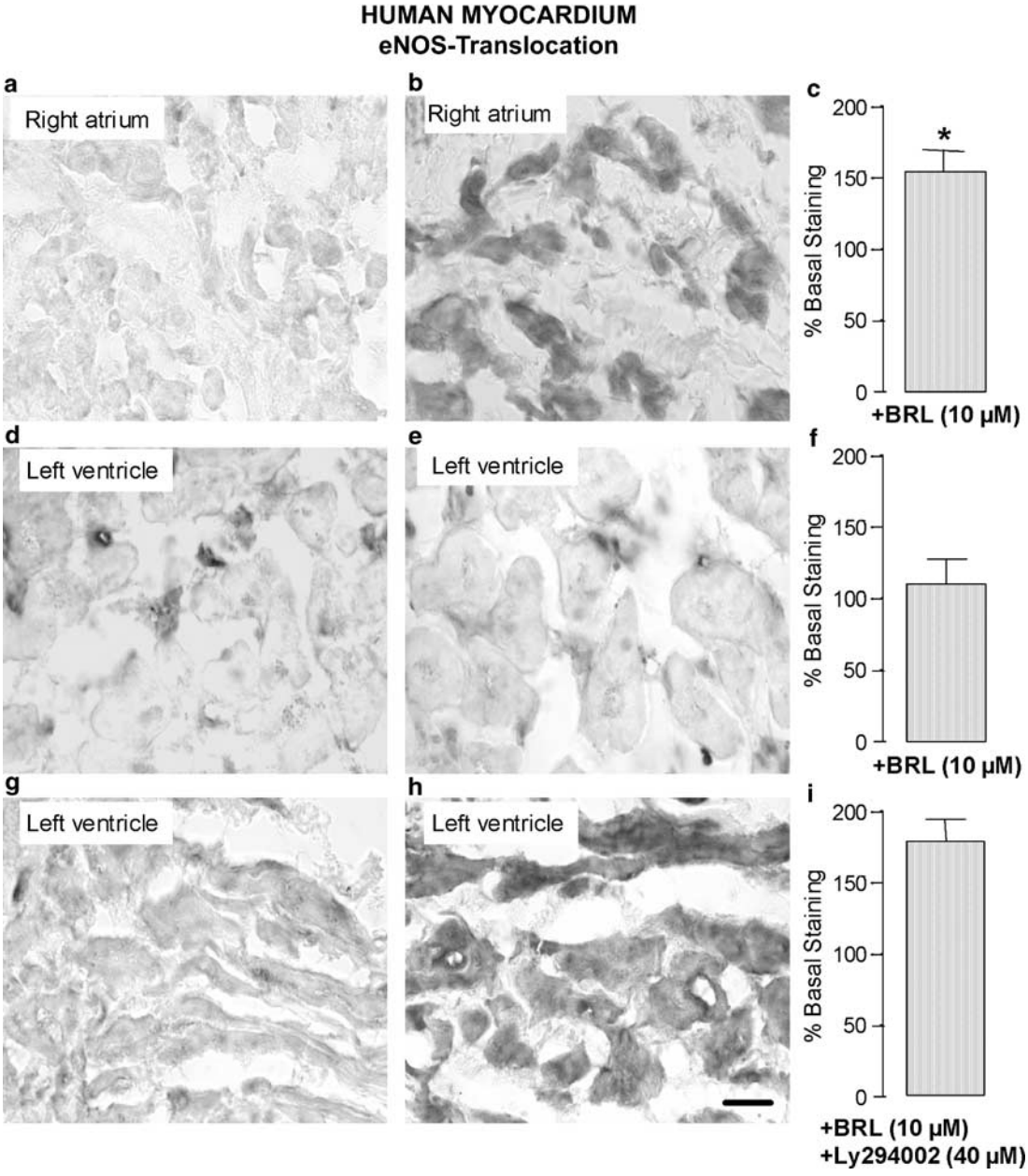

Figure 2 Immunohistochemical detection of activated eNOS (Bloch *et al*., 2001b) in the human right atrial (a–c, g–i) and left ventricular myocardium (d–f). A distinct increase in immunohistochemical detectable eNOS is visible in right atrial cardiomyocytes after BRL stimulation (b, c) compared to unstimulated right atrial cardiomyocytes (a). In the ventricular myocardium, only a faint staining for activated eNOS can be found in unstimulated (d) and BRL-stimulated cardiomyocytes (e, f). Experiments were repeated in the presence of LY294002 (40 μM) in the right atrial myocardium to exclude that the BRL-dependent increase of activated eNOS originates from phosphorylation of eNOS at Ser1177: Staining was distinctly increased from basal (g) to pretreatment with BRL (10 μM) (h, i) in cardiomyocytes, as observed without LY294002. Bar = 20 μm, $^*P<0.05$.

cardiomyocytes, we also observed increase of DAF-FM DA fluorescence (18.2±1.4%, Figure 1f) after applying BRL to atrial cardiomyocytes ($n=66$, four experiments, Figure 1d, e). Thus, the cell-imaging data demonstrate that BRL leads to NO increase in human atrial and ventricular cardiomyocytes.

eNOS activation

To investigate whether β_3-adrenoceptor stimulation may influence eNOS activity in human myocardium, we performed immunohistochemical eNOS stainings in isolated, electrically stimulated right atrial and left ventricular nonfailing trabeculae under control conditions ($t=0$ min) and after application of BRL (10 μM, $t=5$ min), using an eNOS antibody which has been shown to specifically detect the activated, translocated eNOS protein (Bloch *et al*., 2001b). Figure 2 presents pictures taken from original immunostainings.

Only in human right atrial myocardium, an activation/translocation of eNOS was detected after application of BRL (Figure 2a–c). This eNOS activation was also observed in the presence of LY294002, that is, after blocking PI-3 kinase (Figure 2g–i).

No eNOS activation *via* translocation was observed in human left ventricular nonfailing myocardium (Figure 2d–f).

Phosphorylation-dependent eNOS activation in human myocardium

To investigate the phosphorylation-dependent mechanisms involved in β_3-adrenergic eNOS activation, immunohistochemical studies were performed on the phosphorylation of eNOS^{Ser1177} (Fleming *et al*., 1998), eNOS^{Thr495} (Fleming *et al*., 1998) and eNOS^{Ser114} (Gallis *et al*., 1999) in isolated right atrial and left ventricular trabeculae of human myocardium in the absence and presence of BRL.

A time-dependent prominent phosphorylation of eNOS^{Ser1177} was observed in both right atrial (Figure 3a, b) and left ventricular human myocardium (Figure 3c, d). This was paralleled by a time-dependent increase in the phosphorylation of Akt/protein kinase B (PKB) in atrial (Figure 4a, b) and especially in left ventricular myocardium (Figure 4c, d). Akt/PKB is an enzyme which has been shown to be responsible for the eNOS^{Ser1177} phosphorylation (Dimmeler *et al*., 1999; Fleming *et al*., 2001). The phosphorylation of eNOS^{Ser1177} and Akt/PKB was inhibited by pre-treatment with LY 292004 (Figures 3e, f, 4e, f).

eNOS activity seems to be also dependent on the phosphorylation status of eNOS^{Thr495} (Fleming *et al*., 2001). BRL (10 μM) did not influence eNOS^{Thr495} phosphorylation in both left ventricular and right atrial myocardium (data not shown).

Phosphorylation of eNOS^{Ser114} has been implicated in the agonist-induced activation of the eNOS enzyme (Bauer *et al*., 2003). We, therefore, investigated eNOS^{Ser114} phosphorylation after application of BRL in human right atrial and left ventricular myocardium (Figure 5). In human right atrial myocardium, eNOS^{Ser114} phosphorylation was not altered after application of BRL (Figure 5a, b). In contrast, we observed a dephosphorylation of eNOS^{Ser114} in human left ventricular myocardium 5 min after application of BRL (Figure 5c, d).

Discussion

The present study investigated eNOS activation in human nonfailing myocardium using the preferential β_3-adrenoceptor agonist BRL 37344. Our intention was to study the influence of β_3-adrenergic stimulation in the left ventricular myocardium, since this is the region of the heart in which stroke work is most evident and most important. For comparison, we studied the atrial myocardium. This is the first study to demonstrate that eNOS activity in human nonfailing myocardium can be modulated by receptor-induced PI-3 kinase/Akt/PKB-dependent phosphorylation of eNOS^{Ser1177} and eNOS^{Ser114}. Furthermore, distinct differences in the mechanisms of receptor-dependent eNOS activation could be shown between atrial and ventricular human myocardium.

The present study provides evidence that the β_3-adrenergic signalling pathway and the thereby induced activation of eNOS is differentially regulated in right atrial and left ventricular myocardium. Thus, the modulation of eNOS activity is a very complex mechanism that not only differs between the organs (e.g. endothelium and cardiomyocytes), but also within an organ itself. Using the preferential β_3-adrenoceptor agonist BRL 37344, an activation of a NOS pathway has been demonstrated in human intraventricular biopsies of transplanted human myocardium, and it was suggested that the NOS isoform involved in the β_3-adrenoceptor stimulation is probably eNOS (Gauthier *et al*., 1998). In the present study, we performed experiments with an anti-eNOS antibody, which has been shown previously to be specific for detection of the activated and translocated forms of eNOS (Bloch *et al*., 2001b; Reiner *et al*., 2001). Using this antibody, we observed a translocation-dependent eNOS activation in human right atrial, but not in the left ventricular myocardium. This translocation-dependent eNOS activation was also present after $\beta_{1/2}$-adrenoceptor blockade, indicating that the BRL-induced eNOS activation in human right atrial myocardium cannot be attributed to a $\beta_{1/2}$-adrenoceptor stimulation (Pott *et al*., 2003).

No evidence was found in the present study that a translocation-dependent eNOS activation takes place after application of BRL 37344 in human nonfailing left ventricular myocardium. Besides translocation and subsequent calcium-calmodulin binding, eNOS activity is regulated by phosphorylation at eNOS^{Ser1177} (Dimmeler *et al*., 1999; Fulton *et al*., 1999) and eNOS^{Thr495} (Fleming *et al*., 2001) in endothelial cells. Recently, a stretch-dependent eNOS activation by phosphorylation at eNOS^{Ser1177} could be shown in rat cardiomyocytes (Petroff *et al*., 2001). This study is the first to demonstrate that eNOS activation by phosphorylation at eNOS^{Ser1177} also occurs in human atrial and ventricular cardiomyocytes after receptor-induced stimulation. The eNOS^{Ser1177} phosphorylation went in parallel to a phosphorylation of the Akt/PKB and was inhibited in the presence of LY 292004, indicating that an activation of the PI-3 kinase/Akt kinase-dependent signal transduction pathway may be involved in the BRL 37344-induced eNOS activation.

Evidence is provided that a kind of cross-talk or interrelationship may exist between eNOS^{Ser1177}, eNOS^{Ser114} and eNOS^{Thr495} phosphorylation, at least in endothelial cells (Dimmeler *et al*., 1999; Bauer *et al*., 2003; Flemming & Busse,

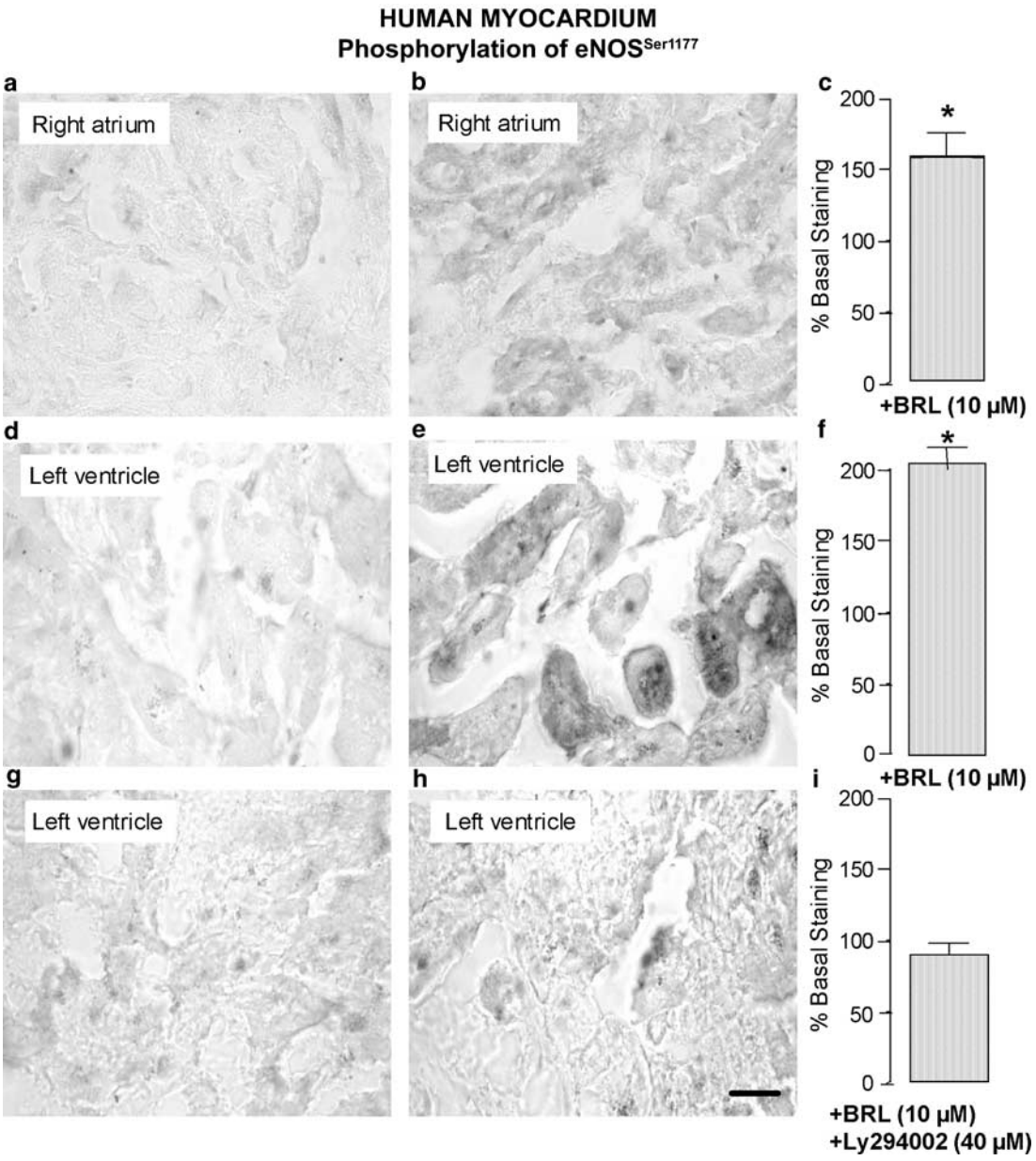

Figure 3 Influence of BRL on intensity of immunostaining for peNOS^{Ser1177} proteins in human right atrial (a–c) and left ventricular (d–i) myocardium. A weak cytosolic staining is observed in myocytes under basal conditions (a, d). An increase can be observed after incubation with BRL in the right atrial myocardium and in the left ventricular myocardium (b, c, e, f). The strongest BRL-induced increase of peNOS^{Ser1177} can be found in cardiomyocytes of the left ventricle (e, f). Experiments were repeated in the presence of LY294002 (40 μM) in the left ventricular myocardium: Staining did not increase from basal (g) to pretreatment with BRL (10 μM) (h, i). Bar = 20 μm, $^*P<0.05$.

2003). In the present study, in which the receptor-stimulated eNOS activation was investigated, no interrelationship between eNOS^{Ser1177} and eNOS^{Thr495} was observed in both right atrial and left ventricular myocardium. Yet, only in the left ventricular myocardium, BRL-induced eNOS^{Ser1177} phosphorylation was paralleled by a decrease in eNOS^{Ser114} phosphorylation. The eNOS^{Ser114} phosphorylation side has been implicated as a negative regulatory site of the eNOS enzyme (Bauer *et al*., 2003). Dephosphorylation of eNOS^{Ser114} and thus a stimulation of eNOS activity has been attributed to protein phosphatase 2B (Kou *et al*., 2002). Thus, it may be speculated that in human nonfailing left ventricular myocardium, BRL-induced NO liberation due to an Akt/PKB-induced eNOS^{Ser1177} phosphorylation may be additionally enhanced by an eNOS^{Ser114} dephosphorylation.

It is generally assumed that NO plays a modulatory role in myocardial excitation–contraction coupling since the eNOS activity depends on $[Ca^{2+}]_i$ (Finkel *et al*., 1995; Kaye *et al*., 1996). The present study provides evidence that there are also Ca^{2+}-independent, but phosphorylation-dependent pathways in cardiac tissue, which predominantly regulate eNOS activity,

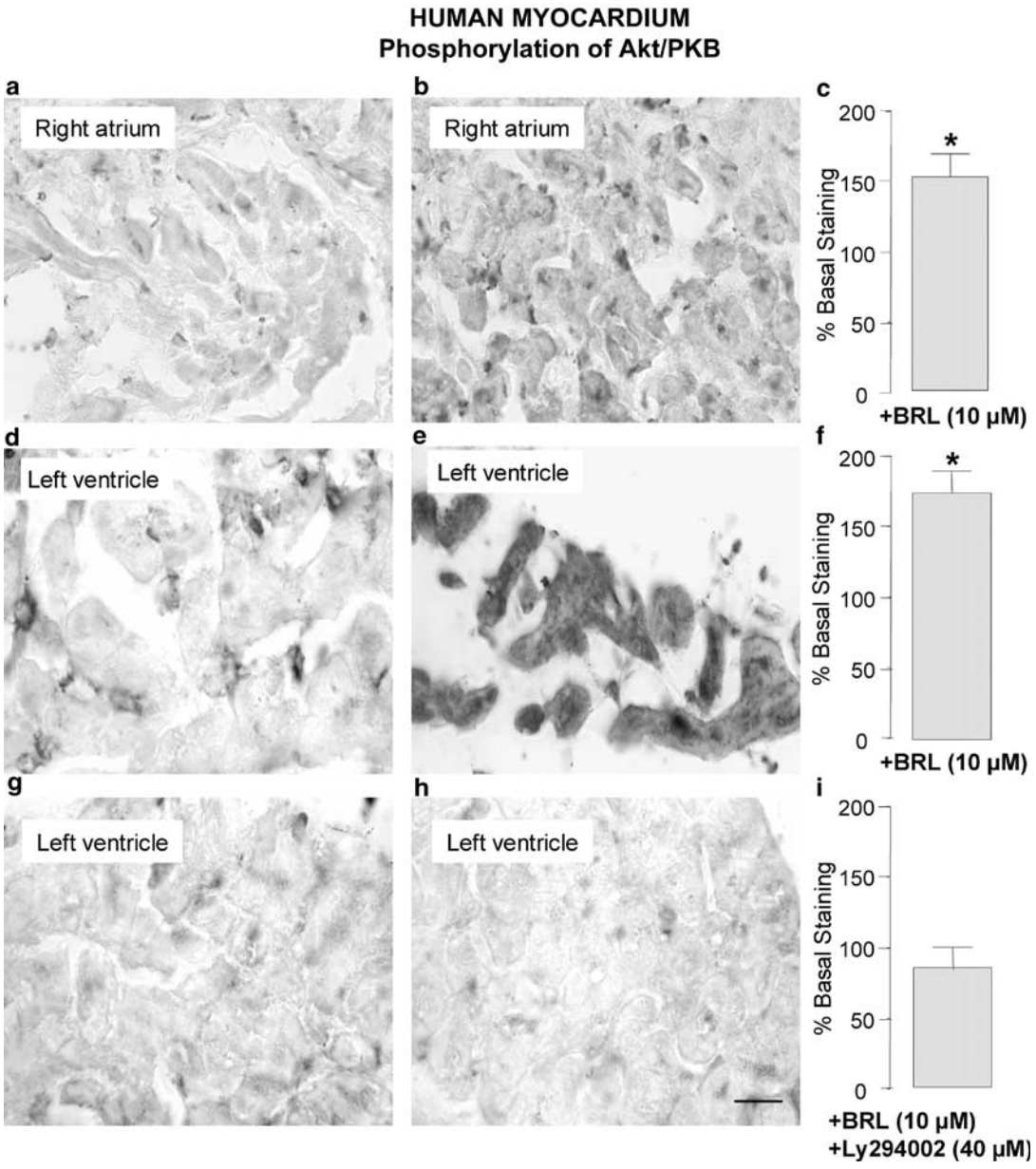

Figure 4 Immunostaining of BRL-dependent activation of Akt/PKB phosphorylation in the right atrial (a–c) and left ventricular (d–i) myocardium. Besides a phosphorylation of Akt/PKB in endothelial cells, a weak basal phosphorylation of Akt/PKB can be seen in cardiomyocytes of the right atrial (a) and left ventricular (d) myocardium. The increase in Akt/PKB phosphorylation can be observed in cardiomyocytes of the right atrium (b, c) and more pronounced in cardiomyocytes of the left ventricle (e, f). Experiments were repeated in the presence of LY294002 (40 μM) in the left ventricular myocardium: Staining did not increase from basal (g) to pretreatment with BRL (10 μM, h, i). Bar = 20 μm, $^*P<0.05$.

especially in the left ventricular myocardium. Our data provide evidence that the activation of eNOS translocation may be obtained for a longer time than eNOS phosphorylation. Accordingly, it could recently be shown that receptor-mediated Ca^{2+}-dependent eNOS translocation first reaches the maximum after 5 min and it can stay up to 20 min; this is longer than our observation time (Prabhakar *et al*., 1998), while a transient phosphorylation of eNOS at Ser1177 is known for not more than 5 min (Harris *et al*., 2001). Besides excitation–contraction coupling, different myocardial cellular and intracellular targets of NO necessary for myocardial maintenance and function could be shown, such as nerve fibers, resistance vessels and capillaries, as well as mitochondria (for a review, see Bloch *et al*., 2001a).

In conclusion, the present study provides evidence that eNOS activation differs between right atrial and left ventricular myocardium. In human atrial myocardium, the phosphorylation-independent eNOS activation is the predominant eNOS regulatory mechanism, whereas in the left ventricle eNOS activity is preferentially modulated *via* phosphorylation. The situation may change under pathophysiological conditions.

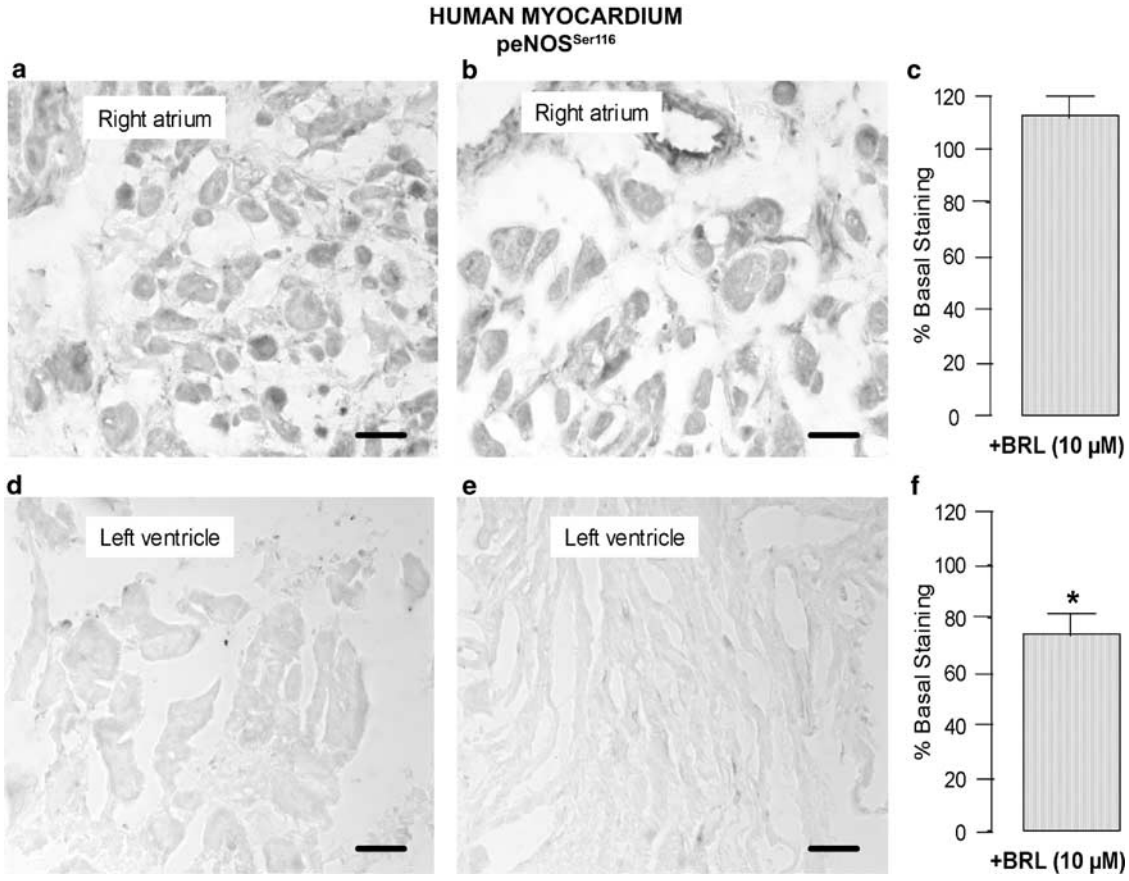

Figure 5 Immunostaining of BRL-dependent activation of Ser^{114}-eNOS phosphorylation in the right atrial (a–c) and left ventricular (d–f) myocardium. No alterations of eNOS Ser^{114}-phosphorylation were observed in the right atrial myocardium following application of BRL 37344; in the left ventricular myocardium, application of BRL 37344 induced a dephosphorylation of Ser^{114}-eNOS. Bar = 20 μm, $^*P < 0.05$.

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