Phosphorylation of myocardial eNOS is altered in patients suffering from type 2 diabetes

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Submitted 19 January 2011; accepted in final form 19 December 2012

Streit U, Reuter H, Bloch W, Wahlers T, Schwinger RHG, Brixius K. Phosphorylation of myocardial eNOS is altered in patients suffering from type 2 diabetes. J Appl Physiol 114: 1366–1374, 2013. First published December 20, 2012; doi:10.1152/japplphysiol.00011.2011.—The present study investigated whether endothelial nitric oxide synthase (eNOS) activation may be dysregulated in cardiac tissue of patients suffering from type 2 diabetes (T2D). We performed immunohistochemical measurements of translocated eNOS activation as well as eNOS phosphorylation at Ser1177, Thr495, Ser635, Ser114, and of the protein kinase B (Akt) in isolated right atrial trabeculae of patients undergoing cardiac bypass surgery with (n = 12, 68.1 ± 2.5 yr) and without T2D (n = 12, 64.7 ± 2.7 yr). In addition, we investigated oxidative (8-isoprostane) and nitrosative stress markers (nitrotyrosine) as well as the effect of pharmacological stimulation of angiotensin (AT)-receptors on eNOS-phosphorylation. Translocation-dependent eNOS activation was similar in both groups. The same holds true for eNOS phosphorylation at Ser114. eNOS phosphorylation at Ser635 was significantly increased, whereas eNOS phosphorylation of Ser1177 was significantly decreased in the diabetic group paralleled by a decrease in phosphorylation of Akt and Thr495. These alterations were accompanied by a significant decrease in nitrotyrosine. After application of angiotensin II (10 μM, 2 min) for investigation of the AKT-receptor-dependent eNOS stimulation, the AT-receptor-dependent eNOS stimulation, we did not find differences between the increases in eNOS Ser1177-phosphorylation in the non-diabetic (+39.7 ± 23.5%) and in the diabetic group (32.22 ± 11.45%). A simultaneous increase in Akt phosphorylation could not be observed. The present study indicates that T2D goes along with a decrease in eNOS phosphorylation at Ser1177 under basal conditions in cardiac tissue. Whether this may be attributed to the insulin resistance of cardiac muscle has to be further investigated. Receptor-stimulated eNOS activation still works at least for angiotensin II-dependent eNOS activation.

Ser1177 has been identified as the most important regulatory phosphorylation site so far (27). At basal cellular state Ser1177 is not phosphorylated but reacts to various stimuli like shear stress (4), insulin (33), bradykinin (8), or HMG-CoA-reductase inhibitors (statins) (7) with phosphorylation and eNOS activation. One of the main pathways involved in eNOS Ser1177-phosphorylation is the phosphatidyl-inositol-3-kinase/protein kinase B (PI3K/Akt) pathway.

Opposed to Ser1177, Thr495 reduces eNOS activity upon phosphorylation, e.g., by a constitutively active protein kinase C (PKC) (11, 29). There is evidence for an interdependent coordination between Ser1177-phosphorylation and Thr495-dephosphorylation (17, 26, 29, 45). Ser635 is another positive regulatory phosphorylation site, although apparently calcium independent. Phosphorylation at this site often parallels Ser1177-phosphorylation (4, 30). The role of the phosphorylation site Ser114 has not been clearly identified yet. Newer studies point to a negative regulatory effect of phosphorylation (25).

Normal insulin signaling results in an activation of two parallel pathways, i.e., the PI3K/Akt and the Ras/Raf/MAP kinase pathway. Whereas the PI3K/Akt pathway stimulates the expression of GLUT4-transporter proteins in skeletal muscle and adipose tissue, less is known whether activation of the insulin receptor may also influence eNOS activation in cardiac muscle similar to as has been described in endothelial cells (52).

Insulin resistance goes along with a decreased sensitivity of the various tissues toward insulin. There is evidence that the balance between the two downstream pathways is altered under these conditions. In endothelial cells it has been described that the PI3K/Akt pathway is reduced so that insulin mainly results in cellular reactions mediated by the Ras/Raf/MAP kinase pathway. Thus reduced NO formation and less insulin-mediated vasodilation occurs (7, 21, 24). Again it has to be questioned whether this holds true also in cardiac muscle.

eNOS seems of great importance in the diseased myocardium. Insulin as well as HMG-CoA-reductase inhibitors have been shown to exert cardioprotective effects after an ischemic insult to the myocardium via PI3K/Akt-mediated increased Ser1177-phosphorylation and NO production (2). A study by Sasso et al. (43) showed that in individuals with chronic ischemic heart disease, as a compensatory mechanism, Ser1177-phosphorylation does increase, an effect that could not be shown for individuals suffering from type 2 diabetes.

ANG II levels are elevated in pathological conditions like diabetes mellitus and heart failure. ANG II conducts its various actions in the heart mostly via the two prominent receptor subtypes (AT1R). Multiple studies with ANG II have already confirmed that eNOS activation can be conducted via both its AT1-
and AT_2-receptors (5, 42, 44, 50). Looking at eNOS phosphorylation, Suzuki et al. (44) could show that phosphorylation of Ser1177 is conducted via the AT_1-receptor. The underlying intracellular signaling pathway has yet to be elucidated.

The data on the role of eNOS in the myocardium as well as data on the possible alterations of regulation mechanisms by the diabetic metabolism are still limited.

The present study investigates whether eNOS-activation mechanisms are altered in atrial myocardium of type 2 diabetic patients under basal conditions and after ANG II stimulation. Therefore, we performed immunohistochemical measurements of human atrial myocardium to investigate eNOS translocation and phosphorylation. Receptor-stimulated Ser1177- and Akt-phosphorylation were also investigated by time-dependent ANG II stimulation.

MATERIALS AND METHODS

Patients and Cardiac Tissue

We obtained right atrial myocardium from 12 patients suffering from type 2 diabetes undergoing cardiac bypass or valve surgery. The mean age of the diabetic patients was 68.1 ± 2.5 yr. Myocardium from 12 patients without diabetes served as our control. The mean age of the nondiabetic patients was 64.7 ± 2.7 yr.

The patients were carefully selected to minimize differences between the nondiabetic and diabetic patient group regarding cardiac risk factors and medication (Tables 1 and 2). Patients with NO-donor medication or AT_1-receptor antagonist were excluded from the patient collective. Preoperatively in both groups cardiac failure was excluded by measuring the left ventricular ejection fraction by catheterization and echocardiography, and atrial arrhythmia was excluded by ECG. Immediately after intraoperative excision, the myocardial tissue was placed into a ice-cold cardioplegic Custodiol solution and delivered to the laboratory within 10 min.

The study was approved by the local ethics committee and conforms to the Declaration of Helsinki. The patients gave their written informed consent to the procedure.

Immunohistochemistry (IHC)

Shortly after the excision, single trabeculae were isolated from the tissue, and pretreatment procedures were conducted in order to investigate activated and phosphorylated molecules.

For tissue preparation the right atrial trabeculae were suspended in separate organ baths with aerated Tyrode’s solution for at least 20 min at 37°C (Tyrode’s solution in mmol/l: 119.8 NaCl, 5.4 KCl, 1.8 CaCl_2, 1.05 MgCl_2, 0.42 Na_2HPO_4, 22.6 NaHCO_3, 0.05 Na_2EDTA, 0.28 ascorbic acid, 5 glucose, continuously gassed with 95% O_2 and 5% CO_2, pH 7.4, temperature 37°C). Some trabeculae were incubated with ANG II (10 μM) for 2 or 10 min in order to investigate receptor-mediated eNOS activation. Afterward the trabeculae were immediately placed in 4% paraformaldehyde for fixation (4 h), then rinsed in 0.1 mol/l phosphate-buffered saline [PBS (in mmol/l): 0.08 Na_2HPO_4, 0.019 NaH_2PO_4, 0.15 NaCl] (4 × 10 min) and subsequently stored in PBS solution with 18% sucrose for cryoprotection (12 h). Finally they were frozen at −80°C with Tissue Freezing Medium (LEICA).

For the immunohistochemical stains, the tissue was cut into 10-μm slices with a cryotome, brought onto specimen slides, and repeatedly stored at −80°C.

As a priming procedure for the immunohistochemical staining, the specimens were placed in a bathing solution of 3% H_2O_2 and methanol for 30 min and then permeabilized with 0.5 M ammonium chloride and 0.25% Triton X in 0.1 mol/l TBS. This was followed by a treatment with 5% bovine serum albumin (BSA) solution in TBS.

Continuously after each step, TBS buffer was used for rinsing up to three times and more. A primary antibody (e.g., anti-phospho-eNOS Ser1177) in combination with a TBS-based solution of 0.8% BSA was used to incubate the specimen slides, followed by its corresponding secondary biotinylated goat anti-rabbit, biotinylated goat antimouse, or biotinylated rabbit anti-goat antibody (1 h at room temperature). Negative control slides were prepared in the absence of the primary antibody. Again, a rinsing procedure with the TBS solution was conducted between the steps. As a detection system, streptavidin-horseradish peroxidase complex (1:150 dilution) was then applied for 1 h. For the final step, development, 3.3-diaminobenzidine tetrahydrochloride and 0.25% Triton X in 0.1 mol/l TBS. This was followed by a treatment with 5% bovine serum albumin (BSA) solution in TBS.

Immunohistochemical staining was performed to detect basal eNOS translocation-activation. Furthermore, alterations of eNOS phosphorylation at serine 1177, threonine 495, serine 635, and serine 114 and Akt as well as nitrosative and oxidative stress markers

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F, female; M, male; ACE inhibitor, angiotensin-converting enzyme inhibitor.

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F, female; M, male; CCB, calcium channel blocker; NSAID, nonsteroidal anti-inflammatory drug.
nitrotyrosine and 8-isoprostane were investigated. In addition, the effect of ANG II stimulation (t = 2 or 10 min) on Ser1177- and Akt-phosphorylation was studied. Tissue under basal conditions with- out ANG II treatment (t = 0) served as our control.

Ultimately, computer-based densitometric analysis (ImageJ analysis software, Toronto Western Research Institute, University Health Network, version 1.33) was performed. Photographs of the IHC stains were captured and transmitted using a camera-linked (Sony Trinicon) light microscope (Leitz Orthoplan). The density or gray value of each of 30 cardiomyocytes was measured randomly selecting cardiomyocytes from three random areas of each slide, and then subtracted from the background gray value (mAryG). The mean background value was determined by measuring the gray value of three random cell-free areas on the slide. A mean gray value was determined for each specimen. Staining and densitometry were performed as described previously (37).

Materials

Solutions. For preparation of the isolated trabeculae, stock solutions were prepared and added to the organ bath. All added chemicals were of analytical grade or the best grade commercially available. All compounds were dissolved in twice distilled water and did not change the pH of the medium.

Primary antibodies. We used rabbit anti-eNOS antibody against the bovine eNOS-peptide plus additional COOH-terminal Cys conjugated to KLH (PYNSSPREQHKSYKC) (Biomol, Hamburg, Germany), which according to Bloch and coworkers et al. (3) specifically detects the activated/translocated form of eNOS by binding to eNOS after dissociation from caveolin.

In addition, we utilized anti-phospho-eNOSSer1177 (Upstate, Lake Placid, NY) corresponding to the amino acids 1172–1183 of the human eNOS; anti-phospho-eNOSSer116 corresponding to the amino acids 106–118 of the human eNOS (Upstate) which shares 12/13 amino acids with bovine and pig eNOS; anti-phospho-eNOSSer635 (Upstate) corresponding to the amino acids surrounding phosphorylated Ser635 of bovine eNOS; and anti-phospho-eNOSThr405 (Upstate) corresponding to amino acids 489–501 of human eNOS, as well as anti-phospho-Akt/PKB (Upstate) corresponding to amino acids 301–312 of mouse pAkt/PKB. We used anti-nitrotyrosine (Upstate) to investigate nitrosative and anti-8-epi-PGF2α (8-isoprostane) (Oxford Biomed) to investigate oxidative stress.

Secondary antibodies. Biotinylated goat anti-rabbit, biotinylated goat anti-mouse, or biotinylated rabbit anti-goat antibodies (Dako, Biomed) to investigate oxidative stress.

RESULTS

Translocation-Dependent eNOS Activation

To investigate eNOS activation by translocation, immuno- histochemical studies on isolated right atrial trabeculae from diabetic and nondiabetic patients were performed using an eNOS antibody that has been shown to specifically detect the activated, translocated eNOS protein (3). No significant difference in translocation-dependent eNOS activation could be observed between the two groups (T2D: 12.57 ± 0.94 densitometric units (DU) vs. control 13.26 ± 0.92 DU).

Phosphorylation-Dependent eNOS Activation

Phosphorylation-dependent mechanisms involved in eNOS regulation were also investigated using immunohistochemical studies of the phosphorylation sites. Phosphorylation of Ser1177 was found to be significantly decreased in the diabetic group in comparison to the control group (T2D 10.08 ± 0.82 DU vs. control 12.8 ± 0.71 DU; P = 0.0013; Fig. 1A). The same holds true for the kinase involved in one of the main signal transduction pathways leading to Ser1177-phosphorylation of Akt/PKB (T2D 8.4 ± 0.34 DU vs. control 13.37 ± 0.51 DU, P = 0.00027; Fig. 1B), as well as for the phosphorylation site Thr495 (T2D 10.37 ± 0.73 DU vs. control 14.48 ± 0.9 DU; P = 0.025; Fig. 1C). In contrast, phosphorylation of Ser635, a site that is also thought to increase eNOS activity, was significantly increased (T2D 10.82 ± 0.77 DU, control 8.03 ± 0.72 DU; P = 0.047; Fig. 2), whereas the phosphorylation of Ser114 was found to be similar in both groups (T2D 10.38 ± 0.8 DU vs. control 10.19 ± 0.81 DU; P = 0.88).

Oxidative and Nitrosative Stress

Since dysregulation of eNOS phosphorylation may be par- alleled by an increase in the generation of oxygen- or nitrogen-dependent radical species (31, 46, 49), we performed immuno- staining of 8-isoprostane as an indicator for oxidative stress and nitrotyrosine indicating nitrosative stress (Ref. 1). 8-Isoprostan staining showed no significant increase of oxidative stress in right atrial tissue of the diabetic group (T2D 10.24 ± 0.72 DU vs. control 9.33 ± 0.78 DU, P = 0.31). However, we discovered a significant decrease of the nitrotyrosine signal in the diabetic group (T2D 9.12 ± 0.83 DU vs. control 14.14 ± 0.84 DU; P = 5.5 × 10−6; Fig. 2).

ANG II-Stimulated phosphorylation of eNOS and Akt/PKB

To investigate the ANG II-dependent activation of eNOS by Ser1177-phosphorylation in type 2 diabetic cardiac tissue, immunohistochemical studies were performed before (t = 0; control) and after incubation of the specimens with ANG II (10 μM) for 2 and 10 min. The specimens without ANG II stimulation (t = 0) served as our control, and basal Ser1177-phosphorylation was defined at a 100%.

A similar increase in Ser1177-phosphorylation and presum- ably eNOS activity could be seen in both groups after 2 min of stimulation at which point also the maximum signal of the time curve could be detected. This finding suggests that ANG II receptor-dependent eNOS activation still works in diabetic patients (t = 2 min: T2D 132.22 ± 11.45%; control 139.7 ± 23.3%; Figs. 3 and 4). Ser1177-phosphorylation decreased equally in both groups after 10 min of ANG II stimulation (t = 10 min: T2D 114.76% ± 10.82%; control 114.25 ± 21.24%, Fig. 3).

The same test set up as above was conducted to detect ANG II-stimulated Akt phosphorylation. No significant increase could be shown for phosphorylated Akt after 2 or 10 min of incubation in both groups, suggesting that Akt is not the kinase involved in the ANG II receptor-dependent Ser1177-phosphorylation (t=2 min: T2D: 102.76% ± 11.33%; control: 111.35% ± 12.37%; t= 10 min: T2D: 105.11% ± 8.34%; control: 100.95% ± 9.55; Figs. 5 and 6).

DISCUSSION

Cardiovascular integrity has been strongly linked to the endothelial NO synthase, for which multiple cardioprotective effects, including vasodilatation, suppression of smooth muscle proliferation, and platelet adhesion, have been demonstrated

J Appl Physiol • doi:10.1152/japplphysiol.00011.2011 • www.jappl.org
More and more attention is now also paid to the role of eNOS in the myocardium, especially in the diseased state. Two main mechanisms of eNOS activation have been identified, eNOS activation via translocation (3) and via phosphorylation. Our aim was to investigate eNOS translocation and eNOS phosphorylation in diabetic compared with nondiabetic human atrial myocardium.

**Translocation-Dependent eNOS Activation in Diabetic Human Atrial Myocardium**

Our results provide evidence that eNOS-translocation-dependent mechanisms in atrial myocardium do not seem to be affected by diabetic metabolic abnormalities. eNOS translocation is primarily calcium dependent. There is evidence that in type 2 diabetes calcium homeostasis in cardiomyocytes is affected by prolonged release of calcium from the sarcoplasmic reticulum. However, the total calcium concentration in systole and diastole remains unchanged and therefore does not seem to affect eNOS translocation (40).

**Insulin and eNOS Phosphorylation in Type 2 Diabetes**

The present study provides evidence that eNOS phosphorylation of Ser1177 is downregulated in human atrial myocardium of patients suffering from type 2 diabetes. Since insulin is a stimulus for the activation of eNOS by phosphorylation at Ser1177 via the insulin receptor and PI3K/Akt pathway (14), the decreased insulin signaling might be the link to explain our finding of decreased Ser1177-phosphorylation in the diabetic state.

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**Fig. 1.** A and B are taken from original immunostains, showing eNOS-Ser1177-phosphorylation (top), Akt-phosphorylation (middle), and eNOS-Thr495-phosphorylation (bottom) in right atrial myocardium. C: data from densitometric evaluation. T2D, type 2 diabetes. *P < 0.05 vs. control.
group. In line with this assumption, we observed a significant decrease in Akt/PKB phosphorylation. Our hypothesis that insulin resistance leads to a downregulation of Ser1177-phosphorylation and hence NO production, would concur with the work of Kobayashi and co-workers (23), who showed that in diabetic mice aortas the PI3K/Akt pathway and therefore NO production is impaired.

Other mechanisms that may interfere with eNOS phosphorylation in diabetic myocardium may be attributed to a direct inhibition of the PI3K/Akt pathway by toxic products or metabolites of the diabetic metabolism (20). Du and co-workers (8) suggest that in bovine aortic endothelial cells hyperglycemia directly leads to a reduction of Ser1177-phosphorylation and eNOS activity through the hexosamine pathway.

Wang and coworkers (47) also observed a slight decrease in basal Akt phosphorylation (Akt protein expression was unchanged) paralleled by a decrease in eNOS phosphorylation in human atrial myocardium of diabetic compared with nondiabetic persons. This indicates that eNOS dysregulation seems to be a general feature in diabetic patients which is not only limited to the endothelium but also affects other tissues.

At the moment the reasons responsible for the downregulation of Akt phosphorylation can only be speculated on. Wang and coworkers measuring the oxidative stress in the coronary sinus blood hypothesized that the increase of the reactive oxygen species in the coronary sinus blood may be responsible for an upregulation of phosphatase and tensin homologue on chromosome 10 (PTEN), the principal negative regulator of the PI3K/Akt pathway (47). On the other hand a decrease of the insulin-receptor-mediated Akt phosphorylation is also feasible [overview by Yu et al (51)]. Thus a dysregulation of multiple signal transduction pathways may be involved in the downregulation of Ser1177-phosphorylation during the pathogenesis of type 2 diabetes.

The phosphorylation site Ser1177 is also known to interact with Thr495 in a highly coordinated fashion. Phosphorylation of Ser1177 is accompanied by dephosphorylation of Thr495 (12). Conversely, in our study we found Thr495-phosphorylation to be significantly decreased as well in the diabetic tissue.
compared with the controls. Because of the interdependence of the two regulatory sites (11, 17, 26, 29, 45) this could imply a compensatory mechanism for the downregulation of Ser1177-phosphorylation, granting sufficient NO production. Isolated Thr495-dephosphorylation has been shown to be sufficient to increase NO production and to have superior influence on the amount of NO produced (11, 17). An enzyme responsible for Thr495-dephosphorylation is the serine threonine protein phosphatase 2A (PP2A), which also seems to be associated with Ser1177-dephosphorylation (34). It could be speculated that there is a mechanism that activates PP2A in type 2 diabetes and leads to common dephosphorylation of Ser1177 and Thr495. Hojlund and colleagues could provide evidence that in insulin-resistant states, i.e., type 2 diabetes downregulation of PP2A-Cα is impaired (19). However, not much is known about the consequence for Ser1177- and Thr495-phosphorylation at this point.

The increase of Ser635-phosphorylation in the diabetic group also supports the idea of a compensatory augmentation of eNOS activity. The kinase responsible for Ser635-phosphorylation is PKA and interestingly not Akt as for Ser1177-phosphorylation. As a result phosphorylation of Ser635 should be independent of insulin resistance, and increased activity may be attributed to an increased neurohumoral stimulation that has been described in diabetic patients.

Ser114-phosphorylation was similar in both groups. The latest studies associate Ser114-phosphorylation with inhibition of eNOS activity (25). However, at this point there is no evidence that this phosphorylation site is affected by the metabolic changes in type 2 diabetes mellitus.

**Type 2 Diabetes and Myocardial Oxidative Stress**

Hyperglycemia and diabetes have been associated with an increased mitochondrial generation of oxidative stress and reactive oxygen species (ROS) (8) as well as increased superoxide generation by eNOS (6) in bovine aortic endothelial cells, which potentially leads to further eNOS uncoupling by depleting the storages of BH4, the essential cofactor for eNOS (31, 46, 49). We could not confirm this finding for diabetic myocardial cells with our immunohistochemical markers 8-isoprostan e and nitrotyrosine.

Paxinou et al. (36) suggest protective effects of NO against oxidative stress by regulating mitochondrial respiration. In neuronal cells NO protects against oxidative stress by stimulation of superoxide dismutase (16). If this idea is transferrable to cardiomyocytes, this might be proof of a well compensated NO production, e.g., by Thr495-dephosphorylation, and should be further investigated by quantitative measurements of NO.

An alternative role for dephosphorylation of Thr495 has been suggested by Lin et al. (26), who propose it leads to eNOS uncoupling and consequently increased oxidative stress. We saw no increase of 8-isoprostan e or nitrotyrosine in the type 2 diabetic group and thus no evidence for eNOS uncoupling or increased oxidative stress and O2 availability to form peroxynitrite with NO and as a result nitrosative stress (35).
This finding that oxidative stress was unchanged and nitrosative stress was decreased in cardiac tissue of the diabetic patients compared with the control group is unexpected but is in line with previous results of our group in erythrocytes of type 2-diabetic patients (32). It has to be taken into account that tissue samples were obtained under sedentary and fasting conditions, i.e., without any additional glucose nutrition or physical exercise. From the present study performed in people with non-insulin-dependent diabetes, it cannot be excluded that oxidative stress may increase during the further progression of the disease. In addition, the oxidative stress situation may vary in different tissues. So oxidative stress may be increased in the blood due to the autoxidation of glucose but decreased in skeletal muscle due to the reduced glucose uptake.

**Angiotensin and eNOS Activation in Type 2 Diabetes**

eNOS can be activated by numerous humoral or physical stimuli. One mechanism is the receptor-mediated activation and Ser1177 phosphorylation by ANG II, which we subsequently looked at. The underlying mechanisms by which ANG II-dependent Ser1177-phosphorylation is conducted have not been elucidated yet. Also it has not yet been determined if one or both of the AT1- or AT2-receptors are responsible for the signal transduction. Evidence currently points toward involvement of both receptors.

Our results confirm studies by Suzuki et al. and Yayama et al. who were able to demonstrate that eNOS may be activated by ANG II via the AT1- (44) and AT2-receptor (50) and that activation takes place via Ser1177-phosphorylation.

Furthermore, we could show that eNOS can be equally activated by ANG II stimulation in both diabetic myocardium as well as myocardium of nondiabetic individuals. Thus it can be deduced that eNOS can still be recruited for activation in diabetic myocardium despite decreased Ser1177-phosphorylation under basal conditions.

In both groups maximum activation was seen after 2 min of stimulation with ANG II. However, Ser1177-phosphorylation does not seem to be conducted via Akt, as no significant parallel increase of P-Akt could be seen in both groups.

This finding is consistent with Saito et al. (42), who showed that Akt is not involved in ANG II-mediated Ser1177-phosphorylation and Suzuki et al. (44), who suggest that the protein kinase A seems to be the kinase involved. Furthermore, Saad et al. (41) and Folli et al. (13) propose that ANG II even inhibits the PI3K/Akt pathway via the AT1-receptor.

At this point, it cannot be determined whether the AT1- or AT2-receptor is responsible, because both receptors have been linked to eNOS activation, and too little is known about the signal transduction ways involved. However, so far none of the receptors has been implicated to be dependent on the PI3K/Akt pathway, so neither can be excluded from involvement in Ser1177-phosphorylation in our studies at this point. Therefore tests with selective blockage of each receptor subtype could be a next step. Additional tests could also investigate ANG II-dependent phosphorylation of Ser635 and activation of protein kinase A.

The possibility that our finding of increased Ser1177 phosphorylation could only be indirectly associated with ANG II stimulation and merely a by-product of crossed signaling pathways in the cell should also be considered. To our knowledge at present not much is known about eNOS activation through ANG II in human cardiomyocytes, and the exact signaling pathways have also not been elucidated yet.

To sum up, the present study indicates that type 2 diabetes goes along with a decrease in eNOS phosphorylation at Ser1177 under basal conditions in cardiac tissue. This may be attributed to insulin resistance of cardiac muscle and consecutive impairment of the insulin-mediated Akt/PKB-dependent phosphorylation of eNOS at Ser1177. Further, receptor-stimu-
lated eNOS activation still works at least for ANG II-dependent eNOS-activation.

**Limitations and Perspectives of the Study**

The present study was performed in only a small number of patients. We cannot exclude that differences of the underlying medication or the clinical situation may influence the results. From the present study there is evidence that insulin may also affect eNOS activation in cardiomyocytes. This has to be investigated in further studies.

The cardioprotective effects of insulin, e.g., after reperfusion injury, have always been investigated by Western blot technique using tissue homogenates (14, 43). Thus a clear distinction between cardiac and endothelial reaction was not possible. In our opinion there is no difference between endothelial and cardiac eNOS signaling pathways. Therefore, we suggest that the current schemes of insulin signaling should be changed that the insulin-dependent PI3K/AKT pathway activates eNOS and Glut4-transporters in both, endothelial cells and cardiomyocytes. The investigation of the mechanism of dysregulation of nitric oxide in the setting of decreased insulin sensitivity is beyond the scope of this study but should be addressed in the future.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

Author contributions: U.S. performed experiments; U.S. analyzed data; U.S. and K.B. edited and revised manuscript; U.S., W.B., M.B., T.W., R.H.S., and K.B. approved final version of manuscript; H.R., W.B., and K.B. conception and design of research.

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