The Effect of Wall Compliance on Bradykinin-induced eNOS Phosphorylation in Isolated Porcine Aortic Endothelial Cells

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17 February 2006

BMTE 06.11

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Abstract

This study evaluates the effect of wall compliance on BK-induced eNOS phosphorylation in isolated porcine aortic endothelial cells. An *in vitro* perfusion system is used in which stiff and compliant cell-seeded tubes are subjected to unidirectional shear stress and pulsatile flow. It is hypothesized that reduced wall compliance causes an impaired BK-mediated eNOS activation, resulting in an altered phosphorylation state of the enzyme. Phosphorylation of eNOS is studied at Ser^{1177} and Thr^{495}. Furthermore the phosphorylation of protein kinase B/ Akt is studied. The results suggest that a reduced wall compliance might induce changes in the BK activation pathway, resulting in a decreased $\text{P}\text{Ser}^{1177}$ and $\text{P}\text{Akt}$ expression.
1. Introduction

Arterial stiffness, caused by aging, hypertension, obesity, hypercholesterolemia, and diabetes, strongly increases the risk for target organ damage and cardiovascular morbidity and mortality [11, 12, 13]. Although the precise mechanism of this age or disease-related loss of cyclic stretch is still poorly understood, there is a growing consensus that a reduced function of the vascular endothelium plays a critical role in this process [9, 11, 13].

Vascular endothelial cells mediate the vascular tone by the release of endothelial factors, such as nitric oxide (NO) [3, 4, 9]. NO plays a critical role in the maintenance of normal vascular function by regulating important vascular mechanisms such as vasorelaxation, platelet aggregation, monocyte adhesion, and smooth muscle cell proliferation and migration [3, 5, 6]. The major source of NO in the vascular endothelium is endothelial nitric oxide synthase (eNOS). When active, this enzyme triggers the reaction in which L-arginine is transformed into L-citruline and NO•. An altered activation state of eNOS thus changes the production and/or bioavailability of NO and is therefore believed to be one of the most important effectors of endothelial dysfunction [2, 3, 6 14].

It is established that NO production is stimulated by mechanical forces such as shear stress and cyclic strain [3, 7, 9, 11]. In vitro experiments have shown that normal haemodynamic conditions, such as unidirectional flow and high shear stress, promote the activation of eNOS, while abnormal conditions, such as oscillatory flow and low shear stress, lead to a decreased eNOS activity [2, 5, 6, 7, 13]. Other experiments have shown that a reduced cyclic stretch results in a decreased eNOS activity [9,11]. Activation of eNOS is regulated by many mechanisms of which phosphorylation is believed to be critical [3, 10]. Specific sites for phosphorylation of eNOS and specific protein kinases that mediate the phosphorylation have been identified. It is known that the phosphorylation of Serine at position 1177 (Ser1177) and dephosphorylation of Threonine at position 495 (Thr495) may result in activation of eNOS [2, 6, 8, 9 11]. In addition, phosphorylation of Ser1177 is induced by the activation/phosphorylation of protein kinase B/ Akt [3, 7, 10, 13].

The aim of this study is to determine the activation state of eNOS under reduced cyclic stretch conditions. For this purpose, an in vitro perfusion system is used, in which compliant and stiff tubes, seeded with porcine endothelial cells, are subjected to unidirectional shear stress and pulsatile flow. Production of NO is stimulated by treating the cells with Bradykinin (BK), a humoral stimulus that is known to induce NO production [1, 3, 8]. Activation of eNOS is studied by determining the phosphorylation state of the enzyme. It is hypothesized that reduced wall compliance causes impaired BK-mediated eNOS activation, resulting in an altered phosphorylation state of the enzyme.
2. Materials and Methods

2.1 Groups and Sampling

ENOS activity was analysed for two test groups: stiff tubes (silicone, 60 shore A), and compliant tubes (silicone, 40 shore A). At pulse pressure of 100 ± 20 mmHg, compliant tubes showed 4-5% cyclic stretch; cyclic stretch of stiff tubes under these conditions was less than 1%. Compliant tubes had a stretch similar to physiological conditions and served as control. Every group comprised four tubes: two flow and two static. From both flow and static, one tube was treated with BK to induce activation of eNOS, the others were kept as control.

2.2 Cell Culture and Seeding

Porcine aortic endothelium cells (PECs, passage 2-8) were cultured in Dulbecco’s Modified Eagle Medium (DMEM, Gibco) supplemented with 5% fetal calf serum (FCS, Amined), 2.4% HEPES (Gibco), 1% penicillin/streptomycin (Gibco), and 0.5% gentamycin (Gibco). Cells were trypsinized and seeded on the inside of cylindrical silicone tubes (inner diameter 6 mm, length 7.5 cm, Statice Sante). To stimulate the generation of a confluent monolayer, tubes were gently rotated at 2-3 rpm over night. Prior to the seeding, tubes were coated with 150 µL human serum fibronectin (1 µg/mL, Chemical International) in 7 mL culture medium without FCS. Before and after exposure to flow, tubes were observed using light microscopy to confirm the formation/maintenance of a confluent monolayer.

2.3 In Vitro Perfusion System

The in vitro perfusion system, represented in figure 2.1, is described in more detail previously [6, 15]. Briefly, the system consists of two cell-seeded tubes, a pump (Ismatec SA), a function generator (Stanford Research Centres), and a reservoir. Tubes were mounted in specially designed fittings. The medium in the reservoir consisted of Dulbecco’s Modified Eagle Medium special (DMEM special, Amined) containing 2% Dextran, to increase the viscosity up to blood viscosity levels. It was supplemented with 1% penicillin/streptomycin (Gibco) and 0.5% gentamycin (Gibco). The medium was constantly gassed with air (0.03% CO2) to keep the pH at 7.2. Both the reservoir and the tubes were placed in a water bad kept at 37°C. Cells were exposed to unidirectional pulsatile flow (sinusoidal, 1 Hz) for 24 h. Shear stress was 0.6 ± 0.3 Pa, pulse pressure 100 ± 20 mmHg.
2.4 ENOS Activation and Protein Extraction

After perfusion, tubes were washed twice with PBS (37 °C, Gibco) and exposed to $10^{-6}$ mol/L BK (Fluka) for 1 minute to induce BK-mediated eNOS activation. To block the BK, tubes were washed with ice-cold PBS immediately after stimulation. Optimal exposure time and concentration of BK were determined previously on static cultured PECs. After BK treatment, proteins were extracted from the tubes using RIPA lysis buffer (1 % Nonidet P-40, 1 % sodium deoxycholate, 0.1 % SDS, 0.15 M sodium chloride, 0.01 sodium phosphate pH 7.2, 2 mM EDTA, 0.2 mM sodium vanadate, 50 mM sodium fluoride, and 1 protease inhibitor cocktail tablet) by standard protocol [6]. The amount of protein was quantified using Bradford assay (Biorad).

2.5 Western Blot

Western blot analyses were performed by standard protocol [6]. Proteins (15 µg/ lane) were separated using a 7 % SDS-PAGE gel and transferred onto a nitrocellulose membrane. Membranes were probed with first antibodies to $^{\text{P}}$Ser$^{1177}$ (rabbit, Cell Signalling), $^{\text{P}}$Thr$^{495}$ (rabbit, Cell Signalling), and $^{\text{P}}$Akt (serine 473, rabbit, Cell Signalling) at a dilution factor of 1:500 and incubated at 4 °C overnight. Second antibodies were species specific and incubated at a dilution factor of 1:1000 at room temperature for 1 h. Signals were visualised by ECL treatment (Eurolone), and intensities were measured using a Kodak Image Station (2000 R). Membranes were stripped and probed for total eNOS (mouse, Cell Signalling) or Akt (rabbit, Cell Signalling), to normalize the $^{\text{P}}$eNOS and $^{\text{P}}$Akt protein signals on the total eNOS and Akt signals.
2.6 Determination of L-Citruline

Activation of eNOS can be determined indirectly using a NOS assay kit (Calbiochem). With this kit it is possible to calculate the production of NO\(\bullet\) by measuring the amount of citruline formed by the endothelium cells. Cells were treated with BK and proteins were extracted using homogenisation buffer. Cellular extracts were incubated with radioactive labelled arginine. After incubation, the reaction was stopped by inactivation the eNOS with a buffer containing EDTA. Equilibrated resin, binding to arginine, was added to the samples and the activity of eNOS was determined by quantifying the radioactivity in the eluate using a liquid scintillation counter (Hidex). All solutions and protocols were included in the manufacturer’s kit. It appeared that the measured amount of radioactive citruline was not related to the amount of protein present in the samples. Therefore, the NOS assay kit is considered not specific enough for the determination eNOS activity is this study.

2.7 Data Analysis

Data are shown as mean ± standard deviation. When possible a paired t-test with a 95 % confidence interval was performed. Probability values were considered significant when p ≤ 0.05.
3. Results

3.1 $^{\text{PSer}}$1177 Expression

To investigate the hypothesized change in the activation state of eNOS under reduced cyclic stretch, the expression of Ser$^{1177}$ phosphorylation is determined. Figure 3.1 shows the results of the Western blot analysis for $^{\text{PSer}}$1177 on PECs in compliant and stiff tubes after 24 h of perfusion, and in static tubes. The ratio of $^{\text{PSer}}$1177 on total eNOS protein levels is represented in figure 3.2. BK treated static and compliant tubes showed a significant higher $^{\text{PSer}}$1177 level than untreated tubes, which indicates that BK treatment indeed stimulates cells to produce NO. BK treated compliant tubes showed a higher $^{\text{PSer}}$ level than BK treated static tubes (2.38 ± 0.51 respectively 1.16 ± 0.13). However this difference is not significant. The stiff tube showed no difference in $^{\text{PSer}}$ expression between BK treated and untreated samples. In addition to this, the level of $^{\text{PSer}}$ in the BK treated stiff tube seems to be slightly lower than the $^{\text{PSer}}$ level in BK treated static tubes (1.17 respectively 1.46 ± 0.13).

Figure 3.1: Western blot showing phosphorylation of Ser$^{1177}$, and total eNOS protein levels after 24 h of perfusion. BK-mediated eNOS activation is marked with ‘+”; untreated samples are marked with ‘-‘.

Figure 3.2: Quantification of the ratio of $^{\text{PSer}}$1177 on total eNOS protein levels after 24 h of perfusion. Data are expressed as mean ± SD, *: p < 0.05.
3.2 \( ^p\)Akt Expression

Phosphorylation of \( ^p\)Ser\(^{1177}\) is induced by the activation/phosphorylation of Akt [3, 7, 10, 13]. Therefore, \( ^p\)Akt (at serine 473) levels are studied and expected to change with the compliance of the tubes. The Western blot analysis is represented in figure 3.3. Figure 3.4 shows the quantification of the ratio of \( ^p\)Akt on total Akt protein levels. BK treated static and compliant tubes (1.15 ± 0.28 respectively 1.05 ± 0.45) showed a higher level of \( ^p\)Akt than untreated tubes (1.00 ± 0.28 respectively 0.45 ± 0.24). However, this difference is not significant. BK treated static and compliant tubes showed no differences in \( ^p\)Akt expression. \( ^p\)Akt levels in the BK treated stiff tube seemed to be lower than in the untreated tube (0.30 respectively 0.72). In addition to this, the BK treated stiff tube seemed to have a lower level of Akt phosphorylation than BK treated static and compliant tubes (0.30 respectively 1.15 ± 0.28 and 1.05 ± 0.45).

![Western blot showing phosphorylation of Akt (Ser\(^{473}\)), and total Akt protein levels after 24h of perfusion. BK-mediated eNOS activation is marked with ‘+’; untreated samples are marked with ‘-’.](image)

**Figure 3.3:** Western blot showing phosphorylation of Akt (Ser\(^{473}\)), and total Akt protein levels after 24h of perfusion. BK-mediated eNOS activation is marked with ‘+’; untreated samples are marked with ‘-’.

![Quantification of the ratio of \( ^p\)Akt (Ser\(^{473}\)) on total Akt protein levels after 24h of perfusion. Data are expressed as mean ± SD.](image)

**Figure 3.4:** Quantification of the ratio of \( ^p\)Akt (Ser\(^{473}\)) on total Akt protein levels after 24h of perfusion. Data are expressed as mean ± SD.

3.3 \( ^p\)Thr Expression

Dephosphorylation of eNOS induces activation of eNOS and is therefore expected to change with decreasing compliance. \( ^p\)Thr levels were undetectable in all BK treated and untreated static, compliant, and stiff tubes (results not shown).
Discussion

Results have shown a non-significant increase in Ser\textsubscript{1177} phosphorylation in compliant tubes. This may indicate that shear stress and pulsatile flow result in an activation of eNOS. In addition to this, \(\text{P} \text{Thr}\) levels were undetectable in compliant tubes, which also suggests that eNOS is activated. The expression of \(\text{P} \text{Akt}\) in compliant tubes remained constant, which may suggest that sub maximal levels of \(\text{P} \text{Akt}\) can cause a maximum phosphorylation of eNOS at Ser\textsubscript{1177}. Conversely, the stiff tube seemed to show a slight decrease in both Ser\textsubscript{1177} and Akt phosphorylation, which might suggest that reduced wall compliance results in an inactivation of eNOS. \(\text{P} \text{Thr}\) expression was undetectable in stiff tubes, which may indicate that the inactivation of eNOS is not strong enough to cause phosphorylation of Thr. Altogether, these results might indicate that cyclic stretch plays an important role in the regulation of eNOS and may contribute to a better understanding of the role of endothelium in the mechanisms underlying arterial stiffness.

Earlier studies have already demonstrated that reduced wall compliance results in a decrease in Ser\textsubscript{1177} and Akt phosphorylation [9, 11]. However, in these studies cell seeded-tubes were perfused for 4 respectively 2 h, which made it possible to study only transient changes in the activation state of eNOS. In the present study, tubes were subjected to flow for 24 h in order to study the long-term changes in the activation state of eNOS.

Experiments were performed on isolated endothelial cells to have enough protein to detect BK-induced eNOS phosphorylation. Consequently, important cell-cell and cell-matrix interactions may be absent. This might have an effect on the response of endothelial cells to mechanical and haemodynamic stimuli, especially at long term.

Endothelial cells were subjected to a shear stress of 0.6 ± 0.3 Pa, which is significantly lower than physiological shear stress levels (approximately 1.5 Pa). Due to technical restrictions of the perfusion system, it was not possible to increase shear stress values more, which might have had an effect on the activation of eNOS. However, earlier research on \textit{ex vivo} perfused porcine carotid arteries demonstrated that a shear stress of 0.6 ± 0.3 Pa was sufficient to cause an increased eNOS activation [6].
5. Conclusion

The aim of this study was to evaluate the BK induced activation state of eNOS in compliant and stiff cell-seeded tubes under unidirectional shear stress and pulsatile flow conditions. The activation state was studied by determining the phosphorylation of eNOS at Ser\textsuperscript{1177} and Thr\textsuperscript{495}, and the phosphorylation of protein kinase B/Akt. Compliant tubes showed an increased phosphorylation of Ser\textsuperscript{1177}. This may indicate that high shear stress in normal wall compliance results in an increased eNOS activity. Thr\textsuperscript{495} levels were undetectable, which confirms the active state of eNOS in compliant tubes. Phosphorylation of Akt was unchanged in compliant tubes, which suggest that a submaximal level of Akt can induce a maximum phosphorylation of Ser\textsuperscript{1177}. The stiff tube seemed to show a decreased level of Ser\textsuperscript{1177} and Akt, which might suggest that a reduced wall compliance results in a decreased eNOS activation. Phosphorylation of Thr\textsuperscript{495} could not be detected, which may indicate that the deactivation of eNOS in the stiff tube is not strong enough to result in phosphorylation of Thr\textsuperscript{495}.

Due to time restrictions it was not possible to perform all experiments in triplets and no significant differences could be demonstrated between groups. Therefore, it is of primary interest to investigate further the phosphorylation state of eNOS at Ser\textsuperscript{1177} and Thr\textsuperscript{495}, and of Akt under reduced wall compliance. Future work is also necessary to elucidate the precise mechanisms responsible for the suggested loss of eNOS and Akt phosphorylation under reduced cyclic stretch conditions.
References


