Original Article

Contrasting Effects of Angiotensin Type 1 and 2 Receptors on Nitric Oxide Release under Pressure

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This study was designed to test the hypothesis that increased pressure itself could cause endothelial dysfunction and lead to decreased nitric oxide (NO) release, partly through effects on the tissue renin angiotensin system in hypertension. Cultured endothelial cells (ECs) isolated from the aortas of WKY rats were continuously exposed to a pressure of 150 mmHg in a CO₂ incubator for 72 h using a pressure system, and the NOₓ (NO₂ and NO₃) and angiotensin II (Ang II) concentrations in the supernatant were measured. An Ang II type 1 receptor (AT₁-R) antagonist (losartan) and an Ang II type 2 receptor (AT₂-R) antagonist (PD123319) were added to the medium. The expression of AT₁-R and AT₂-R mRNAs was also examined. Pressure loading significantly decreased the NO release from ECs. Concomitant administration of losartan restored NO release to the level before the application of pressure (p<0.001). This effect of losartan was blocked by simultaneous administration of PD123319, bradykinin type 2 receptor antagonist, and NO synthase inhibitor (p<0.05). The Ang II concentration was increased by pressure and was further increased by losartan. The gene expression of AT₁-R was not changed by pressure, but AT₂-R mRNA was increased almost 2-fold. These results indicate that high pressure itself attenuates NO release from ECs, and that losartan improves NO release by activating the bradykinin system via AT₂-R stimulation. In addition, the increase of AT₂-R gene expression in ECs during exposure to pressure may compensate for the reduction of NO.

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Key Words: nitric oxide, endothelium, pressure, receptors, angiotensin II

Introduction

NO is a well-known biological modulator with numerous antiatherogenic functions, including modulation of vascular tone and the suppression of vascular smooth muscle proliferation, platelet aggregation, and white blood cell adhesion (1). Several investigations have demonstrated that endothelium-dependent vascular relaxation (EDR) of the forearm arteries and NO bioavailability are impaired in patients with hypertension (2). It has long been controversial whether endothelial dysfunction, such as a decrease of NO production, represents a cause or a result of hypertension. However, EDR is decreased in patients with secondary hypertension (3), and antihypertensive therapy can improve EDR (4, 5); moreover, we have demonstrated that lowering pressure improves NO release from the ECs of hypertensive rats (6). Thus high blood pressure itself may promote endothelial dysfunction.

There are two very important modulators of endothelial NO release; one consists of direct hemodynamic changes and the other of humoral factors such as the renin-angiotensin system (RAS). Endothelial cells (ECs) lining the vessel lumen are exposed to various mechanical forces, including shear stress, stretch, and transmural pressure, which are produced by blood pressure and blood flow. These hemodynamic forces are known to elicit many important physio-
logical responses and to activate various signaling pathways in ECs. Wall shear stress and stretch are well known to increase NO release from ECs (7), but the other hemodynamic force, namely, the transmural pressure that acts vertically on vessel walls, has only been investigated by a few researchers (8, 9). In the present study, we examined the direct effect of pressure on NO release using cultured ECs, so that we could exclude other factors which might influence NO release.

Several investigators have shown that NO and angiotensin II (Ang II) are antagonistic regulators of blood pressure or vascular responses. The abnormal vascular remodeling induced by hypertension has been suggested to arise from an imbalance among Ang II, NO, and other substances (10). We previously reported that pharmacological blockade of Ang II production or activity could markedly reduce the development of hypertension in rats receiving long-term treatment with NO synthesis inhibitors (11). Several researchers have reported a relationship between mechanical overload and the Ang II level in blood vessels (12, 13). In addition, activation of the RAS, including an increase of tissue Ang II levels, is well known to occur in hypertensive cardiac hypertrophy (14) as well as in vascular wall hypertrophy when pressure is applied (12). Taken together, these findings suggest that pressure loading might attenuate NO release from ECs along with RAS activation. Therefore, we hypothesized that transmural pressure may influence not only endothelial NO release but also the RAS; i.e., pressure loading could activate the RAS and cause endothelial dysfunction. As far as we know, few studies have investigated the influence of transmural pressure on the interaction between endothelial NO release and the local RAS under pressure. Here we investigated how the application of pressure affects endothelial NO release and how it modifies the local RAS with respect to such activities as Ang II formation and Ang II receptor function.

Materials and Methods

Cell Culture
ECs were isolated from 6-week-old male Wistar Kyoto rats by the primary explant technique described previously (15). All rats were cared for in accordance with the rules and regulations governing animal research of Kyoto Prefectural University of Medicine and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 15% fetal bovine serum (FBS), EC growth supplement (100 µg/ml), and antibiotics (50 µg/ml ampicillin, 15 µg/ml gentamicin, 1 µg/ml minomycin, and 1 µg/ml amphotericin B). Rat tail collagen (type 1)-coated 35 mm tissue culture dishes (Biocoat Cell Ware; Becton Dickinson Labware, Bedford, USA) were used for the EC culture, and cells from passages 2 and 3 were used for the experiments. Endothelial cells were characterized by their cobblestone morphology and staining with a specific antibody to von Willebrand factor (rabbit IgG; Sigma, St. Louis, USA). Purity of culture was verified by the lack of staining with a monoclonal antibody for smooth muscle alpha actin (mouse IgG; Dako Japan Co., Ltd., Kyoto, Japan) to exclude smooth muscle cell contamination. After reaching 90% confluence, ECs were incubated in conditioned medium (DMEM containing 1.0% bovine serum albumin [BSA] and antibiotics) for 24 h before the experiments to induce quiescence, and then the conditioned medium was exchanged for new medium at the start of the experiments.

Pressure System
Figure 1 shows a schema of the pressure system. The pressure apparatus had two main parts, a cylindrical pressure chamber made of acryl glass and a pump with a pressure sensor. The chamber was placed in a CO2 incubator and the pump remained outside. Two vinyl tubes were connected to the pump, with one tube running into the chamber and the other leading to the incubator through a hole in the wall. Five percent CO2 gas from the incubator was aspirated into the other leading to the incubator through a hole in the wall. Five percent CO2 gas from the incubator was aspirated into the pump and then pumped into the pressure chamber to raise the internal pressure to 150 mmHg. Several culture dishes were placed inside the chamber, which was then closed and placed in the incubator. The chamber had a small screw valve that continuously discharged a small amount of gas to keep the internal gas fresh. In the bottom of the chamber, a small pool of water was maintained to keep the humidity constant. Pressure of 150 mmHg was applied for 6 to 96 h. Other dishes were incubated for the same periods without pressure loading.

Cell Counting
To examine the effect of pressure on cell growth, the number of ECs in each 35 mm culture dish was counted. Dishes were removed from the incubator at 6, 12, 24, 48, 72, and 96 h after the start of pressure application. ECs were freed from the plates using trypsin-EDTA in PBS and were collected by centrifugation at 150 x g for 5 min. Then the number of cells was counted by hemocytometry. Cell viability was con-
firmed by the absence of trypan blue staining.

Detection of Apoptosis
After 72 h, ECs (4 × 10^5/well) were trypsinized, digested in lysis buffer (10 mmol/l Tris-HCl, pH 7.4; 10 mmol EDTA; 0.5% Triton X-100; and 40 µg RNase) for 1 h at 37°C, and treated with 100 µg proteinase K for 1 h at 37°C. The chromosomal DNA was analyzed by agarose gel electrophoresis (1.5%), followed by staining with ethidium bromide.

Dissolved Oxygen
The dissolved oxygen concentration in the culture medium was measured by polarography using a Clark oxygen electrode. An aliquot of medium (1.1 ml) was added to the cuvette of the device (Oxigraph type 9; Central Science Co., Tokyo, Japan), which was covered with a cap to protect it from the air. The medium was stirred during measurement.

Treatment with Drugs
Losartan was kindly provided by Merck and Co. Inc. (Rahway, USA). The effects of the following drugs on NO release from EC were examined: losartan (10^-5 mol/l), an Ang II type 1 receptor (AT1R) antagonist; PD123319 (10^-6 mol/l; Research Biochemicals-Sigma, Milan, Italy), an Ang II type 2 receptor (AT2R) antagonist; HOE140 (10^-5 mol/l; Peptide Institute, Osaka, Japan), a bradykinin (BK) type 2 receptor (BK2R) antagonist; and N^G -monomethyl- L-arginine (L-NAME; 5 mol/l; Nacalai Tesque, Kyoto, Japan), an eNOS inhibitor. These drugs were added just before the culture dishes were placed in the pressure chamber.

Measurement of Total Nitrates and Nitrites (NOx; NO-199µ/l)
At 72 h from the start of the experiment, 200 µl of culture medium was harvested and stored at -20°C until measurement of NOx. The concentration of total nitrates and nitrites (NOx) in the medium was determined by the Griess method with an NO autoanalyzer HPLC system (ENO-10; Eicom Co., Tokyo, Japan); absorbence at 540 nm was measured.

Radioimmunoassay (RIA) of Ang II
Similarly as for NOx measurement, 600 µl of culture medium was harvested and stored at -20°C until the Ang II RIA was performed. All samples were adsorbed with Florisil and eluted with 1 ml of ethanol, after which the ethanol was evaporated. The sample was then incubated with angiotensin II antibody in buffer overnight at 4°C, after which [125I]Ang II was added and incubation was done for 4 h at 4°C. Separation was subsequently performed using PEG.

Reverse Transcription (RT) and Polymerase Chain Reaction (PCR)
At 72 h from the start of the experiment, total RNA was prepared from ECs by the acid guanidinium-phenol-chloroform method, as described previously (16). In order to examine whether levels of the cDNA fragments of AT1R and AT2R mRNA reached saturation under our RT-PCR conditions, we amplified them at different cycles using two different concentrations of mRNA samples from control cells. The densitometric intensity of the bands was measured by using NIH imaging software. RT was performed in a 20-µl reaction mixture containing 1 µg of RNA using the SuperScript Pre-amplification System for First Strand cDNA Synthesis (Gibco-BRL, Gaithersburg, USA) according to the manufacturer's protocol. One microliter of the cDNA was used to amplify rat AT1R and AT2R mRNAs. The gene expression level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also evaluated to prove that an equal amount of cDNA was charged for PCR (17). The sequences of these primers were as follows: AT1R, 5’ GTAGCCAAAGTCACCTGCAT -3’ (sense) and 3’ TATCGAATAAAATTGTTAACCCTGACT -5’ (antisense); AT2R, 5’ AACTGCTATGAGTGTTGATTAGG -3’ (sense) and 3’ ACTTCAATATCGTCAGTAACTGGAC -5’ (antisense); and glyceraldehyde phosphate dehydrogenase (GAPDH), 5’ ATGTGACATCAAGAGGCTG-3’ (sense) and 3’ ATGTGCTGTGTGCCACCCAC-5’ (antisense). The PCR consisted of 35 cycles of denaturation at 95°C for 30 s, annealing at 57°C for 30 s, and polymerization at 72°C for 30 s. PCR products were run on 1.5% agarose gel for 1 h at 9V/cm and were visualized by transillumination with UV light. Densitometry was used for semiquantitative analysis.

Data Presentation and Analysis
Data are expressed as the mean ± SEM. Differences among the groups were tested by 1-way ANOVA combined with Scheffe’s post hoc test, and p < 0.05 were considered to indicate statistical significance.

Results

Dissolved Oxygen Concentration
Application of pressure at 150 mmHg had no significant effect on the dissolved oxygen in the culture medium. The dissolved oxygen level in the medium was 19.45 ± 0.87% in the control cultures and 19.85 ± 0.16% in the pressure-treated cultures (n = 6 each).

Cell Numbers and Apoptosis
There were no differences of cell numbers between cultures with or without pressure at 6, 12, 24, 48, 72, and 96 h from the beginning of application. Cell numbers increased about two-fold by 72 h and decreased slightly thereafter (Fig. 2A).

Effect of Pressure on NOx Release
NOx release was significantly decreased when ECs were cultured under a pressure of 150 mmHg for 72 h when compared with NOx release by cells without pressure (18.61 ± 1.26 vs. 27.87 ± 1.99 pmol/10 µl for cells cultured with and without pressure, respectively; p < 0.001; n = 19; Fig. 3).
Effect of Blockade of AT1R on NO Release under Pressure

Figure 3 shows that a blockade of AT1R inhibited the decrease in NO release elicited by pressure. In the absence of pressure, NO release was almost identical between the vehicle and losartan treatments (28.76 ± 1.38 and 27.87 ± 2.07 pmol/10 μl, respectively; n = 12–15). After application of pressure, NO release was decreased in the vehicle treatment but not in the losartan treatment culture (19.39 ± 1.04 and 27.02 ± 2.15, respectively; p < 0.001 vs. vehicle; n = 10–18).

Ang II Concentration
Pressure increased the Ang II concentration in the medium, but the change was not significant (from 9.42 ± 1.13 to 12.01 ± 1.23 pg/ml; p = 0.133). Adding losartan to the culture medium significantly increased the Ang II concentration when ECs were cultured under pressure (14.20 ± 1.83 pg/ml; p < 0.05 vs. control; n = 12; Fig. 4).

Role of AT1R and AT2R in NO Release under Pressure
To investigate the role of the AT1R and AT2R in NO release from ECs cultured under pressure, losartan and PD123319 were added to the medium individually or simultaneously. During exposure to pressure, NO release was further decreased by PD123319 (11.89 ± 2.15 pmol/10 μl; p = 0.01 vs. vehicle under pressure; n = 10), but was restored to the level before pressure exposure by losartan (27.02 ± 2.15
Losartan restored NO\textsubscript{x} release to the level before the application of pressure, while this effect was completely blocked by simultaneous administration of HOE140 and L-NAME (18.63 ± 1.09, 18.53 ± 2.64 pmol/10 µl, respectively; \(p < 0.05\) vs. losartan; \(n = 8\); Fig. 5).

**Gene Expression of AT\textsubscript{1}R, AT\textsubscript{2}R, and GAPDH**

Figure 6A demonstrates that, under the present RT-PCR condition of 35 cycles, the expression levels of AT\textsubscript{1}R and AT\textsubscript{2}R genes could be semiquantitatively assessed. We also confirmed that there was no detectable contamination of genomic DNA in our RNA samples by performing PCR directly using RNA samples as a target (data not shown). Figure 6B shows the mean ± SEM amounts of AT\textsubscript{1}R and AT\textsubscript{2}R mRNA, expressed as a ratio to GAPDH mRNA. AT\textsubscript{1}R mRNA expression in ECs cultured without pressure.

**Role of the AT\textsubscript{1}R and BK system in NO\textsubscript{x} Release Stimulated by Losartan**

Losartan restored NO\textsubscript{x} release to the level before the application of pressure, while this effect was completely blocked by simultaneous administration of HOE140 and L-NAME (18.63 ± 1.09, 18.53 ± 2.64 pmol/10 µl, respectively; \(p < 0.05\) vs. losartan; \(n = 8\); Fig. 5).
showed similar expression by ECs irrespective of pressure (0.086 □ 0.002 vs. 0.083 □ 0.005 with and without pressure, respectively). In contrast, AT-R mRNA was significantly increased by exposure to pressure (0.031 □ 0.005 vs. 0.101 □ 0.016 with and without pressure, respectively; p = 0.01). Representative results from three independent experiments are shown.

Discussion

The present study suggested that an increase of transmural pressure may decrease NO release from ECs via the RAS and that blocking of the AT-R can reverse this decrease via the AT-R. Since endothelial function is impaired in hypertensive patients (2) and recovers during antihypertensive therapy (3–5), and since the RAS is activated in impaired vessels (12, 14), we hypothesized that pressure itself may be harmful to ECs and have an adverse effect on endothelial NO release via the RAS.

The endothelium is the interface between the circulating blood and the vascular wall, so ECs are continuously exposed to mechanical forces such as shear stress, cyclic stretch, and transmural pressure. There is increasing evidence that both shear stress and cyclic stretch modulate EC gene expression, structure, and function (7, 18); however, very little is known about the influence of transmural pressure. In the hypertensive state, intravascular pressure is markedly increased and the transmural pressure on ECs might be very high. We therefore considered it important to assess the effects of transmural pressure on ECs.

In this study, we devised an apparatus to apply pressure to cultured ECs. Some investigators have previously applied pressure by pumping air into a container (19, 20). In the present study, in order to minimize the differences in conditions between pressure and control cultures, we placed the pressure chamber in a CO2 incubator and used 5% CO2 gas to raise the pressure via an external pump while keeping the gas fresh and humidified. There was no difference in the dissolved oxygen level of the medium between EC cultures with and without pressure. Since cell numbers reached a maximum at 72 h and decreased thereafter, we adopted 72 h as the period for pressure application. There were no differences in cell count between cultures with and without pressure.

The first finding of the present study was that NO release decreased during the application of pressure. Hishikawa has previously reported that the application of pressure to cultured ECs for a shorter period could decrease NO release (9), and Bolz has also reported that NO-dependent vasodilation was decreased by increasing the intravascular pressure using an arterial perfusion system (8). These results are consistent with our hypothesis that pressure itself can be harmful to endothelial function.

The second finding was that the decrease of NO release under pressure was reversed by blocking the AT-R. When cells were cultured in the absence of pressure, application of an AT-R antagonist had no effect on NO release, suggesting that the RAS was involved in the decrease of NO release by pressure. Several lines of evidence have suggested that both the circulating RAS and the local RAS are involved in the regulation of vascular remodeling, especially in the case of cardiac hypertrophy as a response to pressure overload (12, 21). Tissue RAS has been reported to exist in ECs (22). Stall et al. demonstrated that rat coronary endothelial cells express both AT-R and AT-R (23), and Xiao et al. revealed both the expression of renin and formation of Ang II in primary culture ECs (24). To investigate whether Ang II production by ECs was altered by the application of pressure, Ang II levels were determined in the culture medium before and after exposure to pressure. It was found that the Ang II concentration increased after the application of pressure, and was significantly increased by the addition of losartan. Similarly, Bardy et al. reported that the Ang II concentration of the culture medium was increased by the application of transmural pressure to isolated vessels (13). Although relationships between apoptosis and a decrease of NO (25), and between apoptosis and AT-R stimulation (26) have been reported, no evidence of apoptosis (DNA laddering) was seen in our experimental system.

The Ang II receptor has two major subtypes, the AT-R and AT-R, and these are both expressed in ECs (22, 23). The AT-R modulates various well-known cardiovascular and renal processes, such as vasoconstriction, cell proliferation, aldosterone secretion, catecholamine secretion, and cardiac hypertrophy (27). On the other hand, the physiological role of the AT-R has just begun to be elucidated, but it has been suggested to include inhibition of cell proliferation (23), a hypotensive effect (28), and induction of apoptosis (25). AT-R antagonists can markedly increase the blood level of Ang II, and the increased Ang II appears to stimulate the AT-R (29, 30). Accordingly, to investigate the role of the RAS in regulating NO release from ECs under pressure, we used AT-R and AT-R antagonists. In a preliminary experiment, these drugs did not have any effect on cell number under pressure. An AT-R antagonist (losartan) was found to markedly increase NO release from ECs under pressure, while an AT-R antagonist (PD123319) attenuated NO release under pressure. Thus there are two opposing mechanisms, a decrease of NO release via the AT-R and an increase of NO release via the AT-R. The application of pressure apparently resulted in relative predominance of the former action, the decrease of NO release by an increase in stimulation of AT-R. When an AT-R antagonist and an AT-R antagonist were added to EC cultures simultaneously, NO release was intermediate between that in vehicle-treated cultures and that in losartan-treated cultures under pressure. This suggests that the decrease of NO release caused by AT-R stimulation during the application of pressure may have been partly reversed by AT-R stimulation.

It has been well established that BK exerts a protective effect against endothelial dysfunction (1, 31). Promotion of
NO release by the AT:R through the BK:R, which is also expressed in ECs, has been reported by many researchers (32, 33). Since kininogen and kinin are released from the endothelium (34), we added a BK:R antagonist (HOE-140) and an eNOS inhibitor (L-NAME) to EC cultures together with losartan in order to investigate the mechanism of NO release through the AT:R. Although administration of losartan alone restored NO release to the control level before the application of pressure, co-administration of HOE-140 and L-NAME with losartan abolished this restoration. These results suggest that NO release can be restored by AT:R antagonists under pressure because Ang II that is increased by both pressure and AT:R antagonist activates the BK system via the AT:R.

We also investigated the influence of pressure on Ang II receptor mRNA expression. When AT:R and AT:R mRNA expression were assessed by RT-PCR before and after the application of pressure, the AT:R mRNA was markedly increased while the AT:R mRNA showed no change. AT:R mRNA showed a low level of expression in the absence of pressure, but there was a marked increase after pressure was applied. Since it has been reported that the levels of AT:R and AT:R mRNA estimated by RT-PCR are well concurrent with the protein levels of these receptors determined by radioligand assay, it is likely that our results on the gene expression of the two receptors reflect their binding ability. In the future, however, it will be necessary to directly determine the Bmax values for each receptor expressed in ECs. If the AT:R promotes NO release by the above mechanism, it seems reasonable that AT:R mRNA expression would be increased in ECs by the application of pressure. It is thus likely that endothelial NO release is decreased by transmural pressure, and that a biofeedback mechanism is activated to restore NO levels. It has been reported that AT:R are strongly expressed in the endothelium of arteries, but not expressed, or only weakly expressed, in the aorta in humans (28). However, AT:R expression varies among species and also differs with culture conditions (22). AT:R expression is considered to be increased by cardiac failure and arteriosclerosis (27, 35), and to be increased in vascular lesions during remodeling (28). The increase of AT:R mRNA expression demonstrated in the present study might be compensation for endothelial dysfunction induced by pressure, which triggers vascular remodeling. As far as we know, this is the first report of an increase in AT:R mRNA expression by a physical stimulus such as pressure.

It is interesting that pressure seems to influence the RAS at the local tissue level in addition to causing impairment of endothelial function and a decrease in the bioavailability of NO. Pressure itself has already been suggested to be involved in hypertensive tissue damage and cardiovascular remodeling by influencing the local RAS (12, 13), but our findings provide the first evidence of a link between the activated local RAS and decreased endogenous NO release by ECs cultured under pressure.

There have already been several reports that ACE inhibitors and AT:R antagonists are more effective than other drugs for improving endothelial function and increasing NO release in patients with hypertension (36, 37). This suggests that high blood pressure plays a significant role in endothelial RAS activation, as was shown in the present study. The mechanism of the protective effect of AT:R antagonists seems to be separate from the hypotensive effect of these drugs, since our results were obtained in vitro. It seems that pressure activates the RAS and decreases NO release by ECs, so that endothelial dysfunction in hypertension is restored more effectively by inhibiting the local production and activity of Ang II than by other forms of antihypertensive therapy. However, our experiments did not provide any data about the fundamental mechanism through which NO release is decreased by pressure. As far as we know, there have been no previous reports about this mechanism. In our experimental system, NO release was decreased by the addition of PD123319 during culture of ECs under pressure, suggesting that pressure might inhibit NO release at least partly via the AT:R. On the other hand, several reports have shown that NO release from ECs increased via the AT:R (38). At present, we cannot explain the reason for this discrepancy, but differences in experimental conditions might be involved. Also, some mechanism not involving the RAS might contribute to the regulation of eNOS expression and activity when vascular ECs are exposed to pressure in vivo. Further studies will be needed to elucidate the precise mechanism.

In conclusion, transmural pressure causes a decrease of NO release from the vascular endothelium that is partly due to stimulation of the AT:R by an increase of Ang II in the vessel wall. The respective increase and decrease of Ang II and NO that are elicited by chronic pressure stress might trigger vascular remodeling in hypertension. We also showed that application of pressure caused compensatory activation of the AT:R-mediated pathway promoting NO release. Therefore, AT:R antagonists may offer the best protection of the vascular endothelium, since they not only block the AT:R but also stimulate the AT:R and this protection may be accomplished not only by blocking the AT:R but also by stimulating the AT:R.

References


