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Possibly of Angiotesin II-induced Brain Microvascular Endothelial Cell Injury through Oxidative Stress

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ABSTRACT

An increasing number of studies suggest that angiotensin (Ang) II contributes to the pathology of cerebrovascular disease possibly through injuring of the brain microvascular endothelial cells (BMEC), but the mechanisms involved are not well elucidated. Recent studies reveal the importance of Ang II in reactive oxygen species induction and endothelial injury. Hence, the present study investigated the involvement of oxidative stress in Ang II-induced BMEC injury.

Our results showed that 10^{-6} mol/L Ang II increased lactate dehydrogenase (LDH) leakage and inhibited the *via*bility of primary cultured BMEC. Incubation with 10^{-6} mol/L of Ang II for 48 hr increased the extracellular and intracellular malondialdehyde (MDA) content and decreased the activities of intracellular glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) significantly. These effects were abolished by pretreatment with the selective AT_1 receptor antagonist losartan, but not with the AT_2 selective antagonist PD123319. Combining Losartan with PD123319 neither diminished nor promoted the effect of losartan alone. These findings indicated that Ang II induced injury of BMEC may result from oxidative stress by activating AT_1 receptors. This injury may in turn lead to the development of cerebrovascular disease.

Key words: brain microvascular endothelial cell, angiotensin II, AT_1 receptor, losartan, oxidative stress

INTRODUCTION

The endothelium of the cerebral blood microvessels, which is the major component of the blood-brain barrier (BBB), responds dynamically to the flowing substances, free radical exposure and cytokine generation. Microvascular endothelial dysfunction in the brain is notably the cause of cerebral edema and ischemia injury[1].

Accumulating evidence suggest that angiotensin (Ang) II, the main effector of the renin-angiotensin system (RAS), participates in the initiation and regulation of processes that occur during brain ischemia[2]. Elevated levels of Ang II have been documented during brain ischemic injury[3]. Maeda *et al*. reported that the area of the core lesion after middle cerebral artery (MCA) occlusion was significantly reduced in angiotensinogen-knockout mice[4]. Our laboratory previously proved that Ang II-injured brain microvascular endothelial cells (BMEC) participate in the development of cerebrovascular disease[5]. However, the underlying mechanism of Ang II-induced cerebrovascular endothelial cell injury is not full understood.

Oxygen free radicals (OFR) are increasingly implicated as key mediators of cerebral injury. Antioxidant defenses such as free radical scavengers and antioxidant enzymes including superoxide dismutase (SOD), catalase, and glutathione peroxidase (GSH-Px), are considered a promising approach to limit the extent of damage that results from the pathogenesis of cerebrovascular diseases[6]. Griendling *et al*. demonstrated Ang II activated NAD(P)H oxidases in smooth muscle cells[7]. In addition, Ang II has been shown to stimulate the activity of membrane-bound NAD(P)H oxidase in endothelial cells[8,9]. Activated NAD(P)H oxidase by Ang II can produce superoxide anion ($\cdot O_2^-$) which probably causes BMEC injury. $\cdot O_2^-$ is converted into H_2O_2 by the enzyme SOD. Then, H_2O_2 can be converted into the highly reactive hydroxyl radical ($\cdot OH$) which can be detoxified by the enzyme catalase and GSH-Px, yielding water and oxygen. Therefore, the activities of antioxidant enzyme are the critical protective factor against BMEC injury. All the OFR can directly damage the BMEC membrane and cause lipid peroxidation. As a result, the formation of malondialdehyde (MDA), the main decomposition product of peroxides derived from polyunsaturated fatty acids, would be increased[10]. Therefore, we hypothesized that Ang II-induced BMEC injury possibly through decreasing the activities of SOD and GSH-Px, then causing serious oxidative stress in BMEC.

Ang II acts mainly *via* type 1 (AT_1) and type 2 (AT_2) receptors. However, the effect of the AT_2 receptor on Ang II-induced BMEC injury *in vitro* has not been explored in detail so far. Our second goal in this study was to identify the role of AT_1 and AT_2 receptor in enhancing the oxidative stress induced by Ang II.

MATERIALS AND METHODS

I. *Drugs and Reagents*

Ang II, PD123319, type II collagenase, trypsin, EDTA

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were from Sigma Chemical Co. DMEM, HEPES, and fetal bovine serum were obtained from Gibco Chemical Co. Losartan was purchased from Merk Co. Specify anti-factor VIII mouse monoclonal antibody was purchased from Santa Cruz Biotechnology.

II. *Cell Culture*

Wistar rats of 2 to 3 weeks old (from Center of Experimental Animal, Shandong University) were used in the experiment. Cerebral microvessels were isolated by Duan' method[11] with modification. Cerebral cortices cleaned of pia matters, large vessels and white matter were rinsed with 4°C D-Hank's solution and homogenized in 10 mL of DMEM by 20 up and down strokes in a glass homogenizer. The homogenate was filtered through a 145 μm pore nylon sieve and rinsed with DMEM. The filtrate containing microvessels were then filtered through a 75 μm pore nylon sieve rinsed with DMEM. The microvessel segments were then collected, suspended in 10 mL of DMEM, centrifuged at 150×g for 10 min, suspended in 10 mL of PBS containing 10 mg of collagenase (type II) and 10 mg of bovine serum albumin for 45 min at 37°C and then centrifuged at 150×g for 10 min. The supernatant was discarded and the collagenase-treated microvessels were collected. The feeding medium was DMEM supplemented with 20% fetal bovine serum (FBS), 1% L-glutamine, $10U \cdot mL^{-1}$ Heparin, $100U \cdot mL^{-1}$ penicillin, and $100\mu g \cdot L^{-1}$ streptomycin. The medium was adjusted to pH 7.4 using 2.5% $NaHCO_3$ solution and filter-sterilized using 0.22 μm millipore filter. Next collagenase-treated microvessels were seeded with 3 mL of feeding medium into gelatin-coated flasks at densities of 10-30 pieces/cm^2. Then the primary BMEC were grown at 37°C with 5% CO_2. After 5 days, the culture media were removed and fresh feeding medium was added. Thereafter, the preparation was fed every 3 to 4 days. In about 2 weeks, the cells appeared in a confluent state. Cells were characterized as BMEC by immunofluorescent staining with monoclonal antibody to factor VIII-related antigen.

III. *Experimental Design*

There are 5 groups in the experiments. In the Control group, the BMEC were incubated with normal serum-deprived DMEM (The cell *via*bility test was performed in serum medium.). In the Ang II group, the BMEC were treated with 10^{-6} mol/L Ang II for a specified time. In the losartan group (Los), PD 123319 group (PD) and losartan + PD 123319 group (Los + PD), the BMEC were pretreated with the specific AT_1 receptor antagonist losartan (10^{-5} mol/L) or the specific AT_2 receptor antagonist PD 123319 (10^{-5} mol/L) or both losartan (10^{-5} mol/L) and PD 123319 (10^{-5} mol/L) for 30 min. Afterwards the cells were exposed to Ang II to determine the specificity receptor effect of Ang II.

IV. *MTT Assay for Cell Viability*

The MTT assay was used to measure cell *via*bility. The principle of this assay is that the compound 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) undergoes cellular reduction by the mitochondrial dehydrogenase of *via*ble cells into a blue formazan that can be measured spectrophotometrically. Briefly, confluent BMEC monolayers cultured in 96-well plates were incubated with 10^{-6} mol/L Ang II in the presence and absence of 10^{-5} mol/L losartan or 10^{-5} mol/L PD123319 for the selected periods as indicated. After the medium was discarded, the cells were washed twice with PBS and the medium was appended to each well. MTT (0.5 mg/mL) was added to the medium. After an additional 4 hr of incubation, the medium was aspirated and the formazan crystals were dissolved in 200 μL of DMSO. Cell *via*bility was determined to OD values at 570 nm measured by an automatic plate reader.

V. *Measurement of Lactate Dehydrogenase Release*

The extent of BMEC damage was assessed by measuring the rate of lactate dehydrogenase (LDH) release as a marker for membrane disruption and cell death. After 48 hr, the medium and BMEC in each well were harvested respectively for the determination of LDH by LDH kits. Cells were removed by trypsinization, washed and resuspended in 0.1 M phosphate buffer (pH 7.4) to a final concentration of 10^7 cells/mL and then homogenized. The homogenates were centrifuged for 30 min at 3000×g at 4°C, and the supernatant was used.

The percentage of LDH release was deduced by the following formula: LDH release rate (%) = extracellular LDH contents/(extracellular LDH contents+intracellular LDH contents) ×100%.

VI. *Measurement of Lipid Peroxidation*

The medium and BMEC supernatant were prepared as described above. The content of protein in the supernatant was determined by Bradford protein assay kit. The resulting lipid peroxidation was evaluated by the formation of Malondialdehyde (MDA) --the main degradation product of peroxides derived from polyunsaturated fatty acids. The amount of MDA was determined using the chromogenic reagent N-methyl-2-phenylindole with maximum absorbance at 532 nm.

VII. *Measurement of Antioxidant Enzyme Activities*

The supernatant prepared as described above was used for determination of the antioxidant activity of the enzyme superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px). The SOD activity was measured according to the method previously reported by Flohe and Otting[12]. The reduction rate of cytochrome *c* by $\cdot O_2^-$ was monitored

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Table 1. Effect of angiotensin II receptor antagonists on the relative cell *via*bility and lactate dehydrogenase (LDH) release of rat cerebral microvascular endothelial cells injured by angiotensin II

Group	Relative cell *via*bility (%)	LDH release (%)
Control	100.00 ± 1.31	10.01 ± 2.50
10^{-6} mol·L^{-1} Ang II	71.50 ± 1.32[a]	27.53 ± 3.97[a]
10^{-6} mol·L^{-1} Ang II + 10^{-5} mol·L^{-1} Los	91.54 ± 2.72[b]	14.68 ± 4.82[b]
10^{-6} mol·L^{-1} Ang II +10^{-5} mol·L^{-1} PD123319	69.68 ± 2.65[c]	27.36 ± 4.66[c]
10^{-6} mol·L^{-1} Ang II + 10^{-5} mol·L^{-1} Los + 10^{-5} mol·L^{-1} PD123319	93.18 ± 2.40[b]	16.22 ± 3.92[b]

All values are mean ± S.D. (n = 6).
[a]$p < 0.05$ vs. control group.
[b]$p < 0.05$ vs. angiotensin II group.
[c]$p < 0.05$ vs. losartan group.

at 550 nm by using xantine–xantine oxidase system as the source of $\cdot O_2^-$. SOD competes for $\cdot O_2^-$ and decreases the reduction rate of cytochrome *c*. The GSH-Px activity was measured following the method described by Jaskot *et al*.[13]. The supernatant was mixed with 0.1 mol/L phosphate buffer, containing 1 nmol/L GSH, and incubated for 5 min at 30°C. Then, the reaction had been initiated by adding 10 μL of 7 mmol/L H_2O_2 for 15 min, and immediately the GSH absorbance was detected at 412 nm. One unit of GSH-Px was defined as the amount of protein that decreased the concentration of GSH by 1 μmol/L.

VIII. *Statistical Analysis*

Data were presented as mean ± S.D. Groups were compared by One-way ANOVA. Differences were considered significant at $p < 0.05$.

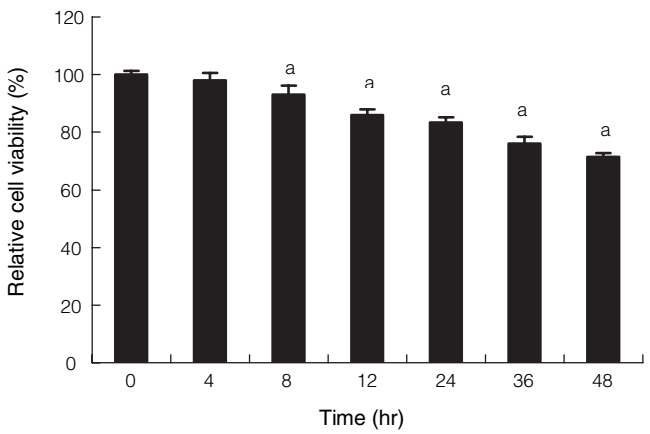

Figure 1. Time course of relative cell *via*bility of rat brain microvascular endothelial cells exposed to angiotensin II. Data are mean ± S.D. of 9 independent experiments that used separate endothelial cell isolates. [a]$p < 0.05$ vs. control group.

RESULTS

I. *Determination of the Viability and LDH Release of BMEC*

As shown in Figure 1, after BMEC were exposed to 10^{-6} mol/L Ang II for 4, 8, 12, 24, 36, and 48 hr respectively, the relative cell *via*bility was reduced in a time-dependent manner. Except for 4 hr, cells incubated with 10^{-6} mol/L Ang II for other times underwent significant damaged. In order to further investigate the extent of damage on BMEC, the LDH release was assayed 48 hr after the addition of 10^{-6} mol/L Ang II. We noted that in relation to the loss of cell *via*bility, LDH release from BMEC also increased markedly. These effects were blocked by AT_1 specific antagonist losartan. Pretreatment with the AT_2 specific antagonist PD123319 did not alter Ang II-induced BMEC injury significantly. Moreover, losartan combinating with PD123319 neither diminished nor promoted the effect of losartan alone. The above results indicate that BMEC injury induced by Ang II apparently mediated through AT_1 receptor stimulation, but not by AT_2 receptor. Furthermore, the protective effect of the AT_1 specific antagonist losartan may not be directly related to Ang II activating unblocked AT_2 receptor *in vitro*. (Figure 1, Table 1).

II. *Determination of Lipid Peroxidation*

Our results demonstrated that the BMEC group treated with Ang II increased MDA, compared with untreated control group which indicated that extensive lipid peroxidation had taken place in these damaged cells. Pretreatment with the AT_1 antagonist losartan alone suppressed the formation of MDA exerted by Ang II significantly; while pretreatment with AT_2 antagonist PD123319 alone exhibited no influence on the formation of MDA induced by Ang II. However there was no significant difference in MDA formation between losartan group and losartan + PD123319 group (Table 2).

III. *Determination of Intracellular Antioxidant Enzyme Activities*

The level of activity of the antioxidative enzymes, SOD and GSH-Px was also determined. As shown in Figure 2, 10^{-6} mol/L Ang II treatment markedly reduced the level of

Table 2. Effect of angiotensin II receptor antagonists on angiotensin II-induced lipid peroxidation in the medium and rat cerebral microvascular endothelial cells

Group	MDA in medium (nmol/ml)	MDA in cells (nmol/mg protein)
Control	0.75 ± 0.058	5.17 ± 1.00
10-6 mol·L^{-1} Ang II	4.64 ± 0.60[a]	28.53 ± 2.01[a]
10-6 mol·L^{-1} Ang II + 10^{-5} mol·L-$^{-1}$ Los	2.02 ± 0.29[b]	13.44 ± 1.31[b]
10-6 mol·L^{-1} Ang II + 10^{-5} mol·L^{-1} PD123319	5.04 ± 0.44[c]	29.03 ± 1.99[c]
10^{-6} mol·L^{-1} Ang II + 10^{-5} mol·L^{-1} Los + 10^{-5} mol·L^{-1} PD123319	2.16 ± 0.45[b]	13.75 ± 1.11[b]

Each value represents the mean ± S.D. of six experiments.
[a] $p < 0.05$ vs. control group.
[b] $p < 0.05$ vs. Ang II group.
[c] $p < 0.05$ vs. losartan group.

activity of SOD and GSH-Px in BMEC by 52% and 42%, respectively. Pretreatment with the AT_1 specific antagonist losartan (10^{-5} mol/L) produced an evident recovery; whereas PD123319 pretreatment did not alter the reduction of SOD and GSH-Px activity in BMEC induced by Ang II. Moreover, no significant change in the level of activity for these enzymes was detected in the additional presence of PD123319 compared with pretreatment with losartan alone.

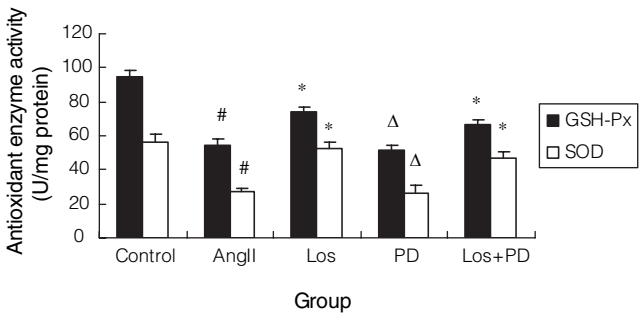

Figure 2. Effect of angiotensin II receptor antagonists on the activities of superoxide dismutase and glutathione peroxidase in angiotensin II-treated rat cerebral microvascular endothelial cells. Cells were pooled and homogenized after being incubated for 48 hr with 10^{-6} mol/L angiotensin II in the presence or absence of 10^{-5} mol/L losartan (Los) or 10^{-5} mol/L PD123319 (PD). The homogenate was centrifuged and the activities of intracellular antioxidant enzymes in the supernatant were determined. Each value represents the mean ± S.D. of six experiments. $^{\#}p < 0.05$ vs. control group, $^{*}p < 0.05$ vs. angiotensin II group, $^{\Delta}p < 0.05$ vs. losartan group.

DISCUSSION

An increasing number of studies suggest that Ang II is involved in the pathology of cerebrovascular disease partly by injuring BMEC[2, 3, 4, 14], but the mechanisms involved are not elucidated. In the present study, the relative cell *via*bility and the LDH release were used to evaluate the extent of BMEC injury. In agreement with previous reports, our result showed that 10-6 mol/L Ang II increased LDH leakage and time-dependently inhibited the *via*bility of BMEC.

Ang II has been shown to stimulate the activity of membrane-bound NAD(P)H oxidase in endothelial cells which participates in endothelial dysfunction[8,9], but not in BMEC. OFR are likely participants in the pathogenesis of cerebrovascular diseases and can significantly increase lipid peroxidation in BMEC, which was evaluated by measuring MDA content in the experiment[10]. It is becoming clear that oxidative stress induces an adaptive antioxidant response within the cell and the level of antioxidant enzymes appears to be lower[15]. In addition to observing lipid peroxidation, we also examined activity of the antioxidant enzymes including SOD and GSH-Px to identify the oxidative stress in BMEC. The results showed that Ang II treatment increased the intracellular and extracellular medium MDA content and decreased the level of activity of intracellular SOD and GSH-Px in BMEC, compared to an untreated control group. These findings indicate that Ang II causes injury to BMEC by increasing free radicals and decreasing endogenous antioxidant defense. They further suggest that oxidative stress induced by Ang II plays an important role in causing or mediating BMEC injury.

Ang II is known to act mainly through the AT_1 and AT_2 receptors. The AT_1 receptor-mediate Ang II actions are best defined in the brain. Longterm treatment with AT_1 receptors antagonists has been reported to improve the neurological outcome and reduce the infarct volume after experimental focal brain ischemia in stroke-prone hypertensive rats before the reduction in blood pressure[16]. Walter *et al*. also reported that a smaller lesion area was observed after MCA occlusion in AT_1 receptor-deficient mice[17]. It also has been reported that AT_1 receptor antagonist prevents brain edema by inhibiting cerebrovascular endothelial cell injury induced by Ang II without lowering blood pressure[14]. However, the function of AT_2 receptors is less well defined; they may counterbalance the effects of AT_1 receptor stimulation in most tissues. Some authors have speculated that when the AT_1 receptor is blocked by an AT_1 receptor antagonist, unbound Ang II acts preferentially on the AT_2 receptor, which may contribute to the beneficial action of AT_1 receptor antagonist[18-20]. Iwai *et al*.[21] reported that significantly larger ischemic area and greater neurological deficit were observed after MCA occlusion

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in AT_2 receptor-deficient mice than in wild-type mice. Therefore, the effect of the stimulation of the AT_2 receptor is becoming increasingly important in both physiological and pathological situations.

In this study we investigated the contribution of the AT_2 receptors in Ang II-induced BMEC injury. We speculated that AT_2 receptors may act to oppose the AT_1-receptor mediated effect in BMEC. Therefore, the PD 123319 (selective block AT_2 receptor) group and the losartan + PD 123319 (block both AT_1 and AT_2 receptor) group were designed to investigate the role of AT_2 receptor in BMEC injury induced by Ang II. Our results demonstrated that pretreatment with the AT_1 selective blocker losartan significantly prevented Ang II-induced BMEC injury, reduced the MDA formation and increased the activity of SOD and GSH-Px. In contrast, the selective blocking of the AT_2 receptor with PD123319 did not markedly bring about these effects. There was no significant difference, in this regard, between the losartan and losartan + PD 123319 groups. Taken together, it appears that BMEC injury and the oxidative stress induced by Ang II seem to involve AT_1 more than AT_2 receptor. This result contrasts with the report[21] that AT_2 receptor stimulation has a protective effect on ischemic brain lesions in mouse focal brain ischemia induced by MCAO, at least partly through the modulation of cerebral blood flow and superoxide production. The reason is perhaps that the conditions (physiological) in BMEC *in vitro* differ from those in brain ischemia model (pathological) *in vivo*.

In conclusion, the present study demonstrates that Ang II injury of the BMEC possibly occurs through induction of oxidative stress by activating AT_1 receptors, which is then involved in the development of cerebrovascular disease.

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