Involvement of Superoxide in Acute Reaction of Angiotensin II in Mesenteric Microcirculation

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Abstract: Superfusion of angiotensin II (Ang II) ceased blood flow in rat mesenteric microcirculation, however, successive reflow occurred. When nitric oxide synthase inhibitor was present, the stoppage of flow occurred by the lower concentration of Ang II. Superoxide dismutase (SOD) significantly delayed the stoppage by Ang II and restored the successive reflow earlier. The acute reaction between Ang II and mesenteric artery induced immediate superoxide ($\cdot\text{O}_2^-$) production

when observed by a chemiluminescence method using the Cypridina luciferin analog. The acute vascular $\cdot\text{O}_2^-$ production on the addition of Ang II contributed to *in vitro* vascular contraction as it was significantly attenuated by SOD. The acute superoxide-producing effect is likely to be specific to Ang II because such significant modification by SOD was not observed for norepinephrine. [Japanese Journal of Physiology, 49, 437–443, 1999]

Key words: angiotensin II, superoxide, nitric oxide, microcirculation, mesenteric artery.

Angiotensin II (Ang II), a physiologically important component of the renin-angiotensin system, plays a critical role in the regulation of blood pressure and homeostasis of body fluids and electrolytes. Ang II produces arteriolar constriction and a rise in systolic and diastolic blood pressure. It is one of the most potent vasoconstrictors known. Although there are many reports suggesting superoxide involvement in vascular contraction, nobody assumed the involvement of superoxide in the Ang II-induced contraction.

The present study aimed to examine the possibility. Superfusion of Ang II ceased blood flow in the microcirculation of the rat mesentery in nearly 16 s, and we have found that superoxide dismutase (SOD) significantly prolongs the ceasing time. We then examined whether the reaction of Ang II with vasculature leads to a rapid release of superoxide ($\cdot\text{O}_2^-$) by the chemiluminescence method, whether it is involved in the contraction of mesenteric artery and whether another pressor agent, norepinephrine, has similar effects on vascular $\cdot\text{O}_2^-$ production.

MATERIALS AND METHODS

Experimental animals. Experiments were performed using male, 8-week-old Wistar Kyoto rats (WKY, Charles River). All rats were handled according to the Guidelines of the Committee on Animal Care and Use of Kagawa Medical University.

Measurements of the time of the stoppage and duration of closure. Rats were anesthetized with pentobarbital (50 mg/kg I.P.). A mid-line abdominal incision was made, and the animal was placed in the lateral position on a microscope stage. The mesentery was prepared for microscopic observation as described previously [1, 2]. Briefly, the mesentery was placed on a thin glass plate and its microcirculation was monitored under a microscope ($\times 40$). The mesentery was trans-illuminated and a single unbranched mesenteric venule of diameter between 15–25 μm and length $\geq 150 \mu\text{m}$ was selected for study. Its microscopic image was monitored with a charge-coupled device (CCD) camera and recorded with a videotape recorder for play-back analysis. During monitoring, the room temperature was maintained

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at 28°C and the mesenteric surface was superfused with Krebs-Henseleit modified solution (mM): NaCl 118.6, KCl 4.75, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 2.54, KH_2PO_4 1.19, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.19, NaHCO_3 12.5, glucose 10.0 and maintained at 37°C. The pH of the solution was adjusted to 7.4 by bubbling with a gas mixture (95% N_2 /5% CO_2). The exposed bowel wall was covered with Saran Wrap except for an observation window. The mesentery was superfused with the buffer alone for 60 min before the experiments. Acute responses of mesenteric microcirculation to direct superfusion of Ang II (10^{-5} – 10^{-7} M), epinephrine bitartrate salts (Epi) (10^{-5} – 10^{-7} M), or norepinephrine bitartrate salts (NE) (10^{-4} – 10^{-6} M) dissolved in saline containing 0.1% bovine serum albumin (BSA) were examined to determine the concentration where the stoppage of flow occurs consistently. Then the consistent stoppages of flow by direct superfusion of Ang II (10^{-5} M), Epi (10^{-5} M), or NE (10^{-4} M) were monitored with or without SOD (50 U/ml dissolved in saline containing 0.1% BSA). SOD was added 5 min before the reagents. To examine whether NO is against the Ang II-induced stoppage of flow, mesenteric rings were superfused with Krebs-Henseleit modified solution containing N^{ω} -nitro-L-arginine methyl ester (L-NAME) (6×10^{-5} M) or L-NAME plus L-arginine (1.2×10^{-3} M) for 30 min before the experiments. CV-11974 (Candesartan, Ang II type 1 (AT_1) receptor blocker [3, 4], 10^{-7} M dissolved in saline containing 0.1% BSA) was superfused directly on the mesentery at 5 min before Ang II superfusion. Each reagent was superfused with a total volume of 100 μl . Vascular diameter and length were measured online using a video caliper system. The time of the stoppage and duration of closure were measured offline during playback of the videotaped images.

Assay of superoxide production. Superoxide ($\cdot\text{O}_2^-$) production was measured according to the chemiluminescence method using 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo-(1,2- α)pyrazin-3-one (MCLA; Cypridina luciferin analog) [5–7]. The mesenteric arteries were removed, cut into rings of 2 mm in length, and kept, prior to use, in ice-cold oxygenated modified Krebs-HEPES (mM: NaCl 99.01, KCl 4.69, CaCl_2 1.87, K_2HPO_4 1.03, MgSO_4 1.20, NaHCO_3 25.0, Na-HEPES 20.0, glucose 11.1, and BSA (1 mg/ml) (pH 7.4, 95% O_2 /5% CO_2) [8]. The rings were equilibrated in the oxygenated Krebs-HEPES buffer at 37°C for 30 min before the experiments. The ring preparations were carefully positioned in a microplate containing 100 μl Krebs-HEPES buffer with 250 μM MCLA. The microplate was placed in a luminometer (Luminoscan, Labsys-

tems, Helsinki, Finland) and agitated by rotation at 37°C throughout the measurements. After 10 min of dark adaptation, the background count was recorded and Ang II (10^{-6} M) or NE (10^{-6} M) was administered in the presence or absence of SOD (50 U/ml). Each dose was selected on the basis of the responses in preliminary experiments. Counts were recorded every 20 s for 15 min, and the respective background counts were subtracted. After measurement, the vascular rings were blotted, dried for 48 h at 60°C and weighed. The specific counts were expressed as counts/15 min/mg dry-tissue weight. The effects of SOD plus catalase (200 U/ml) or indomethacin (10^{-5} M) were further examined for the significant results with Ang II to determine the contribution of H_2O_2 or prostaglandins.

Organ chamber experiments. Isolated vascular rings were studied in organ chambers for measurement of isometric force [9, 10]. Briefly, rats were anesthetized and the mesenteric arteries were removed, taking care not to damage the endothelium. The vessel was placed in ice-cold oxygenated Krebs-Henseleit modified solution (mM: NaCl 118.0, KCl 4.7, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 2.5, KH_2PO_4 1.2, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.2, NaHCO_3 25.0, and glucose 11.1, pH 7.4, 95% O_2 /5% CO_2), cleaned of adhering connective tissues, and cut into 2-mm-long ring segments. A ring was suspended vertically between two stiff stirrups (0.1-mm diameter wire for mesenteric artery) in organ chambers filled with 5 ml of modified Krebs-Henseleit buffer (37°C) aerated with a mixture of 95% O_2 and 5% CO_2 . One stirrup was fixed in the organ chamber while the other was connected to an isometric force transducer (Randonoti, Monrovia, California, USA). The rings were equilibrated under a resting tension of 1 g for 90 min. This tension was optimal for contraction of the preparation as assessed by repeated exposure to a 100 mM KCl solution under various resting tensions. After the equilibration period, the rings were contracted by the KCl solution twice, separated by a 30-min interval. Responses to acetylcholine (10^{-6} M) to relax the rings were examined to check for endothelium damage. After a stable contraction plateau had been reached, the contractility in response to the different concentrations of Ang II (10^{-9} to 10^{-6} M) was measured with isolated rings from different individuals in the presence or absence of SOD (50 U/ml). The effects of SOD plus catalase (200 U/ml) or indomethacin (10^{-5} M) were further examined. Increasing concentrations of NE (10^{-9} to 10^{-5} M) were added in a cumulative fashion to quiescent preparations. Contractions are given as a percentage of the contraction to KCl (100 mM).

Reagents. The following reagents were used

Fig. 1. Effect of angiotensin II (Ang II)-induced stoppage of flow and reflow in mesenteric microcirculation. (A) Schema defining the time of the stoppage and duration of closure. Reagents were directly superfused onto the mesentery. The stoppage of flow was defined as the absence of blood flow for longer than 5 s in a monitored single unbranched mesenteric venule of diameter between 15–25 μ m and length $\geq$ 150 μ m. The reflow was defined as the blood flow starting again and continuing longer than 60 s in the same venule. (B) Effect of Ang II concentration (10^{-7} to 10^{-5} M) on the occurrence of stoppage of flow. The occurrence were given as % of total experimental numbers. CV-11974 (Candesartan, Ang II type 1 (AT_1) receptor blocker, 10^{-7} M) was superfused directly on the mesentery 5 min before Ang II superfusion. N^o-nitro-L-arginine methyl ester (L-NAME, 6×10^{-5} M) without or with L-arginine (1.2×10^{-3} M) was superfused directly on the mesenteries for 30 min before the experiments. Observation was made for at least 5 min.

(from Sigma Chemical Co. unless otherwise stated): pentobarbital (Abbott), Ang II, Epi, NE, CuZn SOD (from bovine erythrocytes), BSA, acetylcholine hydrochloride, L-NAME, MCLA (Tokyo Kasei Kogyo, Tokyo, Japan), catalase (from beef liver, Boehringer Mannheim), L-arginine (Wako, Japan), and indomethacin. AT_1 receptor antagonist CV-11974 [3, 4] was a kind gift from Takeda Chemical Industries, Ltd. Ang II, Epi, NE, SOD and CV-11974 were dissolved in saline containing 0.1% BSA. Indomethacin was dissolved in a 5 mM Na_2CO_3 solution on the day of the experiment [11]. The control study was done using saline containing 0.1% BSA. The concentrations of the reagents used *in vitro* are expressed as final molar concentrations.

Table 1. Effect of angiotensin II, epinephrine and nor-epinephrine concentrations on mesenteric blood flow.

Treatment (M)	Stoppage (%)	n_1/n_0
Ang II 10^{-7}	0	0/4
Ang II 10^{-6}	33	2/6
Ang II 10^{-5}	100	22/22
Epi 10^{-7}	0	0/5
Epi 10^{-6}	40	2/5
Epi 10^{-5}	100	17/17
NE 10^{-6}	0	0/5
NE 10^{-5}	0	0/5
NE 10^{-4}	100	9/9

n_0 , total experimental numbers; n_1 , number of the cases where stoppage of blood flow occurred by Ang II in n_0 . Stoppage (%) was calculated from n_1/n_0 . Measurement conditions were as in Fig. 1.

Data analysis. Data are given as mean $\pm$ SEM. Statistical evaluation was done by unpaired Student's *t*-test or by ANOVA followed by Fisher's protected least significant difference (PLSD) test. Significance was considered to be present when the *p* value was <0.05 .

RESULTS

Direct superfusion of Ang II to the mesentery led to transient stoppage of blood flow followed by a short interval leading to reflow. The former was expressed as the time of the stoppage and the latter as duration of closure (Fig. 1A). The stoppage of flow was defined as the absence of blood flow for longer than 5 s in the monitored mesenteric venule. The reflow was defined as the blood flow starting again and continuing longer than 60 s in the same venules. Observation was made for at least 5 min. With a direct superfusion of 10^{-6} M Ang II, blood flow stoppage occurred in only about 30% of the mesenteric microcirculation and, at 10^{-7} M, no stoppage occurred (Table 1, Fig. 1B).

This is probably because a higher concentration of Ang II is required to occlude the blood vessels completely against relaxing factors such as nitric oxide (NO). We pursued to examine whether the stoppage of blood flow occurs by lower concentrations of Ang II under the presence of NO synthase inhibitor L-NAME. The stoppage phenomenon occurred even with an Ang II of 10^{-7} M (Fig. 1B) and the duration of closure was prolonged (Table 2). These effects were reversed by concomitant addition of 20-fold L-arginine. Because the Ang II concentration of 10^{-5} M stopped blood flow followed by reflow consistently,

Table 2. Effect of angiotensin II on mesenteric blood flow.

Treatment (M)	Time of the stoppage (s)	n_1/n_0	Duration of closure (s)	n_2/n_1
Ang II 10^{-7}	—	0/4	—	—
Ang II 10^{-7} +L-NAME	23±6	9/15	160±43	8/9
Ang II 10^{-7} +L-NAME+L-arg	—	0/5	—	—
Ang II 10^{-6}	25±4	2/6	43±8	2/2
Ang II 10^{-6} +L-NAME	21±5	9/9	15±20	9/9
Ang II 10^{-6} +L-NAME+L-arg	29±6	3/5	66±37	3/3
Ang II 10^{-5}	16±2	22/22	61±6	22/22
Ang II 10^{-5} +L-NAME	0±2	9/9	282±48	5/9
Ang II 10^{-5} +L-NAME+L-arg	20±7	5/5	54±8	5/5

n_0 , total experimental numbers; n_1 , number of the cases where stoppage of blood flow occurred by Ang II in n_0 ; n_2 , number of the cases where reflow occurred after Ang II-induced stoppage in n_1 . Mean±SE. The time of the stoppage and duration of closure were calculated from the data of n_1 and n_2 cases, respectively. Measurement conditions were as in Fig. 1. L-NAME (6×10^{-5} M) and L-arginine (1.2×10^{-3} M) were superfused for 30 min before the experiments. Superfusion of L-NAME alone for 30 min or more did not stop blood flow, as shown in Fig. 1.

this dose was applied for the following experiment with SOD.

Direct superfusion of Ang II (10^{-5} M) to the mesentery led to a transient stoppage of blood flow in microcirculation in 16.5 ± 2.4 s (the time of the stoppage) (Fig. 2A) followed by a rapid reflow in 61.0 ± 5.9 s (duration of closure) (Fig. 2D). The stoppage of flow was completely blocked by Ang II AT₁ receptor antagonist CV-11974 (Fig. 1B). SOD significantly prolonged the time of the stoppage from 16.5 ± 2.4 to 27.8 ± 3.0 s ($p < 0.01$) (Fig. 2A) and shortened the duration of closure from 61.0 ± 5.9 to 17.9 ± 4.4 s ($p < 0.0001$) (Fig. 2D). Because the stoppage by Epi and NE continued much longer and reflow was not observed without washing, we determined only the time of stoppage under the concentration where the stoppage occurred consistently (Table 1). SOD did not prolong the time of stoppage (Fig. 2B, C).

We next aimed to determine vascular superoxide anion production by using MCLA-dependent chemiluminescence in mesenteric rings. Ang II, but not NE, significantly increased luminiscan counts compared with the control (30.7 ± 3.0 versus 15.9 ± 3.9 counts/15 min/mg tissue, $p < 0.05$). SOD significantly decreased the luminescence count to 21.0 ± 2.7 counts/15 min/mg tissue (Fig. 3). Although SOD converts $\cdot O_2^-$ to H_2O_2 , the concomitant addition of catalase was without effect. Indomethacin did not inhibit the effect of Ang II.

We next designed an experiment to examine whether superoxide anion production is involved in the Ang II-induced contraction of isolated mesenteric arteries. As shown in Fig. 4, the contraction increased dependent on Ang II concentrations from 10^{-9} to

Fig. 2. Effect of SOD on angiotensin II (Ang II)-induced stoppage of flow and reflow in mesenteric microcirculation. Effect of (A) Ang II (10^{-5} M); (B) epinephrine (Epi; 10^{-5} M); and (C) norepinephrine (NE; 10^{-4} M) on the stoppage of flow was measured in the presence of SOD (50 U/ml; ■) or a saline control (□). SOD dissolved in saline was superfused 5 min before the experiments. Ang II, Epi and NE doses were selected on the data in Table 1. (D) Effect of SOD (50 U/ml) (■) or saline control (□) on the duration of closure. Data were given as mean±SEM. Statistical analysis was done by unpaired Student's *t*-test. * $p < 0.01$ vs. without SOD. † $p < 0.0001$ vs. without SOD.

10^{-6} M. An Ang II concentration of 10^{-7} M was sufficient to cause maximum contraction and the extent of contraction was similar to that by 10^{-5} M Ang II (data not shown). SOD significantly decreased contractions

Fig. 3. Vascular superoxide anion production measured in mesenteric artery using 2-methyl-6-(*p*-methoxyphenyl)-3,7-di-hydroimidazo-(1,2- α) pyrazin-3-one (MCLA; Cypridina luciferin analog). Angiotensin II (Ang II; 10^{-6} M) and norepinephrine (NE; 10^{-6} M) were examined in the presence (■) or absence (□) of SOD (50 U/ml). The effects of SOD plus catalase (200 U/ml) or indomethacin (indo; 10^{-5} M) were further examined for the significant results of Ang II to determine the contribution of H_2O_2 or prostaglandins. The final MCLA concentration was 250 μ M. The specific chemiluminescence signal was presented as counts/15 min/mg dry-tissue weight. Data were given as mean $\pm$ SEM. Statistical analysis was done by two-way ANOVA, one independent variable was Ang II or NE treatment and the other was with or without SOD. When significance was indicated, comparisons between all groups were done by one-way ANOVA followed by Fisher's PLSD test. * $p < 0.05$ vs. control and with plus SOD.

of the rings induced by Ang II of 10^{-7} and 10^{-6} M (Fig. 4). The concomitant addition of catalase was without effect. Indomethacin did not affect the effect of Ang II. For contraction of the mesenteric artery induced by NE (Fig. 5), there was no significant difference between the saline and SOD groups. Indomethacin or the concomitant addition of catalase was without effect.

DISCUSSION

The present study revealed that SOD significantly delayed the acute effect of Ang II in ceasing blood flow in the mesenteric circulation. SOD also significantly shortened the duration of closure before reflow. Ang II AT_1 receptor antagonist CV-11974 completely blocked the effect of ceasing blood flow. The results indicate the involvement of AT_1 receptor and $\cdot O_2^-$ generation in the acute Ang II effect. We then demon-

Fig. 4. Effect of SOD on contraction of mesenteric rings by angiotensin II (Ang II). The contractility in response to each different concentration of Ang II (10^{-9} to 10^{-6} M) was measured with the isolated rings of mesenteric artery from different individuals using the organ chamber method. Ring segments were pretreated with SOD (50 U/ml) or vehicle 5 min before. The effects of SOD plus catalase (cat; 200 U/ml) or indomethacin (indo; 10^{-5} M) were further examined to determine the contribution of H_2O_2 or prostaglandins. Contractions are presented as % of peak KCl (100 mM) response and as mean $\pm$ SEM. Statistical analysis was done by two-way ANOVA, followed by Fisher's PLSD test. * $p < 0.05$ vs. with SOD.

Fig. 5. Effect of SOD on contraction of mesenteric rings by norepinephrine (NE). Ring segments were pretreated with SOD (50 U/ml) or vehicle 5 min before. The effects of SOD plus catalase (cat; 200 U/ml) or indomethacin (indo; 10^{-5} M) were further examined. Contractions are presented as % of peak KCl (100 mM) response and as mean $\pm$ SEM. Statistical analysis was done by two-way ANOVA followed by Fisher's PLSD test.

strated using the MCLA chemiluminescence method that Ang II rapidly increases vascular $\cdot\text{O}_2^-$. To our knowledge, this is the first report of Ang II acutely increasing vascular $\cdot\text{O}_2^-$ production in such a short period of a few seconds.

These acute $\cdot\text{O}_2^-$ -producing effects are likely to be specific to Ang II because NE-induced effects did not show significant modification by SOD. We also revealed that SOD significantly attenuated the acute contraction response of vascular rings to Ang II. This result may explain why chronic exercise attenuates Ang II receptor-mediated contraction of the rat aorta [12] because exercise training resulted in a significant increase in the activity of SOD [13].

NO is the major endothelium-derived relaxing factor [14, 15]. NO synthase inhibitor L-NAME augmented the Ang II effect on the stoppage of flow (Fig. 1). This may be because Ang II stimulates NO release. Neither losartan nor Candesartan (CV-11974) significantly affected basal NO production, but both of these AT_1 -receptor blockers markedly blunted NO release in response to Ang II in renal arteries [16]. On the other hand, activation of the renin-angiotensin system during sodium depletion increased renal NO production through stimulation by Ang II at the angiotensin AT_2 receptor [17]. The present acute effect of Ang II increasing vascular $\cdot\text{O}_2^-$ production and constriction could be balanced by the NO-mediated vascular relaxation, which could induce a strong and reversible control of circulation.

But when Ang II is chronically elevated in high renin/angiotensin II states, the continuous elevation of $\cdot\text{O}_2^-$ production may cause pathologic disorders, e.g., hypertension and remodeling of blood vessels. Infusion of Ang II, but not NE, to rats for 5 d has been reported to upregulate constitutive $\cdot\text{O}_2^-$ -producing ability in vascular tissues despite both Ang II and NE treatments elevating blood pressure [18]. The site of increased constitutive $\cdot\text{O}_2^-$ generation by the chronic treatment of Ang II is reported to be the smooth muscle [19–21] or adventitial fibroblasts of the aorta [22, 23], where a major source of $\cdot\text{O}_2^-$ was the membrane-bound NADH/NADPH oxidase system. The timing of radical production ascribed to the p22 phox-dependent oxidase by Griendling *et al.* [24] appears too slow to explain the present unknown acute pathway of Ang II inducing rapid $\cdot\text{O}_2^-$ release in blood vessels. This study suggests that H_2O_2 or prostaglandins are not involved in the rapid $\cdot\text{O}_2^-$ release because the concomitant addition of catalase with SOD or the presence of indomethacin did not affect the effect of Ang II. In vascular smooth muscle cells, Ang II is known to activate enzymes such as tyrosine protein kinase, phos-

pholipase C, protein kinase C, and phosphatidylinositol-3-kinase [25]. These signaling pathways may be linked to the present acute production of $\cdot\text{O}_2^-$ by Ang II.

In summary, the acute effect of Ang II stopping blood flow in mesenteric circulation was significantly reduced by SOD and the following reflow was markedly improved by SOD. Ang II rapidly increased MCLA chemiluminescence in the mesenteric artery, which was inhibited by SOD. This superoxide-producing effect seems to be specific to Ang II since the response of blood vessels to NE did not show such significant modification by SOD.

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