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Characterization of Superoxide-dependent Endothelial Relaxing Factor(s)

Gi-Nam Lee, Won-Suk Lee, Byung-Yong Rhim and Ki-Whan Hong[1]

Department of Pharmacology, College of Medicine, Pusan National University, Pusan 602-739, Korea

ABSTRACT

We recently reported a development of an experimental system which can identify the release of a superoxide-dependent vasorelaxant factor from endothelial cells using a two-bath system. In the present work, we further exploited the above system and observed whether the superoxide-dependent relaxing factor(s), released from the porcine coronary artery (PCA) endothelium, was similar in relaxation to those obtained from cat thoracic aortic endothelium and cultured endothelial cells of bovine aorta. However, there was observed a novel difference among the former one and the latter two relaxing factors; the release of relaxing factor from PCA endothelium can be inhibited either by catalase or by superoxide dismutase (SOD), whereas the latter two can be inhibited only by SOD. It was further attempted to characterize the synthetic mechanisms of the relaxing factors: (1) They were readily inhibited by various lipoxygenase inhibitors (gossypol, nordihydroguaiaretic acid, AA 861, and eicosatetraynoic acid). (2) They were not inhibited by cyclooxygenase inhibitor (indomethacin) and by cytochrome P-450 monooxygenease inhibitors (proadifen and cimetidine). Thus, it is likely that these relaxing factors, although obtained from different species, show common functional roles of arteriolar relaxation. It is suggested that they are related to pathophysiological involvement of various tissue ischemia-reperfusion injuries.

Key Words: Superoxide anion, Endothelium, Relaxing factor

INTRODUCTION

The significance of endothelial cells in triggering off vasodilation was first described by Furchgott and Zawadzki (1980). Subsequently, many studies have reported the importance of vascular endothelium in relaxation to a number of vasodilators.

Oxygen stress produced locally in microcirculation has been found to contribute to tissue damage involved in myocardial ischemia-reperfusion injury or head injury (Del Maestro, 1980; Freeman and Crapo, 1982; Kontos, 1985). Also, pathological sources of reactive oxygen radicals (xanthine oxidase system and neutrophils) have been shown to be associated with ischemia or hypoxia (Halliwell, 1989; Mullane *et al.*, 1987; Simpson *et al.*, 1987).

Recently, we reported the release of a relaxing factor from the endothelial cells which is dependent on superoxide-generating systems (Hong *et al.*, 1989a, b). We described this factor distinctly different from the well-known endothelium-derived relaxing factor (Peach *et al.*, 1985; Martin *et al.*, 1986).

However, we have not clarified as to how the relaxing factor is related to cerebral arteriolar dilation which might result from brain injury or to attenuation of coronary flow decrease induced during stenotic ischemia (Kontos, 1985; Laurindo *et al.*, 1988). Thus, in the present work, we designed further experiments, (1) to examine how the relaxing factor released from porcine coronary artery (PCA) endothelium acts on the smooth muscle of PCA, (2) to find species differences in the preventive effect of oxygen radical scavengers, and (3) to determine the effects of arachidonate metabolic inhibitors on the production and release of the relaxing factor(s).

[1] To whom all correspondence should be adressed.

MATERIALS AND METHODS

Organ bath and bioassay experiments

Adult pigs of either sex were killed in a local slaughter house; the hearts were immediately placed into an oxygenated physiological salt solution (PSS) at 10-15°C and brought to the laboratory. The left large anterior descending branch (LAD) and left circumflex branch (LCB) were carefully dissected free from surrounding tissues (average diameter, 3-5 mm).

As previously described by Hong *et al.* (1989a), a two-bath system was used to simultaneously detect the change in muscle tension and to release the relaxing factor from the endothelium. For donor vessel, a 20-mm segment of LAD was opened longitudinally with care taken to prevent damage to the endothelial lining, and then mounted between platinum electrodes. For detector muscle, the coronary artery (LCB) mechanically denuded of endothelium was cut into a helical strip and mounted in the tissue bath. One end of the strip was attached to an isometric transducer (Myograph F-60, Narco Bio-Systems) and allowed to equilibrate under resting tension of 4 g.

Alternatively, cats (2-3 kg) of either sex were sacrificed by guillotine under anesthesia with secobarbital sodium (30 mg/kg, i.p.). In this case, thoracic aorta with endothelium or cultured bovine endothelial cells which were packed within a plastic column with membrane filters (0.45μm pores) was used as the donor vessel, and cat basilar artery denuded of endothelium used as the detector muscle (Hong *et al.*, 1989a). For electrical field stimulation (EFS), the platinum plate electrodes (5 × 5 mm) were used and square-wave biphasic pulse trains were delivered from the stimulator (S6, Grass) for 5 min at frequency of 10-20 Hz with a pulse duration of 2 msec (20V).

PSS was of the following composition (in mM); NaCl 130, KCl 4.7, $NaH_2PO_4 \cdot 2H_2O$ 1.18, $MgSO_4 \cdot 7H_2O$ 1.17, $CaCl_2 \cdot 2H_2O$ 1.6, $NaHCO_3$ 14.9, and dextrose 5.5. The solution was bubbled with 95% O_2-5% CO_2 (37°C). Atropine, propranolol and desipramine (0.2 μM each) and 1-2 μM of indomethacin were introduced into the PSS to rule out the involvement of adrenergic and cholinergic mechanisms and the production of prostaglandins.

Culture of endothelial cells

Freshly prepared bovine aortic segments were obtained from the slaugher house (20-30 cm in length) and washed thoroughly with Dulbecco's phosphate buffer saline. The cells were cultured in a humidified 5% CO_2 incubator. The procedures are similar as described previously (Hong *et al.*, 1989a,b).

Drugs

5-Hydroxytryptamine HCl (5-HT), hypoxanthine (HX), xanthine oxidase (XO, Grade I), superoxide dismutase (SOD), allopurinol, catalase, gossypol, nordihydroguaiaretic acid (NDGA), cimetidine and indomethacin were purchased from Sigma Chemical Co. AA 861, proadifen (SKF 525-A), and 5,8,11, 14-eicosatetraynoic acid (ETYA) were generously donated from Takeda Chemical Ind., Smith Kline & French Labs., and Hoffman-La Roche, respectively.

Statistics

Data are expressed as mean ± S.E. Statistical analysis was carried out by the Student's t-test for paired or unpaired observation. When $p<0.05$, the differences were considered to be statistically significant.

RESULTS

Average contractilities to 0.3 μM of 5-HT for PCA and to 1 μM 5-HT for cat basilar artery were 2.3 g and 0.42 g, respectively.

Assessment of homogeneity

The present study was designed to determine whether the superoxide-dependent relaxing factor released from the endothelium of PCA is similar to that released from the endothelium of cat thoracic aorta. Cat basilar artery was used as a reference detector muscle. Following EFS to either PCA or cat thoracic aorta with endothelium in the donor bath for 5 min, the PSS with added SOD (50 unit/ml) was transferred to the detector muscle bath, in which the strips of either PCA or cat basilar artery was contracted to 5-HT.

As demonstrated in Fig. 1 and Table 1, contractility was reduced by 40-50% when either isolated strip of PCA or cat basilar artery was exposed to the suffusate, whereas the amplitude of contraction was unchanged when the tissue was exposed to the SOD-pretreated suffusate. Similarly, reduced contractility was observed when cat thoracic aorta with endothelium was used as a donor vessel instead of PCA. Alternatively, upon switching from PCA to car basilar

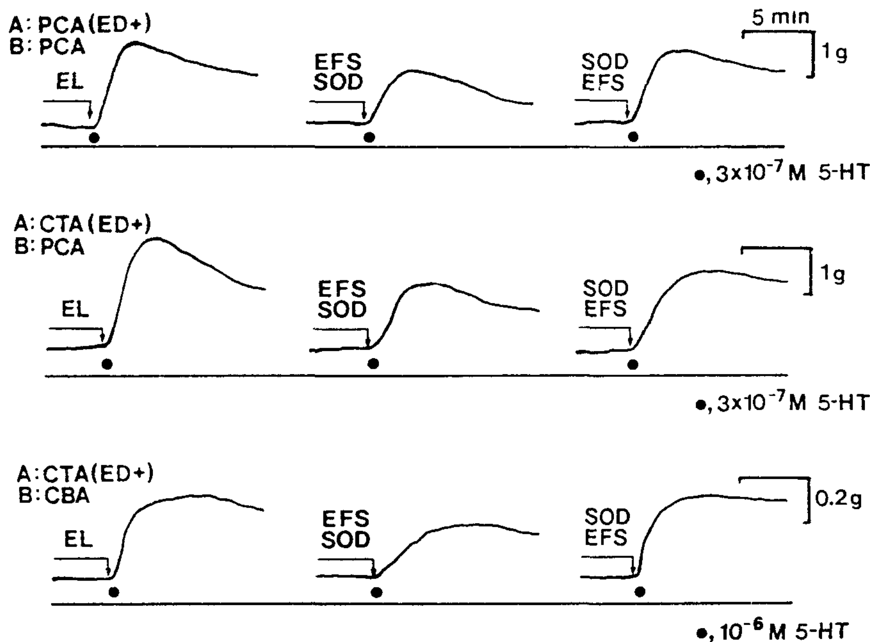

Fig. 1. Tracings of contraction to 5-hydroxytryptamine (5-HT) of two detector muscles denuded of endothelium: top and middle, porcine coronary artery (PCA), and bottom, cat basilar artery (CBA). The donor vessel with intact endothelium was either PCA (top) or cat thoracic aorta (CTA; middle and bottom). Control contraction to 5-HT was obtained after suffusion (⏋) of PSS subjected to electrolysis (EL) in left column. In middle, contraction was evoked in the suffusate previously subjected to electrical field stimulation (EFS) for 5 min followed by treatment with superoxide dismutase (SOD, 50 unit/ml). In right, same as in middle except prior treatment with SOD and thereafter EFS.

Table 1. Comparison of depression of contractions to 0.3 μM 5-HT of porcine coronary artery and cat basilar artery upon exposure to PSS delivered from different donor vessels with intact endothelium

Detector	n	Control (g)[a]	Contraction (%)[b]	
			EFS[c]	SOD + EFS[d]
	Donor, Porcine coronary artery			
Porcine coronary artery	6	2.29 ± 0.37	60.2 ± 4.5	99.9 ± 8.5*
Cat basilar artery	4	0.39 ± 0.03	49.5 ± 3.1	90.5 ± 12.5*
	Donor, Cat thoracic aorta			
Porcine coronary artery	4	2.30 ± 0.73	61.6 ± 12.5	99.6 ± 10.5*
Cat basilar artery	4	0.37 ± 0.04	62.6 ± 1.6	94.4 ± 1.8*

Values are means ± S.E. n, Number of experiments, [a], Control contraction to 5-hydroxytryptamine (5-HT) was obtained after suffusion of PSS subjected to electrolysis. [b], Percentage of contraction was based on deviation from the control value. [c], After cessation of electrical field stimulation (EFS), 50 unit/ml of superoxide dismutase (SOD) was added to circumvent a direct effect of superoxide anion. [d], After pretreatment with 50 unit/ml of SOD, EFS was applied. *, $p<0.05$ vs. EFS.

artery (detector muscle), the results showed little difference. When, instead of EFS, the segment of PCA with endothelium was treated with HX-XO for 5 min, the results were similar to that obtained after EFS (data not shown). The reduction of contraction caused by the suffusate being indirectly exposed to EFS or HX-XO was wholly prevented by pretreatment with SOD in the donor bath.

Table 2. Preventive effect of catalase pretreatment on the depression of contraction when porcine coronary artery with endothelium was used as a donor vessel, as contrasted to that from cultured bovine aortic endothelial cells

	n	EFS		n	HX-XO	
		Posttreatment	Pretreatment		Posttreatment	Pretreatment
		Donor: Porcine coronary artery with endothelium				
Superoxide dismutase (150 unit/ml)	7	62.6±4.5[a]	119.6±10.5***	6	53.1±6.2	113.7±19.8***
Catalase (150 unit/ml)	7	53.4±3.2	110.4± 7.3***	6	50.1±2.7	84.0± 9.5**
		Donor: Cultured endothelial cells				
Superoxide dismutase (150 unit/ml)	6	56.5±2.1	98.6± 4.9***	4	43.6±6.3	94.1±11.3*
Catalase (150 unit/ml)	6	52.8±1.3	53.9± 4.5	4	47.4±6.2	62.7± 6.5

a, Values are means of % contraction ± S.E. Porcine coronary arterial segment denuded of endothelium was contracted to 0.3 μM 5-HT. EFS or hypoxanthine (HX, 50 μM) + xanthine oxidase (XO, 0.02 unit/ml) was applied either to porcine coronary artery with endothelium or to the membrane device with cultured endothelial cells of 5×10^6 cells (average viability, 89.5±1.8%). *, p<0.05; **, p<0.01; ***, p<0.001. See others in Table 1.

Table 3. *Inhibition by different compounds of superoxide-mediated, endothelium-dependent relaxation of cat basilar arterial strips*

Agents	Doses	n	Contraction (%)		n	Contraction (%)	
			EFS[a]	Pretreatment		EFS	Posttreatment[b]
	Antioxidant and lipoxygenase inhibitors						
Gossypol	30 μM	5	59.1±4.1	76.3±2.9*	—	—	—
NDGA	10 μM	5	48.4±7.2	78.4±3.9***	3	56.7±6.5	52.3±2.3
AA 861	25 μg/ml	5	61.7±2.1	93.5±4.8***	3	58.7±4.4	55.5±1.6
	Cyclooxygenase and lipoxygenase inhibitor						
ETYA	0.5 μM	5	60.3±5.8	101.7±6.7**	4	61.2±3.5	72.8±7.5
	Cyclooxygenase inhibitor						
Indomethacin	5 μM	5	54.0±6.2	58.7±6.7	—	—	—
	Cytochrome P-450 monooxygenase inhibitor						
Proadifen	64 μM	4	62.7±6.7	60.9±5.2	—	—	—

Cat basilar arteries were contracted to 1 μM 5-HT by 417mg (95% CL, 399-435mg). [a], EFS was applied to cat thoracic aorta with intact endothelium. [b], Posttreatment represents application of agents after cessation of EFS. Direct depressant effects of NDGA (nordihydroguaiaretic acid), AA 861, ETYA (5,8,11,14-eicosatetraynoic acid), and proadifen were 4.0, 15.4, 18.9, and 24.0%, respectively. *, p<0.05; **, p<0.02; ***, p<0.01 vs. EFS. See others in Table 1.

Differential effect of catalase

To further characterize the involvement of superoxide anion in the release of relaxing factor, catalase effect was compared with SOD (Table 2).

In the experiment with EFS or HX-XO, prior incubation with SOD (150 unit/ml) in the donor bath prevented the reduction of contraction to 5-HT regardless of the sources of endothelial cells. Otherwise, upon pretreatment with catalase (150 unit/ml) in the donor bath with cultured endothelial cells, the prevention of reduced contraction was not evident. However, when the suffusate from PCA with endothelium was exposed to EFS or HX-XO after pretreatment with catalase in the donor bath, this prevented the reduction of contraction of the PCA, suggesting involvement of hydrogen peroxide. The prevention by catalase was not manifested by the suffusate incubated with cat thoracic aorta endothelium or cultured endothelial cells.

Effect of inhibitors of arachidonate metabolism

As summarized in Table 3, prior incubation of endothelium with 30 μM gossypol, 10 μM NDGA and

Table 4. Effect of different inhibitors of arachidonate metabolism on the superoxide-mediated, endothelium-dependent relaxation of porcine coronary arterial strips

Agents	Doses	n	Control (g)	Contraction (%)	
				EFS	Pretreatment
Indomethacin	5 μM	5	2.50±0.33	58.0±6.2	51.5± 7.1
ETYA	0.5 μM	6	2.63±0.23	56.6±3.5	108.2±10.9*
Proadifen	64 μM	9	2.48±0.37	60.1±2.8	79.4± 8.6
Cimetidine	300 μM	3	2.12±0.39	62.9±3.2	64.8± 8.3

EFS was applied to porcine coronary artery with endothelium. As a detector muscle, porcine coronary artery without endothelium was contracted to 0.3 μM 5-HT. *, $p<0.001$ vs. EFS. See others in Table 1.

25 μg/ml AA 861 in the donor bath prevented the reduction of contraction of cat basilar arterial strips caused by indirect exposure to EFS. In this experiment, when NDGA and AA 861 were applied directly they depressed 5-HT-induced contraction by 4.0 and 15.4%, respectively. ETYA (0.5 μM) has completely prevented the depression of contraction as contrasted to indomethacin (5 μM) or proadifen (64 μM). On the other hand, when either NDGA, AA 861, or ETYA was added into the donor bath after cessation of EFS, each exerted little change in the contraction to the 5-HT of cat basilar artery, indicating that these drugs do not interact with the relaxing factor after release.

Likewise, a similar experiment was carried out by using porcine coronary arterial strips as both donor and detector vessels, respectively. In this experiment, indomethacin, proadifen and cimetidine showed little effect on the reduction of contraction, whereas ETYA still exerted the preventive effect (Table 4).

DISCUSSION

In the present study it was demonstrated that the superoxide-dependent relaxing factor, released from the endothelium of PCA, exerted a potent reduction in the contraction of the smooth muscle of PCA as well as cat basilar artery. Also, similar findings were observed for the endothelium from cat thoracic aorta or for cultured endothelial cells of bovine aorta when they were used as a donor. These results were obtained by using either of two superoxide-generating systems: EFS or HX-XO system. The reduction was prevented by prior incubation with SOD irrespective of superoxide-generating systems used and the source of endothelial cells used as donor (McCord and Fridovich, 1969), indicating that the release of the relaxing factor is apparently dependent on the superoxide anion. These findings are consistent with the previous reports (Hong *et al.*, 1989a,b) and support an idea that various relaxing factors are homogenous irrespective of vascular origins.

However, it is noteworthy that there is a novel difference among these relaxing factors. When PCA with endothelium was used as a donor, pretreatment with catalase could prevent the reduction of contraction caused by the relaxing factor derived from endothelium. This is much different from the results of the cultured endothelial cells of bovine aorta or cat thoracic aorta. This experiment indicates that in this system hydrogen peroxide is produced and involved in vascular relaxation. There are two different sources of hydrogen peroxide; one is an endogenous hydrogen peroxide which is translocated from the endothelium (Kellog and Fridovich, 1975; McCord and Fridovich, 1969) and the other is an exogenous hydrogen peroxide generated in the medium extracellularly (Del Maestro, 1980; Halliwell and Gutteridge, 1985). Thus, further study is required to elucidate the origin of hydrogen peroxide and also the different tissue sensitivity toward hydrogen peroxide.

In addition, we found that several lipoxygenase inhibitors such as gossypol (Hamasaki and Tai, 1985; Förstermann *et al.*, 1988), NDGA, AA 861 (Yoshimoto *et al.*, 1983) and ETYA significantly blocked the reduction of contraction induced by the relaxing factor. Cyclooxygenase inhibitor, indomethacin and cytochrome P-450 monooxygenase inhibitor, proadifen (Pinto *et al.*, 1986) showed little change in reduction of contraction. It is difficult to see the products of cyclooxygenase and cytochrome P-450 monooxygenase enzyme pathways to reduce the contraction. ETYA (cyclooxygenase and lipoxygenase inhibitor) and AA 861 (a benzoquinone derivative and 5-lipoxygenase inhibitor) proved to be more potent blockers. The possible actions of these could be the results of; (1) inhibition of the

production and release of the relaxing factor, (2) inactivation of the factor by interaction with the drugs after release, and (3) direct inhibition of the contraction of detector muscle by the drug itself. Upon post-treatment with the above lipoxygenase inhibitors, their preventive effects were not evident, and moreover, their direct depressant effects were not marked, thereby suggesting that the blocking effects can be ascribed to the inhibition of production and release of the factor. Lipoxygenase inhibitors such as gossypol, NDGA, and AA 861 also possess antioxidant property (Förstermann *et al.*, 1988). Thus, suppression by these agents of the reduction of contraction is considered to be attributed to both lipoxygenase inhibition and antioxidant effects (Hong *et al.*, 1989a). Nonetheless, lipoxygenase enzyme is considered strongly responsible for the production of the relaxing factor because ETYA, which has little antioxidant property, potently exerted the preventive effect. Addition of ETYA to the bath with PCA similarly exhibited blockage of the reduction of contraction as contrasted to the effects of indomethacin, proadifen and cimetidine (Pelkonen and Puurunen, 1980).

Interestingly, in the presence of indomethacin, the suffusate subjected to EFS in the presence of endothelium with exogenous arachidonic acid (10 μM) caused an enhancement of the reduction of contraction of cat basilar artery (data not shown). On the basis of the observations that endothelial cells contained some types of lipoxygenase enzyme (Hopkins *et al.*, 1984; Buchanan *et al.*, 1985) and that 15-lipoxygenase products of arachidonic acid exerted relaxing effects of rat aorta (Uotiola *et al.*, 1987), these results provide additional evidence to support the involvement of lipoxygenase in association with arachidonic acid for the production of the relaxing factor. However, leukotrienes (LTC_4 and LTD_4), products of arachidonic acid via 5-lipoxygenase, have been demonstrated to constrict sheep coronary artery (Fauler and Frölich, 1989) and guinea pig basilar artery (Nishiye *et al.*, 1988) as well as PCA of this study (data not shown). Based on these reports, a lipoxygenase product other than leukotrienes is considered to be the relaxing factor. It was reported that in neonatal rat arterial cells and gastric smooth muscle, K^+ selective channels are also activated by arachidonic acid with increases in K^+ conductance (Kim and Clapham, 1989). However, it remains to be determined whether the ability of endothelium to release this relaxing factor in response to superoxide anion can account for an attenuation of vascular reactivity (coronary or cerebral arterioles) in a variety of diseased states such as ischemia-reperfusion injury and shock.

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=국문초록=

Superoxide에 의존하여 내피세포에서 유리되는 이완성 물질의 특성에 대한 실험적 연구

부산대학교 의과대학 약리학교실

이기남·이원석·임병용·홍기환

최근 본 교실에서는 two-bath system을 이용하여 혈관 내피세포에서 superoxide에 의존한 혈관 이완성 물질을 동정하여 발표하였다. 본 실험에서는 상기 system을 이용하여 돼지의 관상동맥 내피세포에서 유리되는 superoxide에 의존한 이완성 물질이 고양이의 흉부 대동맥 내피 및 소의 대동맥 배양 내피세포에서 얻어진 이완성 물질에 의한 이완과 매우 유사함을 관찰하여 다음과 같은 결과를 얻었다.

1. 고양이 흉부 대동맥, 돼지 관상동맥의 내피세포 및 소 대동맥 배양 내피세포 등에서 유리되는 superoxide에 의존한 이완 물질은 모두 유사한 이완 작용을 나타내었다.

2. 돼지 관상 동맥 내피세포에서 유리되는 superoxide 의존성 이완 물질이 고양이의 흉부 대동맥 내피세포나 소의 대동맥 배양 내피세포에서 유리되는 이완 물질과는 다소 다른 점도 있었다. 즉, 돼지 관상동맥 내피세포에서 유리되는 이완 물질의 작용은 catalase나 superoxide dismutase(SOD)에 의하여 억제되었으나, 후자의 두 동맥 내피세포에서 유리되는 이완 물질은 SOD에 의해서만 억제되었다.

3. 이러한 이완성 물질들의 생성은 여러 lipoxygenase 억제제인 gossypol, nordihydroguaiaretic acid, AA 861 및 eicosatetraynoic acid 등의 전처치에 의하여 봉쇄되었다.

4. Cyclooxygenase 억제제인 indomethacin이나 cytochrome P-450 monooxygenase 억제제인 proadifen과 cimetidine에 의하여는 봉쇄되지 아니하였다.

이상의 결과로부터 이러한 이완성 물질들은 비록 각기 다른 종의 동물 모델에서 얻었다고 하더라도 장기에 따라 다소 반응의 차이는 있으나 동질성 이완 물질이며, 나아가 이러한 이완성 물질은 여러 조직의 허혈-재관류 손상에 있어서 병리생리학적으로 관련될 것으로 사료된다.