Assessment of superoxide-mediated release of vascular-inhibitory factor(s) from endothelial cells by using a two-bath system

K. W. Hong, W. S. Lee, B. Y. Rhim and Y. W. Shin[a]

Department of Pharmacology, College of Medicine and [a]Research Institute of Community Medicine, Pusan National University, Ami-Dong, Seo-Gu, Pusan 602-739 (Korea)
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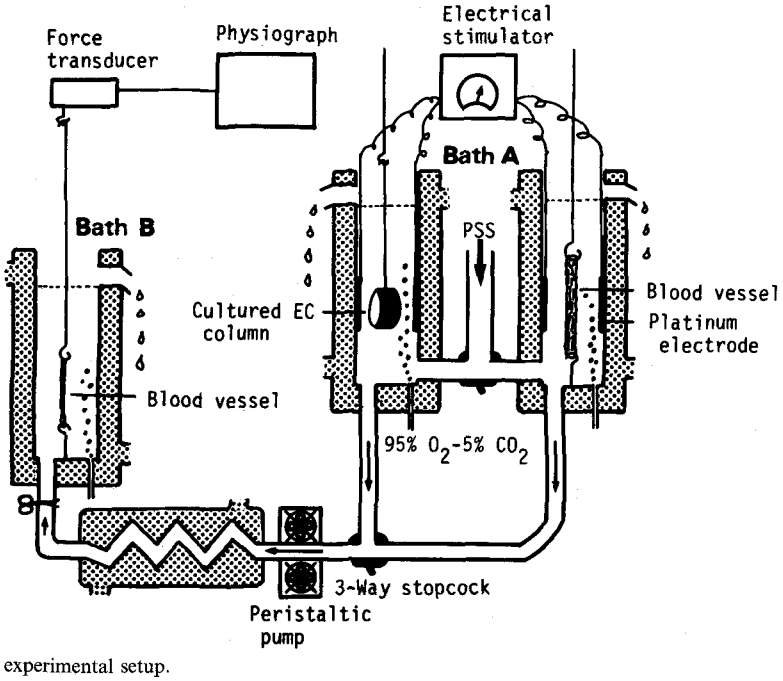
Summary. Release of a vascular-inhibitory factor from endothelial cells (EC), different from endothelium-derived relaxant factor (EDRF), was identified through use of a two-bath system. This two-bath system precluded the effects of oxygen-free radicals that appear when electrical field stimulation (EFS) is directly imposed on detector muscle.
Key words. Two-bath system; vascular-inhibitory factor; superoxide anion.

The relaxation of isolated blood vessels in response to EFS has been ascribed to activation of adrenergic[1] or histaminergic[2] mechanisms or a release of unidentified substance from endothelial cells (EC)[3]. Recently, direct EFS on isolated strips of rat tail artery and dog coronary artery has been demonstrated to cause inhibition of contractile response to noradrenaline, due to the generation of oxygen free radicals[4]. On the other hand, endothelium-derived relaxant factor (EDRF), a labile factor, has been widely reported to be involved in the relaxation of arterial vascular beds through formation of cyclic GMP, and cyclic GMP-dependent phosphorylation[5,6]. In the present study, by employing a two-bath system we identified a release of a vascular-inhibitory factor from the EC by mediation of superoxide anion, which could not be detected by direct EFS.

Materials and methods. The figure shows a schematic diagram of the experimental setup, designed for the two-bath system. In bath A either a thoracic aortic segment or a cultured EC column was suspended. In bath B a detector muscle was mounted. The thoracic aortas were excised from cats (2–3 kg) and cleared of surrounding tissues. Aortic rings (approximately 2 cm long) were opened along their longitudinal axis with special care not to hurt the endothelial lining and then suspended between platinum plate electrodes under a resting tension of 1.0 g. The EC columns were prepared by packing cultured bovine aortic EC into a device covered with membrane filters (0.45-μm pores), which was then installed between platinum electrodes. In bath B, an isolated strip of basilar artery, helically cut and denuded of endothelium, was mounted in a 4-ml tissue bath to serve as a detector muscle.

Throughout the experiment, the physiological salt solution (PSS; mM, NaCl 130, KCl 4.7, $NaH_2PO_4 \cdot 2\ H_2O$ 1.18, $MgSO_4 \cdot 7\ H_2O$ 1.17, $CaCl_2 \cdot 2\ H_2O$ 1.6, $NaHCO_3$ 14.9, dextrose 5.5) was maintained at 37 °C and gassed with 95% O_2 and 5% CO_2. The contraction was evoked by 10^{-6} M 5-hydroxytryptamine (5-HT) after treatment with atropine, propranolol and de-



A schematic diagram of the experimental setup.

sipramine (0.2 μM each) and indomethacin (0.2–1.0 μM). In the control, the PSS (bath A) subjected to electrolysis in the absence of EC was suffused to the detector muscle, and then contraction was evoked by 5-HT[4, 7, 8]. Two kinds of EFS (square-wave biphasic pulse, 2-ms pulse duration at 10 Hz, 50 μA current flow for 5 min) were introduced. In one set of experiments, EFS was directly applied to the detector muscle. In another set, EFS was applied to either the aortic segment with intact EC or to the cultured EC column in the bath A, and the resultant PSS was suffused into bath B after a two-minute delay by means of a peristaltic pump (Harvard Model 1210) with a transit time of less than 10 s. In a third set of experiments, a hypoxanthine (HX, 50 μM) and xanthine oxidase (XO, 0.02 units/ml) system was introduced into bath A to generate superoxide anion instead of EFS.

EC cultures. Freshly-harvested bovine aortic intima cells were seeded in standard culture dishes (Falcon) containing Eagle's minimum essential medium (Eagle's MEM, Gibco) with 20% fetal bovine serum, penicillin G (80,000 units/l) and streptomycin (80 mg/l) and then cultured in a humidified 5% CO_2-incubator. Following 7–9 passages, confluent cells ($1-4 \times 10^6$ per bottle) were detached with trypsin (0.25%)-EDTA (0.01%), collected by centrifugation (1,000 rpm for 10 min), and then sequestered into a plastic column covered with membrane filters.

Statistics. Data are expressed as mean ± SEM. Statistical analysis was carried out by Student's t-test for paired or unpaired observations. When the p-value was less than 5%, contractile differences were considered to be statistically significant.

Results and discussion. Following direct application of EFS to the isolated strip of basilar artery, the tension in response to 5-HT (10^{-6} M) was reduced by 29.9% (table 1) as demonstrated by Lamb and Webb[4]. Likewise, the ED_{50} value for 5-HT was greatly increased (from 0.16 ± 0.03 to 2.90 ± 1.04 μM, $p < 0.01$). Reduced contractility in response to 5-HT was also observed when an isolated strip of basilar artery was exposed to the suffusate in which EFS was applied to either the aortic segment with intact EC or to the cultured EC column, when using the two-bath system (fig. and table 1). In contrast, electrical stimulation of the aortic segment denuded of EC did not result in an inhibitory effect on the detector muscle (table 1). Hence, it can be concluded that reduction of contractility induced by EFS requires intact EC. When, instead of EFS, the aortic segment with endothelium was treated with HX-XO for 5 min, 20 nM allopurinol (an inhibitor of xanthine oxidase)[9] was additionally injected prior to suffusion to preclude any direct effect of XO on the detector muscle. The suffusate exposed to the HX-XO system in bath A resulted in an effect similar to that of EFS, suggesting the release of a vascular-inhibitory substance which is mediated by superoxide anion.

Table 1. Effect of direct system or two-bath system on the 5-HT-induced contraction of cat basilar artery strip in bath B

	Contraction to 5-HT, mg (%)			
	Control[a]	EFS	Control	HX-XO
A. Direct system	402 ± 20	277 ± 36* (70.1)	434 ± 46	333 ± 63* (70.2)
B. Two-bath system				
Aortic segments				
With EC	380 ± 41	228 ± 53* (63.3)	420 ± 51	256 ± 64* (65.6)
Without EC	373 ± 38	348 ± 36 (96.6)	411 ± 31	446 ± 45 (112.5)
Cultured EC[b]	394 ± 36	210 ± 26** (59.0)	350 ± 32	230 ± 11* (66.9)

In the two-bath system, EFS or HX-XO was previously applied to the cat aortic segment or cultured bovine aortic EC in the tissue bath A. In case of HX (50 μM)-XO (0.02 units/ml) system, allopurinol (20 nM) was applied and 2 min later the PSS was delivered to bath B. [a] Control: in A: contraction prior to direct EFS; in B: contraction in the suffusate subjected to electrolysis in the absence of EC. [b] Cultured EC was packed in the device covered with membrane filter (0.45 μm, $5.0 \pm 0.51 \times 10^6$ cells, average viability of 90.4%). Each value represents mean ± SEM of 6–8 experiments. Percent in parentheses was calculated from the corresponding control. * $p < 0.01$; ** $p < 0.001$.

Table 2. Effect of oxygen-free radical scavengers on direct EFS-induced inhibition of contraction in cat basilar artery

Scavengers	n	% Contraction to 5-HT	
		Before treatment	After treatment
Superoxide dismutase (50 units/ml)	6	59.4 ± 8.9	96.8 ± 15.0**
Catalase (150 units/ml)	4	60.6 ± 7.0	100.0 ± 9.3*
Dimethyl sulfoxide (10 mM)	5	72.5 ± 7.7	100.0 ± 9.2*
Glutathione (36 μM)	5	66.3 ± 10.3	103.7 ± 4.9*
Ascorbic acid (500 μM)	5	69.1 ± 6.9	99.9 ± 9.4*

Percent changes were calculated from control contraction to 1.0 μM 5-HT. n, No. of experiments. * $p < 0.05$; ** $p < 0.01$.

In the experiment with direct electrical stimulation (table 2), incubation with superoxide dismutase (SOD)[8], catalase (hydrogen peroxide scavenger) and DMSO (hydroxyl radical scavenger)[10] prevented the inhibition of contraction by EFS. Thus it is suggested that the inhibition by direct EFS can be ascribed to the action of the hydroxyl radical generated. This is consistent with the earlier reports[4, 7]. Nevertheless, in the current study employing the two-bath system (table 3), the inhibition caused by suffusate indirectly exposed to EFS or HX-XO was not reversed by pretreatment with catalase, DMSO and mannitol, but was completely prevented by SOD. Hence, we deduce that the vascular-inhibitory factor from EC may not be mediated by generation of hydroxyl radical[11]. This fact presents a striking contrast to the results from a direct EFS. Glutathione and ascorbic acid are acceptors of electrons and destroy oxygen radicals[12]. Accordingly, these antioxidants are considered to exert similar protective effects on both the direct and indirect two-bath EFS systems. EDRF is another agent that is released from vascular EC; it is very unstable, with a half-life of 6–40 s[13, 14]. It has been demonstrated that

Table 3. Effect of oxygen-free radical scavengers on the contraction-inhibition induced by the suffusate transferred from tissue bath A, in which EFS was applied to the aortic segment with intact EC.

Scavengers	n	% Contraction to 5-HT	
		Before treatment	After treatment
Superoxide dismutase (50 units/ml)	6	65.4 ± 4.8	99.0 ± 3.1 *
Catalase (150 units/ml)	6	62.3 ± 3.1	68.7 ± 3.9
Dimethyl sulfoxide (10 mM)	5	48.4 ± 5.2	63.9 ± 7.9
Mannitol (10 mM)	5	53.8 ± 3.8	67.7 ± 7.8
Gluthathione (36 μM)	5	69.3 ± 10.4	94.7 ± 7.1 *
Ascorbic acid (500 μM)	5	60.5 ± 4.9	89.7 ± 5.6 *

n, No. of experiments. * $p < 0.05$.

superoxide anion is involved in the breakdown of EDRF[15]. We have observed that EFS on the aortic segment with intact EC caused an inhibition of acetylcholine-induced cyclic GMP formation[6], which was prevented by prior treatment with SOD (data not shown). Consequently, both the existence of superoxide anion[15] in bath A and the two-min delay in suffusion of PSS[13, 14] to bath B, preclude the involvement of EDRF in the vascular smooth muscle relaxation.

Based on these considerations, it is presumed that this novel vascular-inhibitory factor is different from the well-known EDRF. However, it remains to be determined whether the ability of EC to release this vasodilator in response to superoxide anion can account for a diminished vascular reactivity in a variety of diseased states such as shock or ischemia-reperfusion injury.

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Flow microcalorimetry as a tool for an improved analysis of antibiotic activity: The different stages of chloramphenicol action

D. Krüger and P. Giesbrecht

Robert Koch Institute of the Federal Health Office, D-1000 Berlin 65 (Federal Republic of Germany)
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Summary. Flow microcalorimetry in combination with photometric mass determination of staphylococci in suspension was used to reveal alterations in the intensity, extent and efficiency of bacterial metabolism during inhibition of protein synthesis by chloramphenicol. It could be demonstrated that these three parameters of metabolic activity were distinctly affected by this drug, and that the method described promises to be a more reliable tool for assaying the degree and the mode of bacteriostatic inhibition than the conventional determination of the minimum inhibitory concentration.
Key words. Microcalorimetry; antibiotics; minimal inhibitory concentration; photometry; staphylococci; metabolic activity; chloramphenicol.

The suitability of flow microcalorimetry for characterizing and quantifying antibiotic-induced alterations in the metabolism of microorganisms has been pointed out in several investigations[1-4]. Because living microorganisms produce heat, characteristic power-time curves (= pt-curves) could be recorded before and after addition of drugs to microbial suspensions[5]. Cultivation of bacteria in a conventional, complex nutrient broth yielded microcalorimetric curves which varied according to the intensity and the extent of the underlying processes, and which proved to be complex[6]; such curves have been considered to reflect a diauxic activity of the microbial