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Electrolysis of Physiological Salt Solution Generates a Factor that Relaxes Vascular Smooth Muscle

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Oxygen-derived free radicals have been implicated in many important functions in the biological system. Electrical field stimulation (EFS) causes arterial relaxation in animal models. We found that EFS applied to neither muscle nor nerve but to Krebs solution caused a relaxation of rat aorta that had been contracted with phenylephrine. In the present study, therefore, we investigated the characteristics of this EIRF (electrolysis-induced relaxing factor) using rat isolated aorta. Results indicated that EIRF acts irrespective of the presence of endothelium. EIRF shows positive Griess reaction and is diffusible and quite stable. EIRF-induced relaxation was stronger on PE-contracted aorta than on KCl-contracted one, and inhibited by the pretreatment with methylene blue. Zaprinast, a cGMP-specific phosphodiesterase inhibitor, potentiated the EIRF-induced relaxation. N^G-nitro-L-arginine, NO synthase inhibitor, did not inhibit the EIRF-induced relaxation. Deferroxamine, but not ascorbic acid, DMSO potentiated the EIRF-induced relaxation. These results indicate that electrolysis of Krebs solution produces a factor that relaxes vascular smooth muscle via cGMP-mediated mechanism.

Key Words: Vascular relaxation, cGMP, Electrolysis, Nitric oxygen, Oxygen radicals

INTRODUCTION

Oxygen-derived free radicals have been proposed as mediators of tissue injury in a variety of disease states. Lamb & Webb (1984) demonstrated that electrical field stimulation (EFS) of isolated rat tail and dog coronary arteries inhibited contractile response to norepinephrine and potassium. Several radical scavengers were effective in reversing the loss of contractile responses, thus implicating a possible role of oxygen-free radicals in the alteration of vascular reactivity. A variety of isolated systemic arterial preparations relax in response to EFS by endothelium-independent mechanisms that appear to involve adrenergic, cholinergic, and nonadrenergic-noncholinergic neurotransmission (Bevan et al, 1982; Cohen et al, 1983; Buga & Ignarro, 1990). Vascular smooth muscle relaxation caused by EFS of mammalian blood vessels entails multiple mechanisms that vary with the vascular bed and species (Hardebo et al, 1989). Isolated canine and bovine coronary arterial preparations relaxed in response to EFS by endothelium-independent mechanisms that did not involve classic neurotransmitters (Feletou & Vanhoutte, 1987; Kalsner & Quillan, 1989; Rooke et al, 1982). EFS of pulmonary arterial rings from rabbit, cat and monkey, however, was reported to cause endothelium-dependent relaxation that did not involve classical neurotransmitter release or arachidonic acid metabolism (Frank et al, 1983). In the rat mesenteric vasculature, nitric oxide (NO) has been shown to enhance release of neural norepinephrine (NE) elicited by EFS (Yamamoto et al, 1993; Yamamoto et al, 1994). Recently, hydroxyl radical has been shown to produce relaxation of aortic strips (Prasad & Bharadwaj, 1996). At the present time, the mechanism of action of EFS-induced vascular smooth re-

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laxation still unclear. During the experiment with EFS-induced relaxation, we found that aliquots of electrolyzed solution caused a relaxation, concentration-dependently, when it applied to the aorta that had been contracted with phenylephrine. Thus, in the present study, we investigated the characteristics of EIRF (electrolysis-induced relaxing factor) using rat aorta.

METHODS

Materials

Phenylephrine HCl (PE), indomethacin, N^{w}-nitro-L-arginine (L-NNA), dimethyl sulfoxide (DMSO), methylene blue (MB), mannitol and deferroxamine mesylate were purchased from Sigma Chemical Co. (St. Louis, USA). Zaprinast was obtained from Bio-Mol (U.K) and ascorbic acid from Handok Pharmaceutical Co., Korea.

Tissue preparations

Male Wistar rats of either sex (250~300 g) were sacrificed by stunning and the thoracic aorta was removed. The tissues were cleaned of adhering fat and connective tissue, and ring preparations were (3~4 mm wide) made as described elsewhere. For relaxation study of organ bath system, othewise indicated, endothelium-denuded ring preparations were used.

Cascade bioassay

The cascade bioassay was set up described previously (Chang et al, 1997). In brief, intact perfused artery was used as the detector of EIRF, and either two endothelium intact or endothelium-denuded arterial rings mounted in series were served as the another detector of EIRF. Electrolysis (90V, 5 msec with varying frequencies and duration) was performed by placing platinum wire electrodes into the inflow tract just above the aorta. The rings were equilibrated at 1g for 50 min. Following submaximal (approximately 80%) pre-contraction of tissues with the PE, vascular responses were recorded on a Grass physiograph (Model 7E) using a force displacement transducer (FT-03).

Condition of the electrolysis of physiological buffer

Electrolysis was performed by placing platinum wire electrodes into the Krebs solution (500 ml), soon after or later. An aliquot (5~100 μl) of the solution was added to the organ bath in which aortic preparations were mounted for the isometric tension study. EFS was conducted at 90 V with a frequency of 2, 5, 10, 20, 30, 100 or 200 Hz in the form of square wave pulses of 5 msec duration. To know the effects of duration of stimulation time, EFS was delivered for 5, 10, 20, 30, 45 and 60 sec. Krebs-Ringer bicarbonate solution was gassed with 95% O_2 −5% CO_2 and had the following composition (mM): NaCl, 136.9; KCl, 5.4; $MgCl_2$, 1.0; $NaHCO_3$, 23.8; $CaCl_2$, 1.5; glucose, 5.5; and EDTA, 0.03.

Relaxation study with electrolyzed-Krebs solution containing different antioxidants

To understand the reactive oxygen species responsible for the relaxation produced from the electolyzed solution (EIRF), endothelium intact or denuded aortas that had been contracted with PE or KCl were used. After reaching plateau contraction, electrolysis was performed in the presence or absence of different antioxidant, which was introduced into the inflow tract just above the aorta or cumulatively to the organ bath, and then relaxation response was constructed as regarding 100% the maximum contraction of PE. The response was also evaluated in the presence of methylene blue to know the mechanism of action of EIRF-induced relaxation. To characterize the EIRF, zaprinast, cGMP-specific phosphodiesterase inhibitor, and L-NNA, NO synthase inhibitor, were included.

Measurement of nitrite/nitrate

In separate experiment, electrolysis was performed by placing platinum wire electrodes into an Effendorf tubes containing 500 μl of Krebs solution with stimulations of varying frequency and time. An aliquot of solution (100 μl) was then taken and measured nitrite contents by adding 100 μl of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediaminene in 5% phosphoric acid). Optical density at 550 nm (OD_{550}) was measured with a microplate reader. Nitrite concentrations were calculated by comparison with OD_{550} of standard solution of sodium nitrite prepared in distilled water.

Statistics

Data are expressed as mean±SEM. Differences between two groups were determined by Student's t-test and were considered significant if $P<0.05$.

RESULTS

Characteristics of electrolyzed solution-induced arterial relaxation

Fig. 1 shows the characteristic of EIRF-induced vascular reactivity at different frequencies. Below 5 Hz stimulation, no relaxation response was observed (data not shown). Addition into the organ bath of electrolyzed solution to the aortic ring that has been contracted with PE caused relaxation, indicating that this relaxation is not result from the direct nerve stimulation. In a bioassay superfusion system, at high frequencies (e.g. 100 or 200 Hz for 5 sec.), the response was changed to initial contraction followed by relaxation. The short lived initial component of contraction was manifested only in donor arteries, but was not detected in recipient rings (Fig. 2). EIRF-induced relaxation was independent of the presence of endothelium. However, repeated infiltration of electrolyzed solution caused damage to endothelium (data not shown).

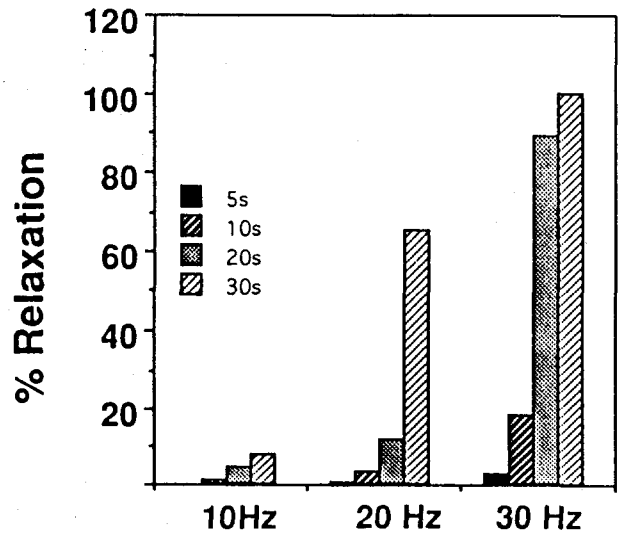

Fig. 1. Relaxation response of electrical filed stimulation (90V, 5 msec) with different frequencies and duration of stimulation. An aliquote (5 μl) of each electrolyzed solution was added to the PE-contracted aorta.

Accumulation of nitrite/nitrate was proportional to EFS intensity

Nitric oxide (NO) was measured as its stable oxidative metabolites. Table 1 shows nitrite/nitrate contents in Krebs' solution electrolysed with different duration and frequencies at fixed voltage (90 V, 5 msec square wave pulse). When used 0.5 msec aquare wave pulse instead of 5 msec, nitrite/nitrate contentents were detected only at high frequency (above 100 Hz) stimulation, which also depended on the duration of stimulation (data not shown).

Effects of methylene blue, L-NNA and zaprinast on the relaxation

In organ bath system, EIRF-induced relaxation was

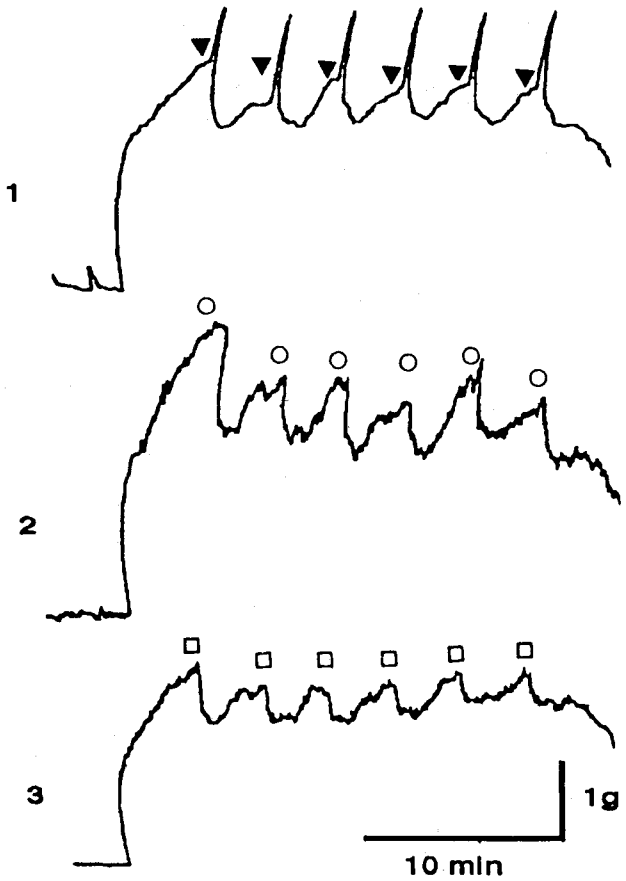

Fig. 2. Typical tracings of EIRF-induced relaxation in the cascade superfusion system. The electrolyis (90 V, 5 msec, 200 Hz) was performed 5 cm above the intact perfused artery (artery 1) and two endothelium denuded arterial rings mounted in series were served as the detector of EIRF (artery 2 and 3).

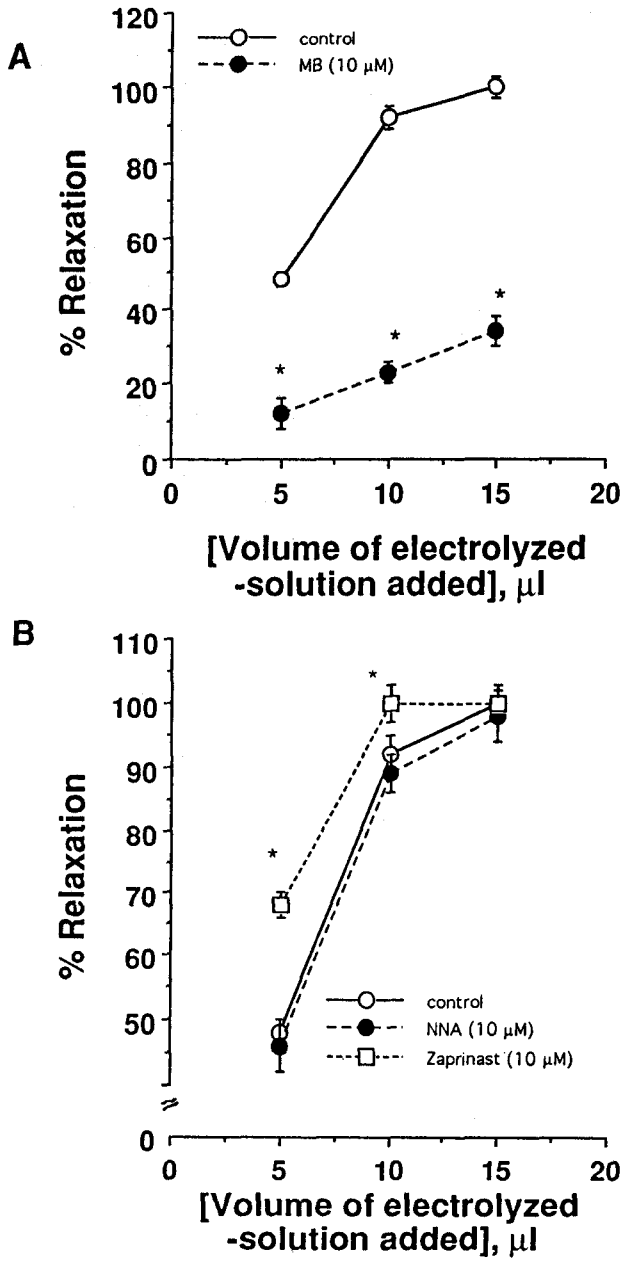

Fig. 3. Effects of methylenblue (MB), NNA and zaprinast on cumulative addition of aliquots of electrolysed solution (90 V, 5 msec, 200 Hz for 5 sec duration) in PE-contracted aorta.

significantly ($p<0.05$) inhibited by the pretreatment with methylene blue for 30 min. L-NNA did not affect the relaxation response by EIRF, but zaprinast significantly ($P<0.05$) potentiated the EIRF-induced relaxation (Fig. 3).

Effects of antioxidants to EIRF-induced relaxation

The relaxation induced by electrolyzed solution

Table 1. Effects of electrical stimulation (90V, 5 msec) on the nitrite/nitrate accumulation depending on the freqency and duration of stimulation (unit: μM)

time / Frequency	Second					
	5	10	20	30	45	60
10 Hz	N.D	N.D	N.D	N.D	N.D	0.46
20 Hz	N.D	N.D	N.D	0.12	3.13	28.1
30 Hz	N.D	N.D	N.D	0.25	47.6	151.6
100 Hz	5.8	20.4	162.3	241.5	285.9	291.9

N.D: not detected.
Nitrie/nitrate contents were measured by Griess reagents

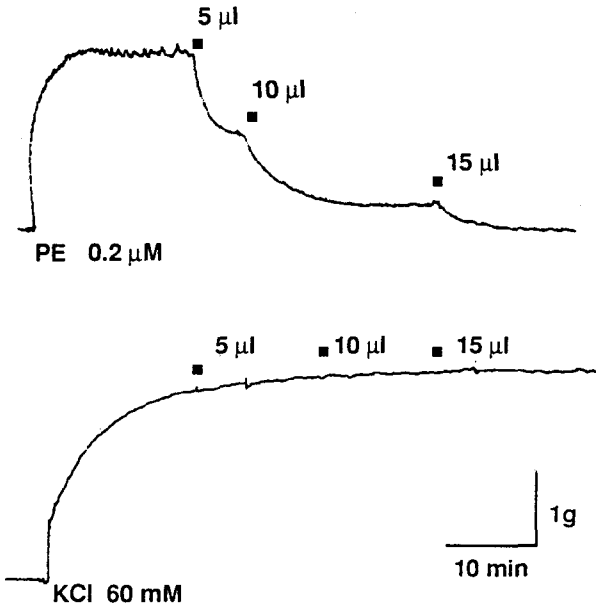

Fig. 4. Typical tracings of EIRF-induced relaxation in PE and KCl-contracted aorta. An aliquot of electrolysed solution (90 V, 5 msec, 200 Hz) for 5 sec duration was added cumulatively.

was pronounced in PE-contracted aorta, but not in KCl-contracted one (Fig. 4). In cascade or organ bath system, as the frequency or stimulating time increases, the relaxation pattern was varied. This indicates that different species of free radicals may be generated depending on the electrical intensity. To scavenge the generated free radicals, some antioxidant, such as ascorbic acid and deferroxamine was added during the electrolysis. As shown in Fig. 5, electrolysis after addition of deferroxamine (30 μM) in inflow tract increased further the relaxation, and the initial component of contraction was disappeared.

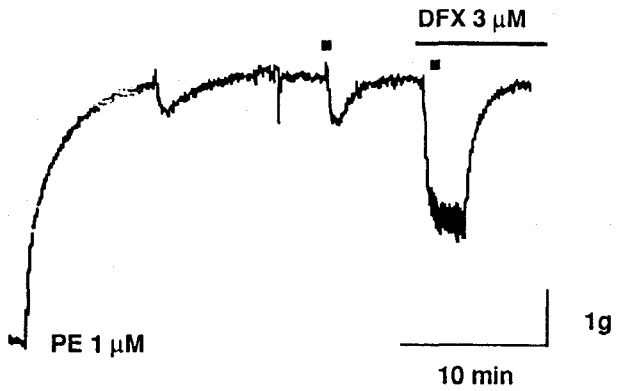

Fig. 5. Typical tracing of relaxation induced by EIRF and potentiation effect of deferoxamine (DFX). Electrolysis (90 V, 5 msec, 200 Hz for 5 sec duration, n) was performed from an inflow tract just 5 cm above the rat aorta reaching plateau contraction by PE. In the presence of DFX (30 μM) in inflow tract, application of electrolysis increased further the relaxation Note that after treatment with DFX, initial contraction component was disappeared.

On the other hand, ascorbic acid (1 mM) and DMSO (10 mM) did not affect the initial component of contraction, but reduced the relaxation, suggesting that iron inhibits EIRF-induced relaxation (data not shown).

DISCUSSION

In the present study, we demonstrated that electrolyzed Krebs' solution caused relaxation of isolated rat thoracic aorta irrespective of the presence of endothelium via EIRF. We purposely used strong electrical stimualtion (high frequencies and high voltage) to see how products of electrolysis can affect the vascular tone. In cascade experiments, the EIRF-elicited vascular response was biphasic pattern depending on the electrical intensity: initial contraction followed by relaxation, which did not show in organ bath system (aliquot experiments), indicating that the species for the initial contraction component are quite labile and this initial contraction component was not diffusible in contrast to EIRF, which is diffusible as measured by cascade system. The species responsible for the relaxation (EIRF) are quite stable, since when aliquot of electrolyzed solution stored in 4°C overnight were added to the aorta that had been contracted with PE, it still have activity to relax the vessel. Electrolysis of Krebs solution produces various kinds of oxygen-derived free radicals. Among the ROS (reactive oxygen species), superoxide anion is of particular interest. It inhibits endothelium dependent relaxation and is a precursor of other reactive oxygen radicals, such as hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$). Electrolysis of Krebs solution produced a positive Griess reaction indicating that nitrites are formed which in turn contributes at least in part to EIRF-induced relaxation. At the present time, from where nitrite/nitrates can be formed is not known. Since electrolysis of distilled water did not respond to Griess reaction, it is quite evident that nitrites species come from the Krebs solution. However, the species is not NO, because it is quite stable, possibly it may be peroxynitrite ($ONOO^-$). On the other hand, H_2O_2 can elicit different and sometimes complex actions on the tone of blood vessels (Rubanyi, 1988). Although in many instances, H_2O_2 causes vasodilatation via activation of soluble guanylate cyclase (Heinle, 1984), at higher concentrations, H_2O_2 can even constrict rabbit carotid artery and bovine intrapulmonary vessels (Wolin et al, 1991). We found that exogenously administered H_2O_2 relaxed the rat aorta irrespective of the presence or absence of endothelium (data not shown). Thus, this unidentified relaxation factor is likely to be H_2O_2. Because positive Griess reaction was detected in the present study, which was proportional to electrical intensity, therefore, it can be highly speculative that nitrogen sources can be oxidized by H_2O_2 possibly to $ONOO^-$. Exposure of isolated or intact vasculature to $ONOO^-$ results in relaxation (Liu et al, 1994; Wu et al, 1994; Villa et al, 1994). Mannitol, $OH^{\cdot}$ radical scavenger (McCord & Fridovich, 1973), was not effective to prevent EIRF-elicited relaxation, indicating that the relaxation factor responsible for the EIRF is not $OH^{\cdot}$ radical (data not shown). We reported the usefulness of photo-induced adequate nitric oxide (PIANO) generating system as a tool for the investigation of the role of NO (Chung & Chang, 1994; Chang, 1995; Chung et al, 1996). The PIANO-mediated relaxation study is effective in vascular smooth muscle, trachea, corpus cavernosum and gastrointestinal smooth muscle and uterine smooth muscle (Chung & Chang, 1994; Chang, 1995; Chung et al, 1996). But it is not effective in isolated electrically driven atrial cardiac muscles (Park et al, 1995). As electrolysis elicits oxygen free radicals, PIANO-generated NO may be converted to other inactive forms

such as peroxynitrite ($ONOO^-$), so this may be the reason why PIANO is not effective in electrically stimulated atrial tissues. Although, we did not measure the cGMP in the present experiment, the mechanism of EIRF-induced relaxation involves guanylate cyclase activation. Because, the relaxation was inhibited by the presence of guanylate cyclase inhibitor, methylene blue, and potentiated further by the presence of zaprinast. The exact nature of EIRF is not elucidated in the present study, but it is certain that mechanism of action of EIRF is mediated by guanylate cyclase activation via NO-like substance(s) or H_2O_2 for the following reasons: (1) the positive Griess reaction, (2) methylene blue-sensitive inhibition of the relaxation and (3) potentiation of the relaxation by zaprinast. In summary, we investigated the relaxation mechansim by factor(s) generated from the electrolysis of Krebs' solution. EIRF is diffusible and quite stable. EIRF-induced relaxation was inhibited by the pretreatment with methylene blue. Zaprinast, a cGMP-specific phosphodiesterase inhibitor, potentiated the EIRF-induced relaxation. Thus, it is concluded that electrolysis of Krebs solution produces factor(s) that relax vascular smooth muscle via cGMP-mediated mechanism. However further study is needed to elucidate the exact nature of ROS responsible for the relaxation.

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