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Loss of contractile activity of endothelin-1 induced by electrical field stimulation-generated free radicals

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1 Electrical field stimulation (EFS; 10 V, 10 Hz, 2 ms) of porcine coronary artery strips precontracted with 10 nM endothelin-1 (ET-1) for 5 min caused a biphasic response, consisting of a slight contraction during EFS and a marked and irreversible relaxation just after EFS. This irreversible relaxation after EFS has never been investigated. In the present study, we have investigated the mechanism of the relaxation after EFS.

2 The EFS-induced response was not affected by the presence or absence of endothelium and was insensitive to 10 μM tetrodotoxin (TTX).

3 In the presence of free radical scavengers (40 u ml^{-1} superoxide dismutase (SOD), 1200 u ml^{-1} catalase or 80 mM D-mannitol), the relaxation after EFS was significantly inhibited. Moreover, relaxation after EFS was not observed in porcine coronary artery strips precontracted with 20 mM KCl.

4 In a cascade experiment, EFS of Krebs-Ringer solution containing 10 nM ET-1 induced marked suppression of the contractile activity of ET-1 in porcine coronary artery strips, which was in accord with the observed decrease in release of immunoreactive ET-1 (ir-ET-1). This effect of EFS was significantly inhibited by each of the free radical scavengers, 3 mM vitamin C, 40 u ml^{-1} SOD, 1200 u ml^{-1} catalase and 80 mM D-mannitol.

5 The exchange of 95% O_2/5% CO_2 gas for 95% N_2/5% CO_2 gas significantly inhibited the EFS-induced decrease in release of ir-ET-1.

6 Neither superoxide anions generated by xanthine (10 μM) plus xanthine oxidase (0.1 u ml^{-1}) nor hydrogen peroxide (10 μM) exogenously added to Krebs-Ringer solution containing 10 nM ET-1 affected the level of ir-ET-1.

7 Generation of hydroxyl radicals was detected in the EFS-applied Krebs-Ringer solution. The EFS-induced generation of hydroxyl radicals was dependent on the period of stimulation and O_2-bubbling, and significant generation of hydroxyl radicals was detectable with stimulation of over 5 min. Moreover, hydroxyl radicals generated in 50 mM NaCl solution containing 10 nM ET-1 by H_2O_2 plus Fe^{2+}, i.e. the Fenton reaction, significantly decreased the level of ir-ET-1.

8 These findings suggest that oxygen-derived hydroxyl radicals generated by EFS of porcine coronary artery strips inactivate ET-1, probably by structural modification. Thus, porcine coronary artery strips precontracted with ET-1 are potently relaxed by EFS.

Keywords: Coronery artery; endothelin-1; electrical field stimulation; hydroxyl radicals

Introduction

Relaxation of various isolated blood vessels from different species in response to electrical field stimulation (EFS) has been described. Such relaxations are generally thought to be caused either by the activation of postjunctional β-adrenoceptors secondary to release of catecholamines from adrenergic nerve endings (Cohen *et al.*, 1983) or by the release of vasorelaxant substances from noradrenergic and noncholinergic nerves (McCulloch & Edvinsson, 1980; Toda, 1982; Fujimori *et al.*, 1989; Verma *et al.*, 1993) or from the endothelium (Buga & Ignarro, 1992; Van Riper & Bevan, 1992). However, EFS-induced responses in isolated blood vessels show wide variation, depending on the segment of artery, species and stimulation conditions (Rooke *et al.*, 1982; Feletou & Vanhoutte, 1989). Some investigators have recently demonstrated that the production of oxygen-derived free radicals by EFS could provoke vasodilator responses (Lamb & Webb, 1984; Greenberg *et al.*, 1986). Especially, prolonged EFS generates enough oxygen-derived free radicals to produce a change in tone of vascular smooth muscle (Feletou & Vanhoutte, 1987).

Endothelin (ET-1) provokes a strong and sustained contraction in various isolated vascular smooth muscle preparations through the stimulation of ET_A receptor (Kasuya *et al.*, 1992; Sakurai *et al.*, 1992). Thus, ET-1 is a suitable tool to investigate the EFS-induced vasorelaxant response.

In preliminary experiments, we investigated the response of porcine coronary artery strips precontracted with 10 nM ET-1 to prolonged EFS, and observed a marked and irreversible relaxation after EFS. Such relaxations has never been investigated. Thus, the present study was designed to elucidate the mechanism of post-EFS relexation in porcine coronary artery strips.

Methods

Measurement of contraction of arterial strips

Right coronary arteries were isolated from fresh adult porcine hearts obtained from a local slaughterhouse. Arterial segments were cut into 2 × 7 mm helical strips, endothelial cells were removed by rubbing the intimal surface with a cotton swab and the segments were suspended in 20-ml siliconized glass organ chambers filled with Krebs-Ringer solution of the following composition (mM): NaCl 113, KCl

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4.8, $CaCl_2$ 2.2, KH_2PO_4 1.2, $MgCl_2$ 1.2, $NaHCO_3$ 25 and glucose 5.5. Depending on the segment used, EFS induced different responses. Thus, we used coronary artery segments restricted to within 2 to 4 cm from the aorta. The solution was maintained at 37°C and gassed with 95% O_2/5% CO_2. Arterial strips were equilibrated at a passive tension of 1.25 g until the contractile tension induced by 50 mM KCl attained a steady state. Isometric contraction was measured by means of a force displacement transducer (Nihon Koden TB-612T) as previously described (Kasuya *et al.*, 1989). In some experiments, artery strips with intact endothelium which showed greater than 75% inhibition of precontraction with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 2 μM) in response to ionomycin (10 nM) were used.

Electrical field stimulation

Electrical field stimulation (EFS) was delivered via two parallel platinum electrodes placed 3 mm apart along both sides of the whole length of the strip. The platinum electrodes were connected to a current amplifier and stimulator (Nihon Koden, SEN-7103). EFS was conducted at 10 V at a frequency of 10 Hz in the form of square wave pulses of 2 ms duration. In most experiments, EFS was applied to the strips for a period of 5 min. These parameters for EFS were selected to obtain reproducible responses by conducting preliminary experiments on the frequency-response and voltage-response relationships etc. Stimulation parameters were measured by displaying the pulse wave on an oscilloscope through a small resistor connected in series (Iwatsu, 01445243). TTX and various free radical scavengers were added into the organ bath 15 min before contracting the strips with ET-1.

Cascade experiment

Two organ baths were set up in series. In the upper bath, EFS was applied to Krebs-Ringer solution containing vasoconstrictors (e.g. ET-1) in the absence of the strips. After EFS, the solution in the upper bath was applied to the coronary artery strips in exchange for the surrounding solution in the lower bath, and contractile activity was estimated. As it took about 30 s to exchange the solution, free radicals generated in the upper bath were reduced and thus did not directly affect the coronary artery strips in the lower bath. Thereafter, appropriate free radical scavengers were added to the upper bath prior to EFS and similar cascade experiments were conducted to assess their effect.

Enzyme-linked immunoassay (EIA) of ET-1

According to the procedure previously described (Suzuki *et al.*, 1989), the immunoreactive endothelin-1 (ir-ET-1) level in the bathing solution (normally Krebs-Ringer solution) was determined by sandwich-EIA. A monoclonal anti-endothelin antibody, AwETN 40, which detects the N-terminal portion of ET-1 was used as the immobilized antibody. The Fab' fragment of rabbit antibodies against endothelin C-terminal heptapeptide (15-21) was used as an enzyme-labelled detector antibody after being coupled with horseradish peroxidase (HRP). AwETN40-coated microtiter wells (98 well, NUNC, Denmark) were prepared by adding 20 μg ml^{-1} (100 μl) AwETN40 to each well, followed by 300 μl Block Ace (Snow Brand Milk Products Co., Japan) diluted 4 fold with PBS. Endothelin-1 at various standard concentrations or a 10 μl sample taken from the bathing solutions dissolved in 100 μl buffer D (0.02 M phosphate buffer, pH 7, containing 10% Block Ace, 0.4 M NaCl, and 2 mM EDTA) was added to each well and incubated at room temperature for 1 day. After being washed with PBS, the plate was reacted with 100 μl anti-endothelin (15-21) Fab'-HRP at a dilution of 1/400 in buffer C (0.02 M phosphate buffer, pH 7, containing 1% bovine serum albumin (BSA), 0.4 M NaCl, and 2 mM EDTA) at 4°C for 16 h. After being washed with PBS, the bound enzyme activity was measured using *o*-phenylenediamine as a chromogen at 492 nm.

Generation and measurement of superoxide anions

According to the procedure previously described (Beauchamp & Fridovich, 1971), the accumulation of blue formazan in accordance with reduction of nitro blue tetrazolium (NBT) at 560 nm was taken as an index of superoxide anion production. The reaction was started by addition of 10 μM xanthine and 0.1 u ml^{-1} xanthine oxidase to a bath filled with Krebs-Ringer solution containing 25 μM NBT maintained at 37°C in the absence of the strips. After 20 min, 3.5 ml of the solution was subjected to spectrum determination with a Hitachi spectrophotometer (model DU-7).

Measurement of hydroxyl radicals

p-Nitrosodimethylaniline (*p*-NMA) was used as an effective scavenger of hydroxyl radicals (Minotti & Aust, 1987), and was bleached at 440 nm in proportion to the generation of hydroxyl radicals (Bors *et al.*, 1979). EFS (10 V, 10 Hz, 2 ms) was applied to Krebs-Ringer solution containing 10 μM *p*-NMA maintained at 37°C for various periods (0, 3, 5, 10 min), and 3.5 ml of each solution with a different incubation period was subjected to spectrum determination with a Hitachi spectrophotometer (model DU-7).

Effect of hydroxyl radicals on ir-ET-1

According to the procedure previously described (Minotti & Aust, 1987), 0.1 mM H_2O_2 and 0.2 mM $FeSO_4$ were added to a bath filled with 50 mM NaCl solution containing 10 μM *p*-NMA maintained at 37°C. The generation of hydroxyl radicals following 5 min reaction was detected by assay of *p*-NMA. Thus, 10 nM ET-1 was added to a bath filled with 50 mM NaCl solution containing 10 mM *p*-NMA just before applying 0.1 mM H_2O_2 and 0.2 mM $FeSO_4$, and 100 μl of the solution was subjected to determination of ir-ET-1 by EIA after 5 min.

Drugs

The following drugs were used: *p*-nitrosodimethylaniline, prostaglandin $F_{2\alpha}$ tromethamine, nitro blue tetrazolium, superoxide dismutase, xanthine, tetrodotoxin (TTX), catalase, D-mannitol, sodium-L-ascorbate (vitamin C) and deferoxamine (Wako Pure Chemicals, Osaka, Japan). Xanthine oxidase was from Boehringer Mannheim Yamanouchi (Tokyo, Japan). Endothelin-1 was from the Peptide Institute (Osaka, Japan). Arg^8-vasopressin and angiotensin II were from Sigma Chemical Co., Ltd (St. Louis, MO, U.S.A.).

Statistical analysis

Values are expressed as mean ± s.e. Comparisons were made by one way analysis of variance (ANOVA) followed by Bonferroni correction or Student's *t* test for unpaired values. Differences with *P* values less than 0.05 were considered statistically significant.

Results

Response to EFS

EFS (10 V, 10 Hz, 2 ms, 5 min) caused a biphasic response in porcine coronary artery strips precontracted with 10 nM ET-1, consisting of a slight contraction followed by a marked and irreversible relaxation (Figure 1a). The response induced by EFS was not affected by the presence or absence of endothelium, and was insensitive to 10 μM TTX (Figure 1b,c).

However, each free radical scavenger, 40 u ml^{-1} superoxide dismutase (SOD), 1200 u ml^{-1} catalase and 80 mM D-mannitol, significantly inhibited the relaxation after EFS (Figure 1d,e,f). Furthermore, relaxation after EFS was not observed in porcine coronary artery strips precontracted with 20 mM KCl (Figure 1g).

Effect of EFS on contractile activity and structure of ET-1

To confirm whether free radicals affect coronary artery strips indirectly, i.e., through modification of ET-1, we investigated the effect of EFS on the contractile activity of ET-1 in cascade experiments. As shown in Figure 2, EFS of Krebs-Ringer solution containing 10 nM ET-1 induced marked suppression of the contractile activity of ET-1 in coronary artery strips, although control Krebs–Ringer solution containing 10 nM ET-1 without EFS induced significant contraction. This apparent effect of EFS was significantly inhibited by each of the free radical scavengers, vitamin C (3 mM), SOD (40 u ml^{-1}), catalase (1200 u ml^{-1}) and D-mannitol (80 mM).

To determine whether the suppression of contractile activity of ET-1 in cascade experiments is associated with a change in the structure of the ET-1 molecule, we measured the concentration of immunoreactive ET-1 (ir-ET-1) in the lower bath by the sandwich EIA method as described in Methods. As shown in Figure 3, after EFS of Krebs-Ringer solution containing 10 nM ET-1, the release of ir-ET-1 in the lower bath was reduced to below the detection limit of EIA.

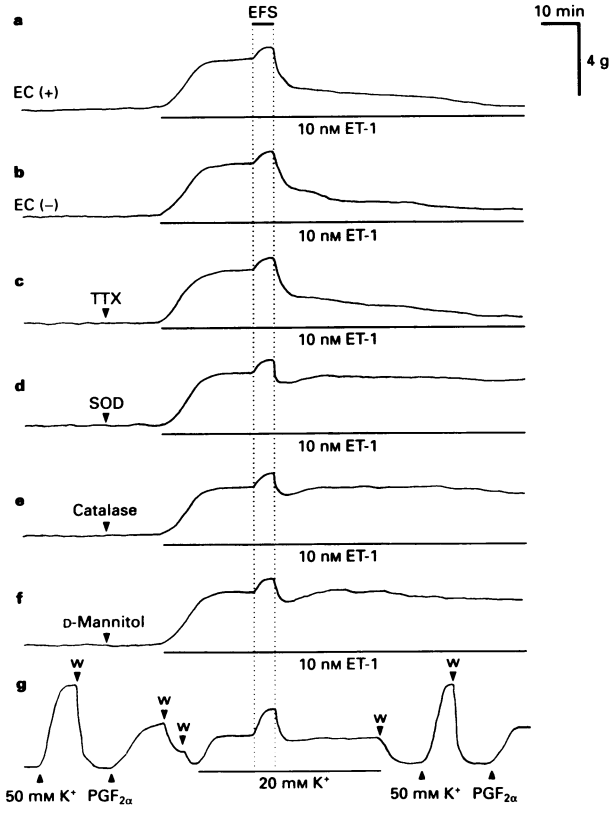

Figure 1 Responses of porcine coronary artery strips precontracted with endothelin-1 (ET-1) or KCl to prolonged EFS (10 V, 10 Hz, 2 ms). EFS was applied to porcine coronary artery strips, with or without endothelium precontracted with 10 nM ET-1 (a,b). The EFS-induced response was not affected by the presence or absence of endothelium. Therefore, porcine coronary artery strips without endothelium were used in the following experiments. EFS was applied to porcine coronary artery strips precontracted with 10 nM ET-1 in the presence of 10 μM tetrodotoxin (TTX) (c) or each radical scavenger (d, 40 u ml^{-1} superoxide dismutase (SOD); e, 1200 u ml^{-1} catalase or f, 80 mM D-mannitol). EFS was applied to porcine coronary artery strips precontracted with 20 mM K^+ (g).

However, the loss of release of ir-ET-1 was significantly inhibited by each free radical scavenger (3 mM vitamin C, 40 u ml^{-1} SOD, 1200 u ml^{-1} catalase and 80 mM D-mannitol).

Measurement of free radicals generated by EFS

To verify the origin of free radicals, we replaced the gas aerating the bath with 95% N_2/5% CO_2 gas to generate a

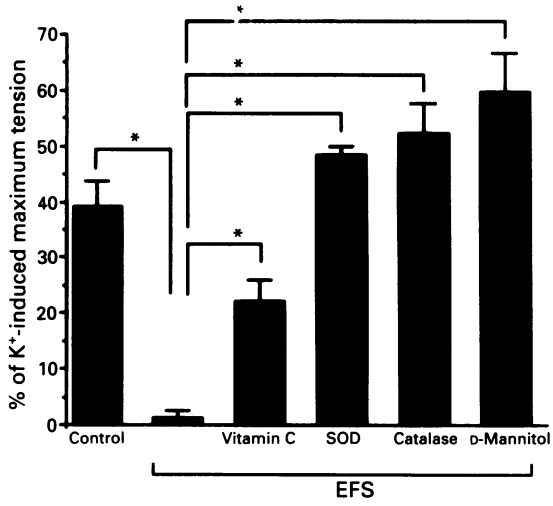

Figure 2 Effect of prolonged EFS on contractile activity of endothelin-1 (ET-1). EFS (10 V, 10 Hz, 2 ms) was applied to Krebs-Ringer solution in the absence or presence of each radical scavenger superoxide-dismutase (SOD (40 u ml^{-1}), vitamin C (3 mM), catalase (1200 u ml^{-1}) or D-mannitol (80 mM)) added just after the addition of 10 nM ET-1. Then, each solution was applied to porcine coronary artery strips. As a control, 10 nM ET-1 was added to Krebs-Ringer solution and left for 5 min without being subjected to EFS, and then the solution was applied to porcine coronary artery strips. The contractile response of porcine coronary artery strips is expressed as a percentage of the maximum tension induced by 50 mM KCl. Mean values ± s.e. ($n = 4–5$) are shown. *Significantly different from values with EFS-applied ET-1 ($P < 0.05$, ANOVA with Bonferoni correction).

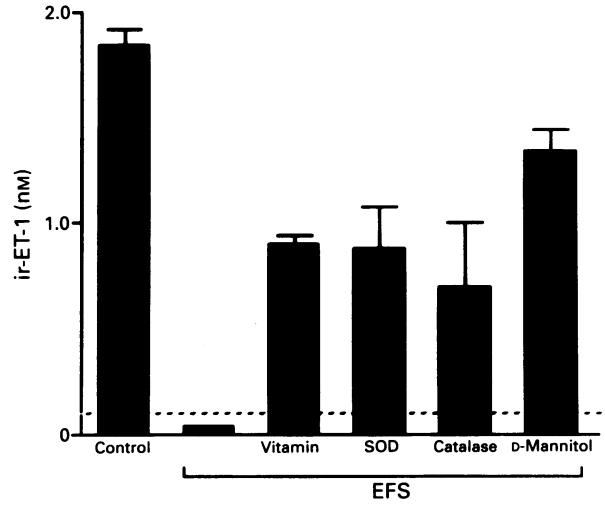

Figure 3 Effect of prolonged EFS on immunoreactive endothelin-1 (ir-ET-1). EFS (10 V, 10 Hz, 2 ms, 5 min) was applied to Krebs-Ringer solution in the absence or presence of each free radical scavenger superoxide-dismutase (SOD (40 u ml^{-1}), vitamin C (3 mM), catalase (1200 u ml^{-1}) or D-mannitol (80 mM)) which was added just after the addition of 10 nM ET-1. Then, ir-ET-1 in each solution was measured by EIA. As a control, 10 nM ET-1 was added to Krebs-Ringer solution, and the solution was subjected to EIA after 5 min. Mean values ± s.e. ($n = 4–6$) are shown. The dotted line represents the limit of detection by EIA.

hypoxic condition. As shown in Figure 4, the EFS-induced loss of release of ir-ET-1 was completely inhibited by hypoxic condition, indicating that oxygen-derived free radicals were generated in the bath by the EFS. To confirm radical species leading to the suppression of contractile activity of ET-1, we investigated the effect of oxygen-derived free radicals, chemically generated or exogenously added, on ir-ET-1. We first investigated the effect of superoxide anions generated by 10 mM xanthine plus 0.1 u ml^{-1} xanthine oxidase on ir-ET-1. As shown in Figure 5a, ir-ET-1 did not alter despite significant generation of superoxide anions in the bath. Secondly, the effect of hydrogen peroxide on ir-ET-1 was examined. As shown in Figure 5b, hydrogen peroxide (1 or 10 μM) did not affect ir-ET-1. We finally investigated the participation of hydroxyl radicals in the suppression of contractile activity of ET-1. As shown in Figure 6, EFS-induced generation of hydroxyl radicals was detected by *p*-NMA assay, and was dependent on the period of stimulation and O_2-bubbling. Significant generation of hydroxyl radicals was detected where EFS was delivered for more than 5 min. Moreover, hydroxyl radicals generated by 0.1 mM H_2O_2 plus 0.2 mM $FeSO_4$ (Fenton reaction, Freeman & Carpo, 1982) decreased ir-ET-1, and this decrease was significantly inhibited by 10^{-4} M deferoxamine, a ferrous chelator, and 1200 u ml^{-1} catalase (Figure 7).

Effect of EFS on contractile activities of other vasoactive agents

To investigate whether prolonged EFS induces suppression of the contractile activity of other vasoactive agents or not, we studied the effect of EFS on the contractile activities of the vasoactive agents, arginine[8]-vasopressin (AVP) and angiotensin-II (AII), since each is a peptide vasonconstrictor like ET-1, and $PGF_{2\alpha}$ which is frequently used for the investigation of EFS-induced relaxation of blood vessels (Rooke *et al.*, 1982; Cohen *et al.*, 1983; Feletou & Vanhoutte, 1989). After applying EFS to Krebs-Ringer solution containing each vasoconstrictor (1 μM AVP, 1 μM AII and 2 μM $PGF_{2\alpha}$), the contractile activity of each vasoconstrictor was investigated on porcine coronary artery strips for $PGF_{2\alpha}$ and rat aorta strips for AVP and AII. As shown in Figure 8a, the contractile activities of AII and AVP were not significantly different before and after EFS, although the contractile activity of $PGF_{2\alpha}$ was suppressed by EFS. This apparent effect of EFS on the contractile activity of $PGF_{2\alpha}$ was significantly

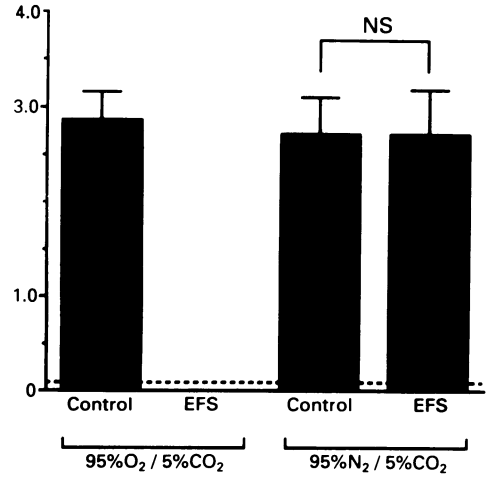

Figure 4 Effect of hypoxic condition on EFS-induced decrease of immunoreactive endothelin-1 (ir-ET-1). EFS (10 V, 10 Hz, 2 ms, 5 min) was applied to Krebs-Ringer solution during bubbling with 95% N_2/5% CO_2 or 95% O_2/5% CO_2 just after addition of 10 nM ET-1. Then, ir-ET-1 in each solution was measured by EIA. As a control, 10 nM ET-1 was added to Krebs-Ringer solution being bubbled with 95% N_2/5% CO_2 or 95% O_2/5% CO_2, and each solution was subjected to EIA after 5 min. The dotted line represents the limit of detection by EIA. Mean values ± s.e. ($n = 4-7$) are shown. NS, not significantly different (Student's *t* test for unpaired values).

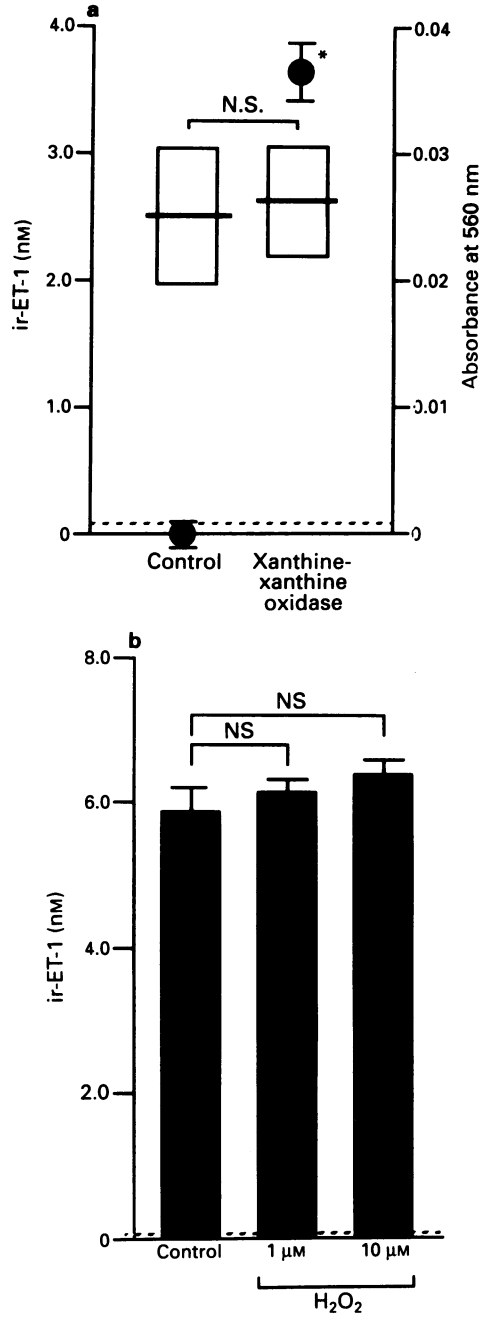

Figure 5 (a) Generation of superoxide anions by xanthine plus xanthine oxidase and its effect on immunoreactive endothelin-1 (ir-ET-1). ET-1 10 nM, xanthine 10 μM and xanthine oxidase 0.1 u ml^{-1} were added to Krebs-Ringer solution containing NBT 25 μM, and the solution was subjected to NBT assay and EIA after 20 min. As a control, 10 nM ET-1 was added to Krebs-Ringer solution containing 25 μM NBT, and the solution was subjected to NBT assay and EIA after 20 min. Histograms represent mean ± s.e. of ir-ET-1 ($n = 3$). Closed circles and bars represent mean ± s.e. of absorbance of blue formazane at 560 nm ($n = 3$). (b) Effect of incubation with H_2O_2 on ir-ET-1: 10 nM ET-1 was added to Krebs-Ringer solution containing 1 μM or 10 μM H_2O_2, and each solution was subjected to EIA after 20 min. As a control, 10 nM ET-1 was added to Krebs-Ringer solution and left for 20 min, and the solution was subjected to EIA. Mean values ± s.e. ($n = 6$). The dotted line represents the limit of detection by EIA. *Significantly different from control value with $P < 0.05$; NS, not significantly different (Student's *t* test for unpaired values).

inhibited by the free radical scavengers, 3 mM vitamin C and 40 u ml^{-1} SOD (Figure 8b).

Effect of O_2-bubbling on structure of ET-1

To investigate whether ET-1 is chemically modified by O_2 bubbling alone, were measured ir-ET-1 by sandwich EIA after incubating 10 nM ET-1 for various intervals in Krebs-Ringer bathing solution with the Po_2 level maintained above 550 mmHg. As shown in Table 1, ir-ET-1 decreased in proportion to the period of incubation and reached a plateau at 10 min. This apparent structural modification of ET-1 caused by O_2 bubbling was fully inhibited in the presence of 40 u ml^{-1} SOD, indicating that this effect may be mediated by free radicals.

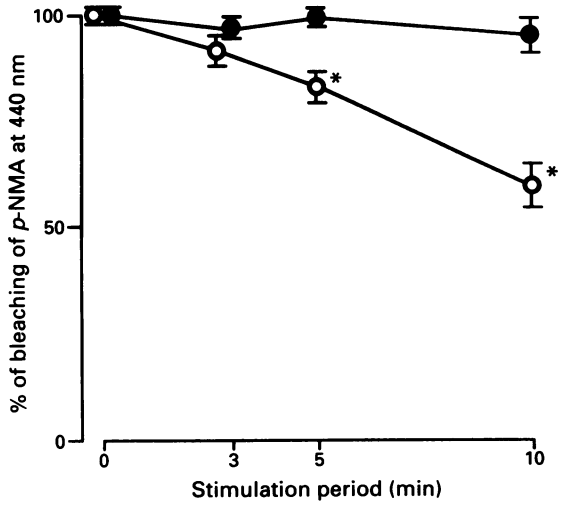

Figure 6 Time-dependent and oxygenation-dependent generation of hydroxyl radicals by EFS. EFS (10 V, 10 Hz, 2 ms) was applied to Krebs-Ringer solution containing 10 μM *p*-nitrosodimethylaniline (*p*-NMA) bubbled with 95% N_2/5% CO_2 (●) or 95% O_2/5% CO_2 (○) for various stimulation periods. Each value is expressed as a percentage of absorbance of *p*-NMA at 440 nm in Krebs-Ringer solution without bubbling. Mean ± s.e. ($n = 3$–6) are shown. *Significantly different from values in hypoxic condition with $P < 0.05$ (Student's *t* test for unpaired values).

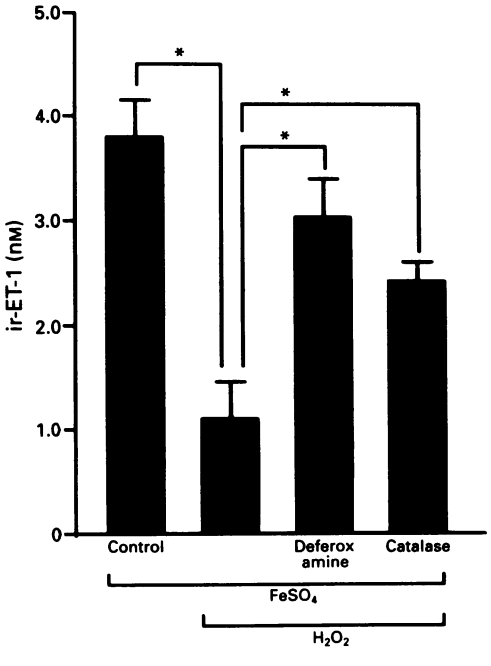

Figure 7 Effect of hydroxyl radicals generated by the Fenton reaction on immunoreactive endothelin-1 (ir-ET-1): 10 nM ET-1, 0.1 mM H_2O_2 and 0.2 mM $FeSO_4$ were added to 50 mM NaCl solution in the absence or presence of each suppressor agent for hydroxyl radicals (10^{-4}M deferoxamine or 1200 u ml^{-1} catalase) and left for 5 min, and each solution was subjected to EIA. As a control, 10 nM ET-1 was added to 50 mM NaCl solution containing 0.2 mM $FeSO_4$ and left for 5 min, and the solution was subjected to EIA. Mean values ± s.e. ($n = 4$) are shown. *Significantly different from values with Fenton reaction-applied ET-1 ($P < 0.05$, ANOVA with Bonferoni correction).

Discussion

The present study demonstrated that prolonged EFS of porcine isolated coronary artery strips precontracted with ET-1 causes a non-neurogenic and endothelium-independent response, which was biphasic with a slight contraction during EFS followed by a long-lasting relaxation after EFS. This relaxation after EFS was significantly inhibited by the free radical scavengers, SOD, catalase and D-mannitol. These results suggest that EFS-generated free radicals are involved in the relaxation response to EFS.

Free radicals show a wide spectrum of action on blood vessels. Hydrogen peroxide causes hyperpolarization and relaxation of pig coronary artery smooth muscle cells (Beny & von der Weid, 1991). Superoxide anions inactivate endoplasmic reticulum Ca^{2+} transport by lowering the activity of the phosphoenzyme which phosphorylates the 100 kDa subunit of the Ca^{2+} pump in endoplasmic reticulum (Grover & Samson, 1988). Superoxide anion and its derivatives, hydroxyl radicals, are responsible at least in part for vasodilatation of cat cerebral artery as well as for damage to the blood-brain barrier (Nelson *et al.*, 1992). Hydroxyl radicals facilitate endothelium-dependent relaxation (Rubanyi & Vanhoutte, 1986). Free radicals generated by EFS induce damage of endothelial cells in the rat tail artery (Lamb *et al.*, 1987).

However, the free radicals generated by EFS is this study are unlikely to have caused relaxation of coronary artery strips in a direct manner or to have damaged the contractile apparatus of smooth muscle cells for the following reasons: (1) the relaxation after EFS was not seen in coronary artery strips precontracted with 20 mM K^+; (2) the contractile response to 50 mM K^+ or 5 μM $PGF_{2\alpha}$ in coronary artery strips was consistent before and after EFS (Figure 1g). Thus, we hypothesized that free radicals generated by EFS might act on ET-1 and decrease its contractile activity, and we examined the effect of EFS on the contractile activity of ET-1 in cascade experiments. EFS of Krebs-Ringer solution containing ET-1 induced marked suppression of the contractile activity of ET-1 in coronary artery strips. This apparent effect of EFS was significantly inhibited by free radical scavengers (Figure 2) suggesting that EFS-generated free radicals suppress the contractile activity of ET-1. A previous study has demonstrated that the C-terminal Trp^{21} as well as the intramolecular loop structure constructed by two disulphide bonds of ET-1 are especially important for its vasoconstrictor activity (Kimura *et al.*, 1988). Thus, it is possible that EFS-derived free radicals suppress the contractile activity via structural modification of ET-1. We examined this point using an enzyme-linked immunoassay recognizing the two important domains of ET-1. As expected, EFS decreased the immunoreactivity of ET-1 (ir-ET-1) and this effect was significantly inhibited by free radical scavengers. Furthermore, the exchange of 95% O_2/5% CO_2 for 95% N_2/5% CO_2 significantly inhibited the EFS-induced decrease of ir-ET-1. These results suggest that oxygen-derived free radicals generated by EFS induce structural modifications of ET-1, thereby suppressing its contractile activity. This phenomenon is not specific for ET-1, because a similar suppression in contractile activity by EFS was recognized in the case of $PGF_{2\alpha}$, although the chemical modification of $PGF_{2\alpha}$ was not clarified in this study (Figure 8a,b). Furthermore, a previous study demonstrated the oxygen metabolites generated by pro-

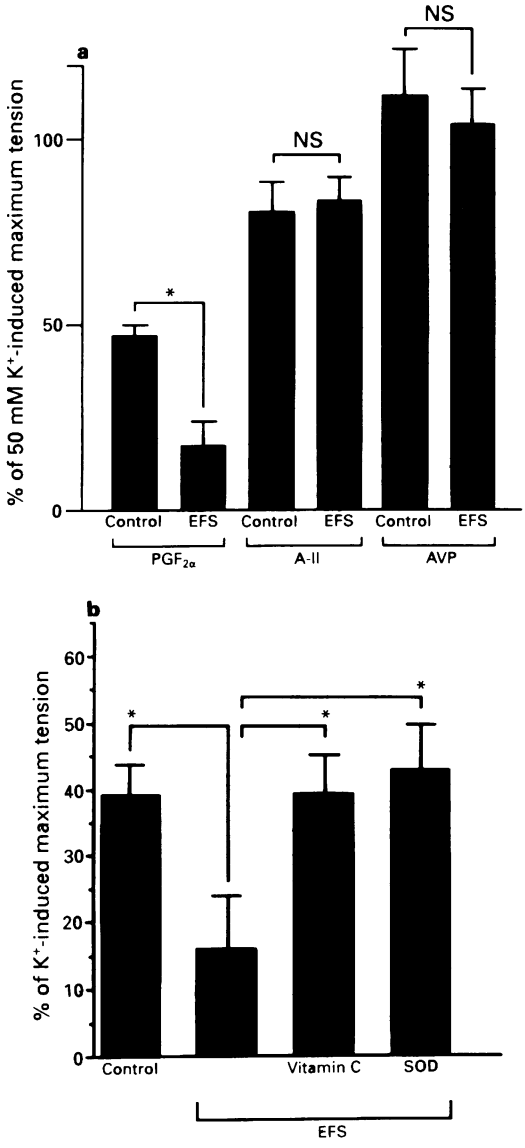

Figure 8 (a) Effect of EFS on contractile activities of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), angiotensin II (AII) and Arg-vasopressin (AVP). EFS (10 V, 10 Hz, 2 ms, 5 min) was applied to each Krebs-Ringer solution containing 2 μM $PGF_{2\alpha}$, 1 μM AII or 1 μM AVP, and each solution was applied to the strips. Rat aortic strips were used for the assay of contractile activities of AII and AVP. As a control, each vasoconstrictor (2 μM $PGF_{2\alpha}$, 1 μM AII or 1 μM AVP) was added to Krebs-Ringer solution, and each solution was applied to the strips after 5 min. The contractile response of porcine coronary artery strips or rat aortic strips was expressed as percentage of maximum tension induced by 50 mM KCl. Mean values ± s.e. ($n = 6$) are shown. (b) Effect of radical scavengers on EFS-induced suppression of contractile activity of $PGF_{2\alpha}$. EFS (10 V, 10 Hz, 2 ms, 5 min) was applied to Krebs-Ringer solution in the absence or presence of each radical scavenger (vitamin C (3 mM) or SOD (40 u ml^{-1})) added just after the addition of 2 μM $PGF_{2\alpha}$. Then, each solution was applied to porcine coronary artery strips. As a control, 2 μM $PGF_{2\alpha}$ was added to Krebs-Ringer solution, and the solution was applied to the strips after 5 min. The contractile response of porcine coronary artery strips was expressed as a percentage of maximum tension induced by 50 mM KCl. Mean values ± s.e. ($n = 6$) are shown. *Significantly different from control level with $P < 0.05$; NS, not significantly different (Student's t test for unpaired values).

Table 1 Effect of O_2 bubbling on immunoreactive endothelin-1 (ir-ET-1) in the absence and presence of superoxide dismutase (SOD)

Incubation time (min)	*ir-ET-1* (nM) *Control*	*SOD*
0	6.04 ± 0.37	6.38 ± 0.57
5	3.93 ± 0.43*	6.19 ± 0.79
10	2.24 ± 0.36*	7.06 ± 0.69
20	2.13 ± 0.48*	5.73 ± 0.76

Krebs-Ringer solution was maintained at 37°C and gassed with 95% O_2/5% CO_2. The Po_2 level in Krebs-Ringer solution was maintained above 550 mmHg, which was monitored by a pH/blood gas analyser (Corning, Model 158). ET-1 (10 nM) was added to Krebs-Ringer solution in the absence or presence of SOD, and the solution was subjected to EIA after various incubation periods. Data are mean ± s.e. ($n = 4$). *Significantly different from control value with ET-1 without incubation ($P < 0.05$, ANOVA with Bonferroni correction).

longed EFS (9 V, 1 ms, 4 Hz, 5 min) oxidized noradrenaline and decreased its contractile activity (Lamb & Webb, 1984), although the degree of decrease in contractile activity of the agonist was modest in their study compared with our results.

To elucidate which free radicals are involved in the inactivation of ET-1, we examined the effect of each free radical, chemically generated or exogenously applied, on ir-ET-1. Neither superoxide anions generated by xanthine plus xanthine oxidase nor hydrogen peroxide exogenously added, affected ir-ET-1 (Figure 5a,b). In parallel with these findings, the contractile activity of ET-1 was not suppressed by these free radicals (data not shown). However, hydroxyl radicals generated by H_2O_2 plus $FeSO_4$ (Fenton reaction) significantly decreased ir-ET-1 (Figure 7). Furthermore, generation of hydroxyl radicals was detected in EFS-applied Krebs-Ringer solution, and their generation was dependent on the period of stimulation and O_2-bubbling. Significant generation of hydroxyl radicals was detectable when the stimulation period exceeded 5 min. Moreover, this generation of hydroxyl radicals was significantly inhibited in the presence of deferoxamine (data not shown). These results strongly suggest that oxygen-derived hydroxyl radicals generated by EFS mediate the suppression of the contractile activity of ET-1. In this regard, the fact that all free radical scavangers, SOD for superoxide anion, catalase for hydrogen peroxide and mannitol for hydroxyl radicals, significantly inhibited the relaxation of coronary artery strips after EFS in this study conforms with this possibility, since hydroxyl radicals are thought to be generated from superoxide anions by way of hydrogen peroxide in the presence of Fe^{2+} (Rubanyi & Vanhoutte, 1986). A possible source of Fe^{2+} in this study may be contamination from reagents comprising the Krebs-Ringer solution.

Previous studies have demonstrated that hydroxyl radicals induce conformational change of β_2 microglobulin via oxidation of tryptophan (Trp) and that hydroxyl radicals mediate glucose-induced protein damage associated with Trp fluorescence quenching (Hunt *et al.*, 1988; Capeillere-Blandin *et al.*, 1991). It has also been reported that hydroxyl radicals, as well as myeloperoxide-derived oxidants, induce inactivation of α-1-proteinase inhibitor by oxidation of the reactive site methionine (Met) (Maier *et al.*, 1989). These studies raise the possibility that Met^7 and Trp^{21} in the two important domains of ET-1 recognized by EIA are sites of oxidation by hydroxyl radicals and that the modification of each or both residues leads to the loss of both contractile activity and immunoreactivity of ET-1. However, the EIA used in the present study can detect Met sulphoxide^7 ($Met(o)^7$) ET-1 as well as ET-1 (Suzuki, personal communication). Moreover, $Met(o)^7$ ET-1 also has similar contractile activity to ET-1 (Kimura, unpublished data). It is therefore tempting to speculate that the modification of Trp^{21} of ET-1 by hydroxyl radicals is critical for EFS-induced inactivation of ET-1. On the other hand, AII and AVP are probably resistant to chemical modification by EFS since they lack both Met and

Trp (Aumelas *et al.*, 1985; Mann *et al.*, 1986). Further study is required to determine whether EFS-derived hydroxyl radicals actually oxidize Trp^{21} of ET-1.

The mechanism for the slight contraction which follows EFS of coronary artery strips is unclear. This response was insensitive to TTX and free radical scavengers. Hence, known vasoconstrictor neurotransmitters (Kalsner & Quillin, 1989) and free radicals (Auch-Schwelk *et al.*, 1989) are not likely to contribute to this response during EFS. It is most likely that activation of voltage-dependent Ca^{2+} channels secondary to EFS-induced depolarization of the smooth muscle membrane may bring about the slight contraction, because this contractile response was significantly larger in 20 mM K^+-depolarized contractions and partially inhibited in the presence of a low concentration of nicardipine. Thus (1) contractile responses during EFS were $35.9 \pm 1.7\%$ in 20 mM K^+-depolarized contractions and $22.3 \pm 2.1\%$ in ET-1-induced contractions, respectively ($n = 4$, contractile response expressed as percentage of maximum tension induced by 50 mM KCl) and (2) The contractile response during EFS in ET-1-induced contractions was reduced to $14.5 \pm 1.0\%$ in the presence of 10^{-8} M nicardipine ($n = 4$, contractile response expressed as percentage of maximum tension induced by 50 mM KCl).

Recently, it has been demonstrated that preincubation of ET-1 in oxygenated Krebs-Ringer solution results in progressive loss of its contractile activity in porcine coronary arterial rings in parallel with loss of its immunoreactivity, dependent on the period of incubation (Balwierczak *et al.*, 1992). We also confirmed this finding in the case of maintaining P_{O_2} level in Krebs-Ringer solution above 550 mmHg, and further verified that the decrease of ir-ET-1 by the incubation was fully inhibited by the free radical scavenger, SOD (Table 1). Thus, it is possible that free radicals may be generated by bubbling only with O_2/CO_2 gas, and in a free state in the solution, inactivate ET-1. However, free radicals generated by O_2-bubbling alone may be less potent in inactivating ET-1 than the hydroxyl radicals generated by EFS in this study. Since, the level of ir-ET-1 was decreased by O_2-bubbling in a time-dependent manner and reached a plateau (about 2 nM) at 10 min, although the level of ir-ET-1 was reduced to below the detection limit of EIA (1 pM) by EFS (Table 1, Figure 3). It has been demonstrated that ET-1-induced contraction of porcine coronary artery is more or less irreversible and lasts for several hours (Yanagisawa *et al.*, 1988), due to the very slow dissociation of ET-1 from its receptor once bound (Hirata *et al.*, 1988). This observation indicates that O_2-bubbling does not inactivate ET-1 bound to its receptor. On the contrary, coronary artery strips precontracted with ET-1 showed marked relaxation to near baseline values just after EFS in this study, indicating that the EFS-generated hydroxyl radicals may potently inactivate not only ET-1 in a free form in the solution but also ET-1 bound to its receptor.

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