Mediators Involved in Decreasing Peripheral Vascular Resistance With Carbachol in the Rat Hind Limb Perfusion Model

Renzo Y. Loyaga-Rendon¹, Shuichi Sakamoto¹, Takeshi Aso¹, Keiko Iwasaki-Kurashige², Ryoko Takahashi², and Hiroshi Azuma²,*

¹Department of Comprehensive Reproductive Medicine; ²Department of Biosystem Regulation, Institute of Biomaterials & Bioengineering; Graduate School, Tokyo Medical & Dental University, Tokyo 101-0062, Japan

Received March 16, 2005; Accepted May 13, 2005

Abstract. We examined the involvement of nitric oxide (NO) and/or endothelium-derived hyperpolarizing factor (EDHF) in decreasing peripheral vascular resistance in the rat hind limb perfusion model and analyzed the identity of EDHF in this model. The potency of carbachol (CCh) to produce relaxation was quantitatively similar to sodium nitroprusside (SNP). CCh-induced relaxation was abolished after endothelial denudation, but resistant to nitroarginine and indomethacin. The relaxation was inhibited by tetraethylammonium, ouabain, charybdotoxin plus apamin, and under depolarization. SNP-induced relaxation was accompanied by increased cGMP production, which was inhibited by ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one). Although CCh produced a similar extent of relaxation to SNP, the cGMP level was 24 times lower than that with SNP. Low KCl produced a definite relaxation, which was inhibited by ouabain, but independent of NO, prostacyclin, and endothelium. 1-EBIO (1-ethyl-2-benzimidazolinone) as an activator of IKCa channel also produced a concentration-dependent relaxation, which was inhibited by charybdotoxin, ouabain, and depolarization, but independent of NO and prostacyclin. Clotrimazole and 17-octadecynoic acid as inhibitors of P450 monooxygenase inhibited the CCh-induced relaxation. Meanwhile, catalase at a concentration sufficient to inhibit H2O2-induced relaxation did not exert definite inhibition of the CCh-induced relaxation. These results suggest that CCh produces an endothelium-dependent, EDHF-dependent, and NO-cGMP-independent relaxation and that K+ ion and metabolite(s) of P450 monooxygenase possibly play an important role for this relaxation.

Keywords: hind limb perfusion model, carbachol-induced relaxation, endothelium-derived hyperpolarizing factor, K+ ion, metabolite of P450 monooxygenase

Introduction

The endothelium plays a crucial role in the maintenance of vascular tone by releasing factors such as nitric oxide (NO) (1), prostacyclin (PGI2), and an endothelium-derived hyperpolarizing factor (EDHF) (2). It is generally accepted that the kind of endothelium-derived relaxing factors varies according to the vessel type. NO appears to be more important in the conduit vessels such as aorta, whereas the importance of EDHF increases as the diameter of the vessel decreases (resistance vessels) (3).

The identity of EDHF remains unresolved, although several candidates have been proposed. H2O2 has been shown to produce endothelium-dependent hyperpolarization in mesenteric arteries of rodents and humans (4, 5). Epoxycosatrieneoic acids (EETAs) have been addressed as EDHF in the coronary vessels of different species (6) and recently in the human mammary arteries (7). K+ ion itself appears to be EDHF in rat hepatic arteries (8) and some other vessel types (9). Anandamide, an endocannabinoid, and endothelial natriuretic peptide (CNP) are also accounted as EDHF in the coronary and mesenteric arteries of rats (10, 11). Another hypothesis suggests that EDHF might not be necessarily a diffusible substance, but the phenomenon...
might be explained by the spreading of the electrical current from the endothelium to the underlying smooth muscle cells through gap junctions (12). An intriguing hypothesis proposes that there might be more than one EDHF and these putative factors might vary according to vessel type even in the same species (13). In spite of the different opinions regarding its identity, a consensus appears to be reached in the fact that the hyperpolarization, which leads to the relaxation produced by EDHF, is due to the opening of $K_{Ca}$ channels either on the endothelium or on the smooth muscle cells, therefore recognizing a critical role of $K_{Ca}$ and its channels (14).

In the skeletal arteries, Wigg et al. (15) and Zygmunt et al. (16) initially suggested that NO was mainly responsible for the agonist-induced relaxation in the small branches of the femoral arteries. However, recently, it has been shown in vivo that EDHF might be similarly important to NO in producing endothelium-dependent relaxation in the rat hind limb circulation (17). Meanwhile, the identity for EDHF in the peripheral resistant vessels of the hind limb was not proposed. Thus, in the present experiments, we used a perfusion model to investigate the contribution of NO and/or EDHF to the endothelium-dependent relaxation and to approach to the identity of EDHF, in the peripheral resistance vessels of the rat hind limb.

**Materials and Methods**

**Chemicals**

The following chemicals were used in the present experiments: phenylephrine hydrochloride (Kowa Pharmaceutical Co, Tokyo); carbamylcholine chloride (carbachol: CCh), sodium nitroprusside (SNP), Nω-nitro-L-arginine (nitroarginine), indomethacin, charybdoxin, apamin, ouabain, 3-isobutyl-1-methylxanthine (IBMX), 1H-[1,2,4]oxadiazolo[4,3-$d$]quinoxaline-1-one (ODQ), catalase, clorimazole, 17-octadecyenoic acid (17-ODA), carbenoxolone, and 11,12-epoxyeicosatrienoic acid (11,12-EETA) (all from Sigma, St. Louis, MO, USA); carbamylcholine chloride (carba-chol: CCh), sodium nitroprusside (SNP), L-$\alpha$-arginine (nitroarginine), indomethacin, charybdoxin, apamin, ouabain, 3-isobutyl-1-methylxanthine (IBMX), 1H-[1,2,4]oxadiazolo[4,3-$d$]quinoxaline-1-one (ODQ), catalase, clorimazole, 17-octadecyenoic acid (17-ODA), carbenoxolone, and 11,12-epoxyeicosatrienoic acid (11,12-EETA) (all from Sigma, St. Louis, MO, USA); Triton X-100 and tetraethylammonium (Nacalai Tesque, Kyoto); H$_2$O$_2$ (Wako, Osaka); and 1-ethyl-2-benzimidazolinone (1-EBIO) (Tocris, Ellisville, MO, USA). Phenylephrine, CCh, nitroarginine, charybdoxin, apamin, tetraethylammonium, catalase, H$_2$O$_2$, SNP, carbenoxolone, and Triton X-100 were dissolved in distilled water, whereas dimethylsulfoxide was used to dissolve ODQ, indomethacin, IBMX, ouabain, clorimazol, 17-ODA, and 1-EBIO. 11,12-EETA was dissolved in ethanol. The drugs were kept as stock solutions until use and were periodically replaced.

**Experimental animals**

This study complied with the Animal Welfare Regulations of Tokyo Medical and Dental University and the Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society. Thirteen to fifteen weeks old Sprague-Dawley female rats were purchased and kept in the Animal Care Center of our university for one week before the experiments.

**Perfusion experiments**

The animals were heparinized (200 IU/kg, i.p.) to avoid coagulation. Under ether anesthesia, rats were killed by dislocation and exsanguinations from carotid arteries. Twenty-one-gauge metal catheters were inserted into the proximal portion of the femoral arteries, and the proximal portion of the femoral veins was opened longitudinally to facilitate the flow. The hind limbs were separated from the rest of the body by scissors, and modified Krebs solution was perfused into the isolated hind limbs with the aid of independent peristaltic pumps at a constant flow rate of 3.5 ml/min. The perfusion solution was prepared by mixing 2 vol Krebs solution with 1 vol Dulbecco’s Modified Eagle’s Medium (DMEM), the ionic composition of which was as follows; 115.16 mM NaCl, 4.89 mM KCl, 24.17 mM NaHCO$_3$, 1.07 mM MgSO$_4$-$\cdot$7H$_2$O, 2.5 mM CaCl$_2$-$\cdot$2H$_2$O, 0.8 mM KH$_2$PO$_4$, 0.636 mM NaH$_2$PO$_4$, 8.45 mM D-glucose, 0.000825 mM Fe(NO$_3$)$_3$-$\cdot$9H$_2$O, 0.4 mM L-arginine-$\cdot$HCl, 0.324 mM L-cysteine-$\cdot$2HCl, 1.3 mM L-glutamine, 0.4 mM glycine, 0.067 mM L-histidine-$\cdot$HCl-$\cdot$H$_2$O, 0.267 mM L-isoleucine, 0.267 mM L-leucine, 0.33 mM L-lysine-$\cdot$HCl, 0.067 mM L-methionine, 0.133 mM L-phenylalanine, 0.133 mM L-serine, 0.266 mM L-threonine, 0.026 mM L-tryptophan, 0.1315 mM L-tyrosine 2Na-$\cdot$2H$_2$O, 0.2678 mM L-valine, 0.0109 mM niacinamide, 0.00954967 mM choline chloride, 0.0030206 mM folic acid, 0.0065484 mM pyridoxal-$\cdot$HCl, 0.00035427 mM riboflavin, 0.00559599 mM D-pantothenic acid (hemicalcium), 0.0039533 mM thiamine-$\cdot$HCl, and 0.4163 mM pyruvic acid-$\cdot$Na. The rate of mixing between Krebs solution and DMEM was determined in order to minimize the occurrence of edema according to the preliminary experiments. The perfusion of a cell- and protein-free solution into an isolated limb was accompanied by significant edema, which was probably associated with endothelial dysfunction. This latter condition was a significant concern in our model. Thus, some manipulations of the perfusion solution and temperature conditions were performed in order to achieve no edema and reproducibility of the experimental results. The magnitude of edema was definitely less at low temperature and by using modified...
Krebs solution. In a preliminary study, we confirmed that the responsiveness of hind leg vascular bed to phenylephrine and CCh did not differ between 22°C and 37°C, thus all experiments were performed at 22°C. All the solutions were constantly aerated with a gas mixture of 5% CO₂ and 95% O₂. Agents were added to parallel chambers at appropriate concentrations and then perfused through the proximal femoral artery. The changes in perfusion pressure were continuously monitored and recorded in the Mac Lab system and on a pen-writing oscillograph for analyses.

Endothelium-dependent relaxation

Thirty minutes after starting the perfusion, a stable basal perfusion pressure was obtained and then contraction with phenylephrine at a concentration of 10 µM was induced. Relaxation was caused by applying 0.1 nM to 100 µM CCh during the phenylephrine-induced contraction. Nitroarginine (100 µM), indomethacin (1 µM), charybdotoxin (100 nM), apamin (100 nM), KCl (60 mM), tetraethylammonium (1 mM), and ouabain (100 µM) were used for pharmacological analyses of the CCh-induced relaxation. These agents were applied 20 min prior to the phenylephrine contraction (except for charybdotoxin and apamin, which were used for 5-min pretreatment). Because some of the inhibitors affected the phenylephrine-induced contraction, the concentration of phenylephrine was adjusted in order to produce similar extent of contractions. The contralateral hind limb was used as a paired time control, where vehicles were applied. Triton X-100 at a concentration of 0.05% was induced. Relaxation was caused by applying 0.1 nM - 100 µM of 37.9 ± 0.4% (n = 4) as compared with the paired time control accordingly. The concentration (EC₅₀) to produce a half of the maximum relaxation and maximum relaxation (Eₘₐₓ) were obtained from concentration-relaxation curves.

Statistics

Data are given as the mean ± S.E.M. When % of relaxation is mentioned, it refers to the % of the phenylephrine-induced contraction. Data were compared using the paired or unpaired t-test or one-way ANOVA accordingly. The concentration (EC₅₀) to produce a half of the maximum relaxation and maximum relaxation (Eₘₐₓ) were obtained from concentration-relaxation curves.

Assessment of the identity of the EDHF-induced relaxation

To evaluate the role of H₂O₂, EETAs, K⁺, and gap junctions as candidates for EDHF, we examined the effects of H₂O₂ (10 nM – 100 µM), 11,12-EET (1 µM), and low concentrations of KCl (2 – 11 mM) during the contraction caused by 10 µM phenylephrine. The effects of carbenoxolone (30 – 100 µM), clotrimazole (10 µM), 17-ODA (3 µM), or catalase (1250 IU/ml, previously filtered) on the CCh-induced relaxation were also analyzed. The low KCl-induced relaxation was evaluated in the presence of 30 µM nitroarginine, 1 µM indomethacin, or 100 µM ouabain. The effects of 1 – 100 µM 1-EBIO, IKCa, channel activator, were assessed in the presence of 60 mM KCl, 100 µM ouabain, 10 µM ODQ, or 100 nM charybdotoxin.

Results

Endothelium-dependent and -independent relaxations

The basal hind limb perfusion pressure, measured 30 min after the beginning of perfusion, was determined to be 37 ± 0.8 mmHg (n = 25). Phenylephrine at 10 µM induced a sustained contraction of 138.8 ± 3.1 mmHg, (n = 24). At 0.1 nM – 100 µM, CCh produced a concentration-dependent relaxation with the maximal value of 93.5 ± 0.4%, with an EC₅₀ of 99 ± 23 nM (n = 6). Similarly, 0.1 nM – 100 µM SNP produced a concentration-dependent relaxation with a maximal value of 92.3 ± 4.1% and an EC₅₀ of 111 ± 38 nM (n = 4) (Fig. 1A). Triton X-100 (0.05%) applied for 1 min decreased the 100 nM CCh-induced relaxation to 2.4 ± 0.2% (n = 4) as compared with the paired time control of 37.9 ± 1.8% (n = 4) (P < 0.0001), without affecting the SNP-induced relaxation (Fig. 1B), indicating the
endothelium-dependence of the CCh-induced relaxation.

Nitroarginine, as an NO synthase (NOS) inhibitor, at a concentration of 100 µM in combination with 1 µM indomethacin, as a cyclooxygenase inhibitor, showed no effect on the basal perfusion pressure (delta increase was 0.2 ± 2.1 mmHg), whereas the phenylephrine-induced contraction was greatly increased. Thus, 3 µM phenylephrine was used to make the magnitude of contraction similar to that produced by 10 µM phenylephrine in the absence of nitroarginine. On the other hand, the addition of 100 nM charybdotoxin in combination with 100 nM apamin, as IKCa and SKCa channel blockers, respectively, increased the basal perfusion pressure by 19.8 ± 5.7 mmHg (n = 4) (P < 0.05) as compared with the control and that in the co-presence of nitroarginine and indomethacin. In addition, the phenylephrine-induced contraction was further increased in the presence of charybdotoxin and apamin; thus, the concentration of phenylephrine was reduced to 1 µM in this case. The concentration-relaxation curve for CCh remained unaffected by the combination of nitroarginine and indomethacin, but there was a tendency to shift the curve leftward. On the other hand, the curve was shifted to the right after the addition of charybdotoxin and apamin (Fig. 2A). The EC50 values of CCh to produce relaxation were determined to be 168.8 ± 14.7 nM for the control (n = 4), 102.7 ± 22.6 nM in the presence of nitroarginine plus indomethacin (n = 7), and 253.3 ± 55.4 nM (n = 4) in the presence of nitroarginine, indomethacin, charybdotoxin, and apamin (P < 0.05 vs nitroarginine plus indomethacin). The magnitude of the maximum relaxation remained similar in the 3 groups. Further characterization of the relaxation was performed in the single dose application of 100 nM CCh. Tetraethylammonium at 1 mM increased the basal perfusion pressure by 7.5 ± 3.8 mmHg (P < 0.05, n = 4) without affecting the phenylephrine-induced contraction. In the presence of the tetraethylammonium, the CCh-induced relaxation was 5.8 ± 3.3% (n = 4), which was significantly (P < 0.005) lower than the paired time control (36.8 ± 4.5%) (n = 4). The combination of 1 mM tetraethylammonium and 30 µM nitroarginine did not show further inhibition than tetraethylammonium alone. In the presence of nitroarginine and indomethacin, 100 µM ouabain increased basal perfusion pressure (delta value was 7.2 ± 2.9 mmHg (P < 0.05)) and inhibited the CCh-induced relaxation (15.2 ± 2.1% (n = 6) vs the paired time control of 25.3 ± 3.5% (n = 6)) (P < 0.05) (Fig. 2B). The CCh-induced relaxation was abolished during the contraction caused by 60 mM KCl (Fig. 2C). When 60 mM NaCl in the modified Krebs solution was replaced with 60 mM KCl to produce contraction, the CCh-induced relaxation was also abolished. On the other hand, a single application of 100 nM SNP produced a definite relaxation, which was significantly inhibited by 10 µM ODQ, partially inhibited by 100 µM ouabain and slightly but not significantly inhibited by 1 mM tetraethylammonium (data not shown).

cGMP production

In the presence of IBMX, the basal perfusion pressure was not affected. Meanwhile, the phenylephrine-induced contraction was reduced to 43.4 ± 3 mmHg
(n = 22) and there was a tendency for the sustained contraction to gradually decrease as the time passed. However, CCh and SNP could produce the relaxation in a concentration-dependent manner under the reduced phenylephrine contraction. SNP in a concentration of 10 µM produced a similar extent of relaxation to 10 µM of CCh (Fig. 3A). Therefore, 10 µM SNP and 10 µM CCh were used to stimulate cGMP production.

In a preliminary study using rings of rat thoracic aorta, we found that the cGMP levels in response to CCh were increased both in the aortic tissue and the incubation medium (Krebs solution). There was a positive and significant (P<0.001) correlation (r = 0.806) between cGMP levels in the aortic tissue and incubation medium. Thus, we assumed that the detected cGMP levels in the perfusate of the hind limb would reflect changes in the tissue cGMP levels.

The basal cGMP level in the perfusate was determined to be 60.3 ± 4.8 fmol/ml (n = 22). A tendency to decrease the value was observed after the addition of 10 µM phenylephrine without significant change. The cGMP level in the perfusate was increased by SNP (100 nM to 100 µM) in a concentration-dependent manner. On the contrary, CCh in concentrations of 100 nM to 100 µM did not follow the same pattern in the change of cGMP levels in the perfusate (Fig. 3B). The levels of cGMP after the stimulation with 10 µM SNP were determined to be 361 ± 25 fmol/ml (n = 5), which was almost completely inhibited by 10 µM ODQ, confirming that activation of guanylate cyclase was responsible for the increase in cGMP levels (data not shown). On the contrary, the determined levels of cGMP in the perfusate of the hind limb was almost completely inhibited by 10 µM ODQ, confirming that activation of guanylate cyclase was responsible for the increase in cGMP levels (data not shown).
cGMP after the stimulation with CCh in a concentration of 10 µM were 70.1 ± 10.2 fmol/ml (n = 6). This value was higher than that of the paired time control (57.8 ± 10 fmol/ml) (n = 6), but significantly (P < 0.001) lower than that observed for SNP (361 ± 25 fmol/ml) (Fig. 3C).

Characterization of EDHF-induced relaxations

H₂O₂ in a cumulative application manner failed to produce any relaxation in endothelium intact or denuded preparations (data not shown). In a single application manner, H₂O₂ at concentrations of 10 and 100 µM produced slow and sustained relaxation reaching the maximum of 30% after 30 min of perfusion. Catalase (1250 IU/ml), which was filtered immediately before use and treated for 30 min, completely inhibited the 100 µM H₂O₂-induced relaxation (Fig. 4A). The Eₘₐₓ caused by CCh was significantly (P < 0.05) decreased by catalase in the presence of nitroarginine and indomethacin (Eₘₐₓ = 87.3 ± 2.5% (n = 5) for the catalase group and 96.9 ± 1.5% (n = 5) for the paired time control) without change in EC₅₀ values (114.8 ± 34 nM for the catalase group and 116 ± 3.1 nM for the paired time control) (Fig. 4B).

On the other hand, clotrimazole (10 µM) and 17-ODA (3 µM) as inhibitors of cytochrome P₄₅₀ monooxygenase showed inhibitory effects on the CCh-induced relaxations with the significantly (P < 0.05) lower Eₘₐₓ value of 78.3 ± 8.1% for clotrimazole (n = 3) and 81.6 ± 1.9% for 17-ODA (n = 3) than the paired time controls (101.5 ± 2%) (n = 6). The EC₅₀ values were determined to be 462.3 ± 35.4 nM for clotrimazole (P < 0.05 vs control), 267 ± 35.4 nM for 17-ODA, and 197 ± 22.8 nM, for the paired time control (Fig. 5A). Meanwhile, 11,12-EETA applied in a single (1 µM) or cumulative (10 nM to 1 µM) manner did not produce a definite relaxation during the contraction caused by phenylephrine (Fig. 5B).

KCl, at low concentrations of 2 to 11 mM produced a concentration-dependent relaxation with the Eₘₐₓ value...
Mediators in Decreasing Vascular Resistance

269

of 26.3 ± 3.5% (n = 5) (Fig. 6A), although KCl at a high concentration of 60 mM produced a contraction. The extent of relaxation was similar under a single and cumulative application manner. KCl in a concentration of 10 mM was used for characterization of the relaxation. The relaxation was significantly inhibited by 100 µM ouabain, but remained unaffected after treatment with Triton X-100 (0.05%) for 1 min (data not shown) or by the combination of 30 µM nitroarginine and 1 µM indomethacin (Fig. 6B).

1-EBIO (1 to 100 µM) produced a concentration-dependent relaxation with the maximal value of 83.3 ± 3.1% (n = 7). The 1-EBIO-induced relaxation was not inhibited but rather increased by nitroarginine in combi-

Fig. 4. 

H_2O_2-induced relaxation (A) and the effect of catalase on the carbachol (CCh)-induced relaxation (B) in the hind limb perfusion model of the rat during the phenylephrine (PE)-induced contraction. Catalase at a sufficient concentration (1250 IU/ml) to inhibit H_2O_2-induced relaxation (A) slightly but significantly (*P < 0.05) decreased the maximum relaxation caused by CCh (10 and 100 µM) (n = 5) (B).

Fig. 5. Inhibitory effects of clotrimazole (CTZ) and 17-octadecinoic acid (ODA) on the carbachol (CCh)-induced relaxation (A) and representative tracing of the effects of 11,12-epoxyeicosatrienoic acid (EET) and CCh during the contraction caused by phenylephrine (PE) (B) in the hind limb perfusion model of the rat. The concentration-relaxation curve for CCh was shifted to the right and upward in the presence of 10 µM CTZ or 3 µM ODA (n = 3) (A). *P < 0.05. 11,12-EET did not change the perfusion pressure during the contraction caused by 10 µM PE (B).
Fig. 6. KCl-induced relaxation and pharmacological analyses of the relaxation in hind limb perfusion model of the rat. Cumulative application of KCl in concentrations of 2 to 11 mM produced a concentration-dependent relaxation as shown in panel A (n = 3). The relaxation caused by 10 mM KCl remained unaffected in the presence of 30 µM N^G-nitro-L-arginine (LNA) in combination with 1 µM indomethacin (IM) during the phenylephrine (PE)-induced contraction as shown in panel B.

Fig. 7. 1-EBIO-induced relaxation and pharmacological analyses of the relaxation in hind limb perfusion model of the rat. Concentration-relaxation curves for 1-EBIO (1 to 100 µM) (n = 7) were compared in the presence or absence of 30 µM N^G-nitro-L-arginine (LNA) in combination with 1 µM indomethacin (IM) as shown in panel A (n = 5). Representative tracings of effects of endothelial denudation with Triton X-100 (0.05% for 1 min) and 100 nM charybdotoxin on the 1-EBIO-induced relaxation are shown in panels B and C, respectively, during the phenylephrine (PE)-induced contraction. Relaxations caused by 20 µM 1-EBIO in the presence of 60 mM KCl (n = 5) or 100 µM ouabain (OUB) (n = 6) were compared with the corresponding time controls without inhibitors (D). *P<0.05, **P<0.005.
nation with indomethacin (Fig. 7A). 1-EBIO in a concentration of 20 \(\mu M\) produced a relaxation (38.3 \(\pm\) 2.7\%, \(n = 8\)). After the treatment with Triton X-100 (0.05\%) for 1 min, the 1-EBIO-induced relaxation was significantly inhibited to 9.7 \(\pm\) 0.1\% (\(n = 4\)) as compared with the time control 38.9 \(\pm\) 1.7\% (\(n = 4\)) (\(P<0.01\)) (Fig. 7B). One hundred nanomolar charybdotoxin (Fig. 7C), 60 mM KCl (7.9 \(\pm\) 1.8\%, \(n = 4\), \(P<0.005\)) and 100 \(\mu M\) ouabain (15.1 \(\pm\) 2.5\%, \(n = 4\), \(P<0.05\)) (Fig. 7D) also showed inhibitory effects on the 1-EBIO-induced relaxation. When 60 mM NaCl in the modified Krebs solution was replaced with 60 mM KCl to produce contraction, the 1-EBIO-induced relaxation was also inhibited to the similar extent.

Carbenoxolone, a gap junction inhibitor (100 \(\mu M\)) abolished the CCh-induced relaxation with an inhibition of the phenylephrine-induced contraction (approximately 70\% of the control) and without change in the basal perfusion pressure. When 30 \(\mu M\) of carbenoxolone was used, the phenylephrine-induced contraction and the CCh-induced relaxation remained unaffected (data not shown).

**Discussion**

Peripheral resistance is controlled by the vascular tone of resistance vessels, in which endothelial cells play an important role. EDHF has been increasingly recognized to be important in the maintenance of vascular tone mainly in the peripheral resistance vessels (18, 19). In the femoral and skeletal arteries of the rat, it is not established which is an important contributor for regulating total peripheral vascular resistance. Some initial reports suggested that NO was more important than EDHF in producing endothelium-dependent relaxation (15, 16). These experiments were performed in the isolated ring specimens and in arteries of bigger size than those controlling peripheral resistance in skeletal muscle (20). On the other hand, using hind limb perfusion models, it was shown that the endothelium-dependent relaxation in response to acetylcholine was completely resistant to the inhibition of NOS or cyclooxygenase (21 – 23). Furthermore, in an in vivo hind limb perfusion experiment using the rat, it was shown that EDHF played an important role in reducing the vascular tone with acetylcholine (17). Thus it seems appropriate that perfusion experiments might be more suitable for investigating the involvement of EDHF.

According to the results presented in this report, the potency and efficacy of CCh to produce relaxation was quantitatively similar to the NO donor SNP. However, the characteristics of the two relaxations were different from each other. CCh induced an endothelium-depen-
Up to now, a clear candidate for EDHF in the hind limb perfusion model has not been established. \( \text{H}_2\text{O}_2 \) has been shown to be an EDHF in the mesenteric vessels of humans and rodents (4, 5). In the present experiments, only high concentrations of \( \text{H}_2\text{O}_2 \) could produce a progressive and sustained relaxation and the characters of the relaxation were different from those produced by CCh. Concordantly, catalase at concentrations which abolished the \( \text{H}_2\text{O}_2 \)-induced relaxation, showed no definite inhibition of the CCh-induced relaxation. Taking these data together, they suggest that \( \text{H}_2\text{O}_2 \) is not a candidate for EDHF in the CCh-induced relaxation.

Since EETAs are also prominent candidates for EDHF in coronary arteries (6, 28), we tried to inhibit the cytochrome P\(_{450}\) monooxygenase in order to decrease the endogenous production of EETAs. Clotrimazole has been widely used for this purpose. In the present experiments, clotrimazole inhibited the CCh-induced relaxation with concomitant decrease in the phenylephrine-induced contraction. These results should be analyzed with caution since it has been shown that clotrimazole inhibits not only P\(_{450}\) monooxygenase but also potassium channels (29). Thus, we evaluated the effects of another P\(_{450}\) monooxygenase inhibitor, 17-ODA, which partially but clearly inhibited the CCh-induced relaxation without affecting the phenylephrine-induced contraction, leading us to speculate that a metabolite(s) of P\(_{450}\) monooxygenase may be involved in the CCh-induced relaxation. Meanwhile, we could not find any relaxation by an application of 11,12-EETA. The lack of relaxation with 11,12-EETA may preclude its proposal as EDHF in this system and seems contradictory with the results obtained by clotrimazole and 17-ODA. However, since it has been shown that in some vascular systems regioisomer specificity of EETAs might exist (30), it is possible to assume that EETA(s) different from 11,12-regioisomer may be involved in the CCh-induced relaxation in our system.

Potassium ion itself was proposed as another possible candidate for EDHF in the rat hepatic arteries (8, 31). It was proposed that K\(^+\) would leave the endothelial cells through the activated SK\(_{Ca}\) and IK\(_{Ca}\) channels, leading to endothelial hyperpolarization. The increased concentration of K\(^+\) in the myoendothelial space would activate the inward rectifier potassium channels and/or Na\(^+\)/K\(^+\) ATPase, resulting in smooth muscle cell hyperpolarization and relaxation. In the current experiments, low concentrations (2 to 11 mM) of KCl both in a single or cumulative application manners produced a definite relaxation under the basal concentration of 4.89 mM KCl in the perfusion medium. The KCl-induced relaxation was independent of endothelium and unaffected by NOS and COX inhibitors, but abolished by ouabain. Furthermore, the CCh-induced relaxation was inhibited by charybdotoxin in combination with apamin or tetraethylammonium. These findings led us to assume that potassium ion possibly plays a role as a candidate for EDHF in the CCh-induced relaxation of the peripheral resistance vessel of the rat. This assumption appears to be supported by previous reports that showed that low concentrations of potassium ion could produce maximal relaxations in small branches of the femoral and gluteal arteries, respectively. According to Savage et al. (9) and De Clerk et al. (32), KCl-induced relaxation was independent of endothelium and sensitive to ouabain.

We further analyzed the possibility of K\(^+\) as a candidate of EDHF by observing the effect of 1-EBIO to produce relaxation. In the present experiments, 1-EBIO produced a concentration-dependent relaxation in the hind limb perfusion model, which was inhibited by endothelial denudation or depolarization with high concentration of KCl, charybdotoxin, and ouabain, suggesting that the relaxation was dependent on endothelium, associated with hyperpolarization and activation of charybdotoxin sensitive channels, and related to Na\(^+\)/K\(^+\) ATPase activity. If these findings are taken together, they appear to support the K\(^+\) theory as the identity for EDHF. However in other types of cells and vessels, it has been described that 1-EBIO exerts actions other than the activation of IK\(_{Ca}\) channels and produces relaxations not associated with hyperpolarization as well (33 – 35). Thus further experiments are required to understand the exact mechanism of 1-EBIO-induced relaxation.

Gap junctions are considered as a main candidate for the transmission either of a putative EDHF or a current generated in the endothelial cells (12). However, gap junction studies are limited due to the inability to selectively block the myoendothelial gap junction. Herein we used the 18-glycyrrhetinic acid derivative carbenoxolone at different concentrations. We showed the concentration-dependent inhibition of the CCh-induced relaxation, suggesting the involvement of myoendothelial gap junctions. However, since a concentration-dependent inhibition of the phenylephrine-induced contraction was concomitantly observed with carbenoxolone, gap junctions within the smooth muscle layer were also possibly inhibited by this agent. Nonetheless, these results suggest the involvement of gap junctions in the coupling of the endothelial cells and smooth muscle cells in the EDHF-mediated relaxation.

Regarding the present report, the temperature under which the experiments were performed might be a concern. It could be argued that some of the analyzed enzymes would not be functional. However, we found
no difference in the vascular response to phenylephrine and CCh at 22°C and 37°C. In addition, we have found that the relaxations induced by certain polyphenols are partially inhibited by nitroarginine and that the addition of catalase abolishes these relaxations (K. Iwasaki-Kurashigke, unpublished observations), showing that under the present experimental conditions, the enzymes are functional and susceptible to pharmacological manipulations.

In conclusion, CCh produces an endothelium-dependent, EDHF-dependent, and NO-cGMP-independent relaxation in the hind limb perfusion model of the rat. Potassium ion and metabolite(s) of P₄₅₀ monooxygenase possibly play an important role for this relaxation.

Acknowledgments

Dr. R.Y. Loyaga-Rendon is a holder of the Scholarship from the Ministry of Education, Culture, Sports, Science, and Technology, Japan. This work was partly supported by Grants-in-Aid for Scientific Research (13671700 to TA and 14572152 to HA) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan; the Smoking Research Foundation, Japan (to HA); and the New Drug Research (NDR) Foundation, Japan (to HA).

References


34 Walker SD, Dora KA, Ings NT, Crane GJ, Garland CJ. Activation of endothelial cell IK(Ca) with 1-ethyl-2-benzimidazolone evokes smooth muscle hyperpolarization in rat isolated mesenteric artery, Br J Pharmacol. 2001;134:1548–1554.