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European Journal of Pharmacology 538 (2006) 132–140
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Mechanisms underlying the impaired EDHF-type relaxation response in mesenteric arteries from Otsuka Long-Evans Tokushima Fatty (OLETF) rats

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Received 27 October 2005; received in revised form 2 March 2006; accepted 3 April 2006
Available online 15 April 2006

Abstract

We previously reported that in mesenteric arteries from streptozotocin-induced diabetic rats, the endothelium-derived hyperpolarizing factor (EDHF)-type relaxation is impaired, possibly due to a reduced action of cAMP. Here, we observed an impairment of acetylcholine-induced EDHF-type relaxation in mesenteric arteries from a type 2 diabetic model, Otsuka Long-Evans Tokushima Fatty (OLETF) rats [vs. age-matched control Long-Evans Tokushima Otsuka (LETO) rats], and we investigated the mechanism underlying this impairment. In the LETO group, this EDHF-type relaxation was attenuated by 18α-glycyrrhetinic acid (a gap-junction inhibitor) and by a protein kinase A (PKA) inhibitor. In both groups (OLETF and LETO), it was enhanced by 3-isobutyl-1-methylxanthine, a cAMP-phosphodiesterase (PDE) inhibitor, but following these enhancements it was still weaker in OLETF rats than in LETO rats. The relaxations induced by cilostamide (a selective PDE3 inhibitor) and 8-bromo-cAMP (a cell-permeant cAMP analog) were reduced in OLETF rats, as was PKA activity. The relaxations induced by two activators of Ca^{2+}-activated K^+ channels (K_{Ca}) [1-ethyl-2-benzimidazolinone (1-EBIO), intermediate-conductance K_{Ca} channel (IK_{Ca}) activator, and riluzole, small-conductance K_{Ca} channel (SK_{Ca}) activator] were also impaired in OLETF rats. We conclude that the impairment of EDHF-type relaxation seen in OLETF rats may be attributable not only to a reduction in cAMP/PKA signaling, but also to reduced endothelial K_{Ca} channel activities.

Keywords: Calcium-activated K^+ channel; cAMP; Endothelium-derived hyperpolarizing factor; Mesenteric artery; Type 2 diabetes; (Rat)

1. Introduction

The endothelium plays a major role in the regulation of vascular tone. It is capable of exerting a profound relaxing influence on the underlying smooth muscle, an effect mediated by at least three different factors, depending on the vascular bed. These factors include nitric oxide (NO) and prostacyclin, both diffusible factors (Furchgott and Zawadzki, 1980; Cohen, 1995). In addition, after blockade of NO- and prostacyclin-synthesis, stimulation of the endothelium is capable of evoking a vascular smooth muscle relaxation that has been attributed to a third factor, endothelium-derived hyperpolarizing factor (EDHF) (Ahluwalia and Hobbs, 2005; Busse et al., 2002; Cohen and Vanhoutte, 1995; Feletou and Vanhoutte, 2004; Griffith, 2004; Sandow, 2004).

Several lines of evidence suggest that endothelial dysfunction could play a key role in the development of both macro-and microangiopathy in diabetes patients and in animal models of diabetes (De Vriese et al., 2000b; Luscher et al., 2003; Pieper, 1998; Poston and Taylor, 1995; Smits et al., 1993). An accumulating body of evidence indicates that endothelium-dependent relaxation is impaired in several blood vessels in type 2 diabetes in animal models and in patients (Kim et al., 2002; Kobayashi et al., 2004; Matsumoto et al., 2004e; Minami et al., 2002; Sakamoto et al., 1998; Ting et al., 1996) as well as in type 1 diabetes (Cohen, 1995; De Vriese et al., 2000b; Kamata et al., 1989; Kobayashi and Kamata, 1999; Kobayashi et al., 2000; Matsumoto et al., 2003a, 2004b; Mayhan, 1992; Pieper, 1998; Poston and Taylor, 1995). Although there is now evidence to suggest that EDHF activity becomes altered both in disease states (such as hypertension and diabetes) and as a result of ageing (De Vriese et al., 2000b; Feletou and Vanhoutte, 2004; Fitzgerald et al., 2005; Hill et al., 2001; Rummery and Hill,

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doi:10.1016/j.ejphar.2006.04.006

2004), the exact nature of its alteration in the diabetic state remains unclear.

We recently demonstrated that acetylcholine-induced EDHF-type relaxation, which proved sensitive to a gap-junction inhibitor, was impaired in the mesenteric arteries of streptozotocin-induced diabetic rats (Matsumoto et al., 2003a). Furthermore, phosphodiesterase (PDE) inhibitors enhanced the EDHF-type relaxation in such arteries isolated from both control and diabetic rats, but the augmentation was greater in the diabetics, with the result that the maximal relaxation amplitude was similar to that in the controls (Matsumoto et al., 2003a,b). Under normal conditions, cAMP appears to facilitate the spread of current through gap junctions, enabling the potentiation and transmission of EDHF-mediated hyperpolarization to regions electrically distant from the endothelium (Chaytor et al., 2002; Griffith, 2004; Griffith et al., 2002). Hence, it is possible that reductions in cAMP activity via increased PDE could contribute to the impaired EDHF-type relaxation observed in diabetic rats. Indeed, we recently suggested that the selective PDE3 inhibitor cilostazol might improve EDHF-type relaxation in mesenteric arteries from streptozotocin-induced diabetic rats via an enhancement effect on the cAMP/protein kinase A (PKA) pathway (Matsumoto et al., 2005a).

In the present study, we investigated (i) whether the contribution made by the cAMP/PKA pathway to EDHF-type relaxation is altered in mesenteric arteries from aged Otsuka Long-Evans Tokushima Fatty (OLETF) rats, an animal model of type 2 diabetes, and (ii) whether the ability of the endothelial cells to hyperpolarize–which involves the activation of two populations of endothelial potassium channels, the small conductance and intermediate conductance K_{Ca} channels (SK_{Ca} and IK_{Ca}, respectively)–is altered in mesenteric arteries from this type 2 diabetic model.

2. Materials and methods

2.1. Reagents

Phenylephrine, indomethacin, N^G-nitro-L-arginine (L-NNA), 3-isobutyl-1-methylxanthine (IBMX), riluzole, apamin, charybdotoxin, glybenclamide, 18α-glycyrrhetinic acid (18α-GA), 8-bromo-adenosine-3′,5′-cyclic monophosphate (8BrcAMP), N^6,O^2-dibutyryl-adenosine-3′,5′-cyclic monophosphate (db-cAMP), EDTA, EGTA, phenylmethylsulfonyl fluoride (PMSF), leupeptin, aprotinin, and sodium nitroprusside were all purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acetylcholine chloride was from Daiichi Pharmaceuticals (Tokyo, Japan). A PKA inhibitor (14–22 amide, cell-permeable, myristoylated) and cilostamide were from Calbiochem-Novabiochem Corporation (La Jolla, CA, USA). Cromakalim was from Toronto Research Chemicals (North York, ON, Canada) and 1-ethyl-2-benzimidazolinone (1-EBIO) from Tocris (Natrick, MA, USA). All drugs were dissolved in saline, except IBMX, riluzole, glybenclamide, cromakalim, 18α-GA and 1-EBIO (which were dissolved in dimethyl sulfoxide). Control experiments confirmed the absence of significant effects on vascular tone at the final vehicle concentration used.

2.2. Animals and experimental design

Five-week-old male OLETF rats and Long Evans Tokushima Otsuka rats (LETO rats, genetic controls for OLETF) were supplied by Tokushima Research Institute (Otsuka Pharmaceutical). Food and water were given ad libitum in a controlled environment [room temperature 21–22 °C, room humidity 50± 5%], until 60 weeks of age. This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals adopted by the Committee on the Care and Use of Laboratory Animals of Hoshi University (which is accredited by the Ministry of Education, Culture, Sports, Science and Technology, Japan).

2.3. Measurements of plasma glucose, insulin, and blood pressure

Plasma parameters and blood pressure were measured as previously described (Kobayashi et al., 2004; Matsumoto et al., 2004a,b).

2.4. Measurement of isometric force

Vascular isometric force was recorded as in our previous papers (Matsumoto et al., 2003a, 2004d,e, 2005a,b). Rats were anesthetized with diethyl ether and euthanized by decapitation at 60 weeks old. The superior mesenteric artery was rapidly removed and immersed in oxygenated, modified Krebs-Henseleit solution (KHS). This solution consisted of (in mM) 118.0 NaCl, 4.7 KCl, 25.0 $NaHCO_3$, 1.8 $CaCl_2$, 1.2 NaH_2PO_4, 1.2 $MgSO_4$, and 11.0 dextrose. The artery was carefully cleaned of all fat and connective tissue, and ring segments 2 mm in length were suspended by a pair of stainless-steel pins in a well-oxygenated (95% O_2–5% CO_2) bath of 10 ml KHS at 37 °C. The rings were stretched until an optimal resting tension of 1.0 g was loaded, and then allowed to equilibrate for at least 60 min. Force generation was monitored by means of an isometric transducer (model TB-611T; Nihon Kohden, Tokyo, Japan). The tissues were equilibrated for 40 min in the presence of 100 μM L-NNA and 10 μM indomethacin (to block nitric oxide synthase and cyclo-oxygenase, respectively) before administration of phenylephrine. In most experiment, various concentrations of phenylephrine (0.1–1 μM) were used to permit matching of precontractions. In experiments involving IBMX, the equieffective concentrations of phenylephrine used were 1–3 μM. Once the phenylephrine-induced contraction had stabilized, relaxation responses were elicited in a cumulative manner [using acetylcholine, cilostamide (PDE3 inhibitor), riluzole (SKCa activator), 1-EBIO (IK_{Ca} activator), or cromakalim (K_{ATP} activator)]. Such concentration–response curves were also generated in the combined presence of L-NNA (100 μM), indomethacin (10 μM), and one of the following: 18α-glycyrrhetinic acid (18α-GA; 100 μM for 40 min), 3-isobutyl-1-methylxanthine (IBMX; 20 μM for 40 min), PKA inhibitor (5 μM for 40 min), apamin (100 nM for 40 min), or charybdotoxin (70 nM for 40 min). In some experiments, when the phenylephrine-induced contraction had reached a plateau

level in a given intact mesenteric ring, sodium nitroprusside (10^{-10} –10^{-5} M) was added in a cumulative manner. After the addition of sufficient aliquots of the agonist to produce the chosen concentration, a plateau response was allowed to develop before the addition of the next dose of the same agonist. In preliminary experiments, we established that the phenylephrine-induced pre-contraction was stable throughout a given experiment. Each relaxation response was expressed as a percentage of the contraction induced by phenylephrine.

2.5. In vitro kinase assay for PKA activity

PKA activity was measured as previously described (Matsumoto et al., 2004d). Mesenteric arteries were pretreated with 20 μM IBMX for 30 min at 37 °C, then, treated with either 100 μM db-cAMP or vehicle (deionized water) for 30 min at 37 °C. Next, they were rapidly frozen in liquid N_2, and stored at −80 °C. The mesenteric tissues were homogenized in a homogenization buffer containing 25 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.3 mM PMSF, 0.04 mM leupeptin, and 0.02 mM aprotinin. The homogenates were centrifuged, and the supernatant (10 μg of protein) was used in a nonradioactive assay for cAMP-dependent protein kinase [PKA assay system; Promega (Madison, WI, USA)]. For the positive control (10 ng of PKA; provided with the kit), we used the same assay conditions as for the artery samples. For the negative control (deionized water), we again used the same assay conditions as for the artery samples. Phosphorylated and nonphosphorylated peptide bands were visualized on a 0.8% agarose gel, and the former were quantitated by spectrophotometry.

2.6. Statistical analysis

Data are expressed as means±SEM. When appropriate, statistical differences were assessed by Dunnett's test for multiple comparisons after a one-way analysis of variance, the minimum probability level regarded as significant being indicated by a P value of <0.05. Statistical comparisons between concentration–response curves were made using a two-way ANOVA, with Bonferroni's correction for multiple comparisons being performed post hoc ($P<0.05$ again indicating the minimum level of significance).

3. Results

3.1. General parameters

At the time of the experiment, all OLETF rats (non-fasted) exhibited hyperglycemia, their blood glucose concentrations (335±49 mg/dl, $n=15$) being significantly higher than those of the age-matched nondiabetic control LETO rats (also non-fasted) (162±5 mg/dl, $n=15$; $P<0.01$). The body weight of the OLETF rats (671±37 g, $n=15$) was greater than that of the LETO rats (565±11 g, $n=15$; $P<0.01$). As expected at age 60 weeks (see Discussion), the plasma insulin concentrations were similar between OLETF rats (1324±170 pg/ml, $n=15$) and LETO rats (1385±104 pg/ml, $n=15$). The systolic blood pressure of the OLETF rats (137±4 mm, Hg, $n=15$) was higher than that of the LETO rats (113±2 mm Hg, $n=15$; $P<0.001$).

3.2. Acetylcholine-induced EDHF-type relaxation and sodium nitroprusside-induced relaxation

In order to investigate the EDHF-type relaxation evoked by acetylcholine in the rat mesenteric artery, we performed a series of experiments in which acetylcholine (10^{-9}–10^{-5} M) was added cumulatively to rings pre-contracted by phenylephrine in the presence of 100 μM L-NNA plus 10 μM indomethacin. As shown in Fig. 1, this relaxation was significantly weaker in mesenteric arteries from OLETF rats, and the EC_{50} values were 77±13 nM and 41±4 nM in OLETF and LETO, respectively ($P<0.05$). The acetylcholine-induced EDHF-type relaxation was completely inhibited by apamin (100 nM) plus charybdotoxin (100 nM) and by 30 mM K^+, and it was absent in rings denuded of their endothelium (data not shown).

The sodium nitroprusside-induced (endothelium-independent) relaxation was not different between the two groups (Fig. 1B).

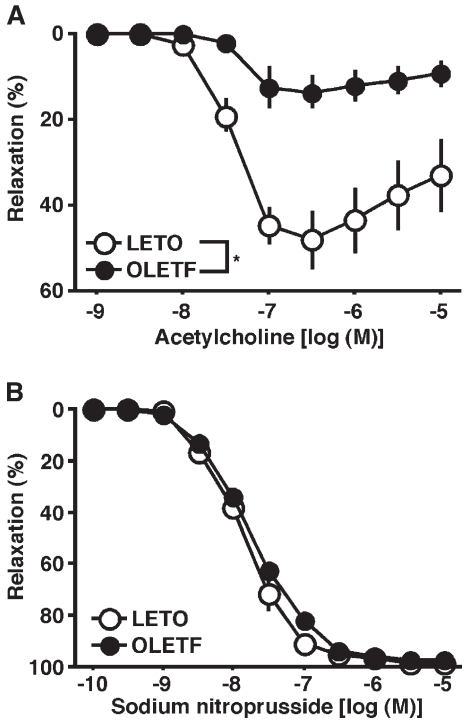

Fig. 1. Concentration–response curves for the endothelium-derived hyperpolarizing factor (EDHF)-type relaxation evoked by acetylcholine (A) and for the endothelium-independent relaxation evoked by sodium nitroprusside (B) in isolated rings of mesenteric arteries obtained from age-matched LETO and OLETF rats. Ordinate shows relaxation of mesenteric arteries as a percentage of the contraction induced by phenylephrine (0% being defined as the stable tonic level of precontraction). In the EDHF experiment, L-NNA (100 μM)+ indomethacin (10 μM) was applied 40 min before the phenylephrine application, and was present thereafter. Each data-point represents the mean± SEM from 8 (A) or 3 (B) experiments; the SEM is included only when it exceeds the dimension of the symbol used. *$P<0.001$, LETO vs. OLETF.

3.3. Effect of 18α-glycyrrhetinic acid on acetylcholine-induced EDHF-type relaxation

To examine the part played by gap-junctional communication in the present EDHF-type relaxation, rings were incubated with 18α-GA, a gap-junction inhibitor (as well as with L-NNA plus indomethacin) for 40 min before administration of phenylephrine. As shown in Fig. 2A, acetylcholine (10^{-9}–10^{-5} M) caused a reduced concentration-dependent relaxation in such rings when they were from LETO rats, but not in those from OLETF rats.

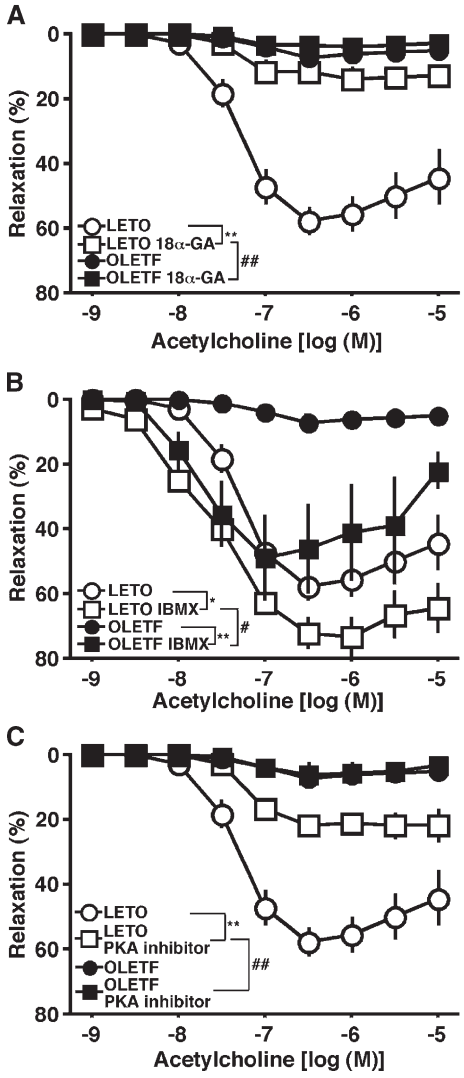

Fig. 2. Effects of the gap-junction inhibitor 18α-GA (A), the PDE inhibitor IBMX (B), and a PKA inhibitor (C) on the EDHF-type relaxations evoked by acetylcholine in mesenteric arteries obtained from age-matched LETO and OLETF rats. In each experiment, a combination of L-NNA (100 μM), indomethacin (10 μM), and one of 18α-GA (100 μM) (A), IBMX (20 μM) (B), or PKA inhibitor (5 μM) was applied 40 min before phenylephrine application, and was present thereafter. Ordinate shows relaxation of mesenteric arteries as a percentage of the contraction induced by phenylephrine (0% being defined as the stable tonic level of precontraction). Each datapoint represents the mean±SEM from 4 or 6 experiments. *$P<0.01$, **$P<0.001$, L-NNA+indomethacin group vs. L-NNA+indomethacin+18α-GA or IBMX or PKA inhibitor group. $^{\#}P<0.01$, $^{\#\#}P<0.001$, inhibitors-treated-LETO vs. inhibitors-treated-OLETF group.

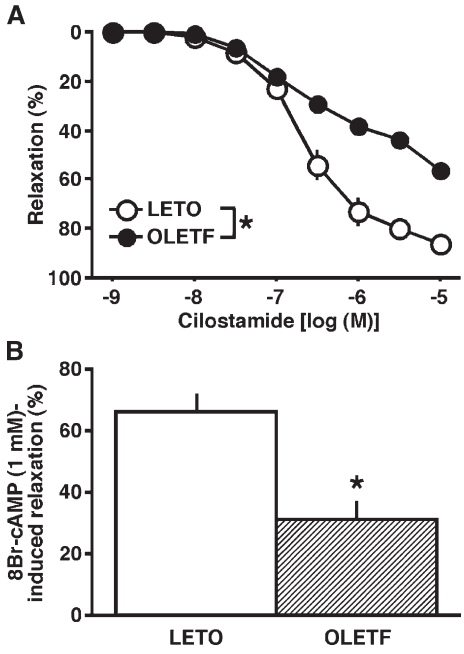

Fig. 3. cAMP-mediated relaxation in mesenteric arteries from age-matched LETO and OLETF rats. (A) Concentration–response curves for the relaxations induced by cilostamide (phosphodiesterase 3-specific inhibitor) in isolated rings of mesenteric artery. Ordinate shows relaxation of mesenteric arteries as a percentage of the contraction induced by phenylephrine (0% being defined as the stable tonic level of precontraction). In each experiment, L-NNA (100 μM) +indomethacin (10 μM) was applied 40 min before the phenylephrine application, and was present thereafter. Each data-point represents the mean ±SEM from 5 experiments; the SEM is included only when it exceeds the dimension of the symbol used. *$P<0.05$, LETO vs. OLETF. (B) Decrease in cAMP derivative-induced relaxation in OLETF mesenteric arteries. 8-bromo-cAMP (8BrcAMP; 1 mM) was added after the phenylephrine-induced contraction had reached a steady-state level. Results are expressed as the mean±SEM from 6 experiments. *$P<0.01$, LETO vs. OLETF.

3.4. Effect of phosphodiesterase inhibitors on acetylcholine-induced EDHF-type relaxation

Since IBMX depressed contraction, the concentration of phenylephrine used in experiments involving this agent was increased to between 1 and 3 μM. The tension developed in response to 1–3 μM phenylephrine in the presence of 20 μM IBMX was 1571±59 mg in OLETF mesenteric rings ($n=6$) and 1483±39 mg in age-matched LETO mesenteric rings ($n=6$, no significant difference). The concentration–response curves for the peak amplitude of the EDHF-type relaxation induced by acetylcholine in rings pretreated with IBMX are illustrated in Fig. 2B, which shows that acetylcholine (10^{-9}–10^{-5} M) caused an enhanced concentration-dependent relaxation in these rings in the presence of IBMX. Unexpectedly, this enhanced EDHF-type relaxation rats was significantly weaker in OLETF than in LETO rats (Fig. 2B).

3.5. Effect of PKA inhibitor on EDHF-type relaxation

To examine the part played by PKA in the present EDHF-type relaxation, rings were incubated with a PKA inhibitor (as well as with L-NNA plus indomethacin) for 40 min before

administration of phenylephrine. As shown in Fig. 2C, under these conditions the acetylcholine-induced EDHF-type relaxation was attenuated by the PKA inhibitor in the LETO group (Fig. 2C), but not in the OLETF group. Moreover, the PKA-resistant EDHF-type relaxation (i.e., under treatment with L-NNA, indomethacin, and PKA inhibitor) was significantly weaker in OLETF rats than in LETO rats.

3.6. cAMP-mediated relaxation

We examined the relaxing effects of a PDE3 inhibitor and a cAMP analog in rings from the OLETF and the LETO rats (in the presence of 100 μM L-NNA plus 10 μM indomethacin). The concentration–response curves for cilostamide (10^{-9}–10^{-5} M), a specific PDE3 inhibitor, showed that the peak relaxation was significantly weaker when the mesenteric arteries were from OLETF rats [56±2% and 86±2% of the phenylephrine-induced tone in OLETF ($n=5$) and LETO ($n=5$), respectively ($P<0.001$)] (Fig. 3A). The EC_{50} values for the cilostamide-induced relaxations were 339±57 nM and 192±23 nM in OLETF and LETO, respectively ($P<0.05$). Furthermore, the relaxation responses induced by 8Br-cAMP (a cell-permeant cAMP analog that is rated as more resistant to PDE) was significantly weaker in the OLETF group than in the LETO group (Fig. 3B).

3.7. Relaxation induced by K^+-channel activators

In order to investigate the involvement of SK_{Ca} and IK_{Ca} channels in relaxation in the rat mesenteric artery, we performed a series of experiments in which either 1-EBIO (10^{-6}–$10^{-4.5}$ M), an activator of IK_{Ca} channels (and possibly SK_{Ca}, but not BK_{Ca}, channels) or riluzole ($10^{-5.5}$–$10^{-4.5}$ M), an activator of SK_{Ca} channels, was added cumulatively to rings pre-contracted by phenylephrine in the presence of 100 μM L-NNA plus 10 μM indomethacin. The relaxation response induced by 1-EBIO–which is blocked by charybdotoxin (a nonselective inhibitor of BK_{Ca} and IK_{Ca} channels, and some voltage-dependent K^+ channels)–was significantly weaker in the OLETF group than in the LETO group (Fig. 4A). Moreover, the relaxation response induced by riluzole–which is blocked by apamin (a selective inhibitor of SK_{Ca} channels)–was also significantly weaker in the OLETF group than in the LETO group (Fig. 4B). On the other hand, the relaxation response induced by cromakalim [activator of ATP-sensitive potassium (K_{ATP}) channels]–which is blocked by glybenclamide (a selective inhibitor of K_{ATP} channels)–was not significantly different between the OLETF and LETO groups (Fig. 4C).

3.8. PKA activity

We examined the effects of db-cAMP treatment on PKA activity in mesenteric artery rings following pretreatment with the PDE inhibitor IBMX (20 μM). As shown in Fig. 5, the level of PKA activity in vehicle-treated mesenteric arteries was significantly lower in the OLETF group than in the age-matched LETO group ($P<0.05$). Moreover, the db-cAMP-stimulated

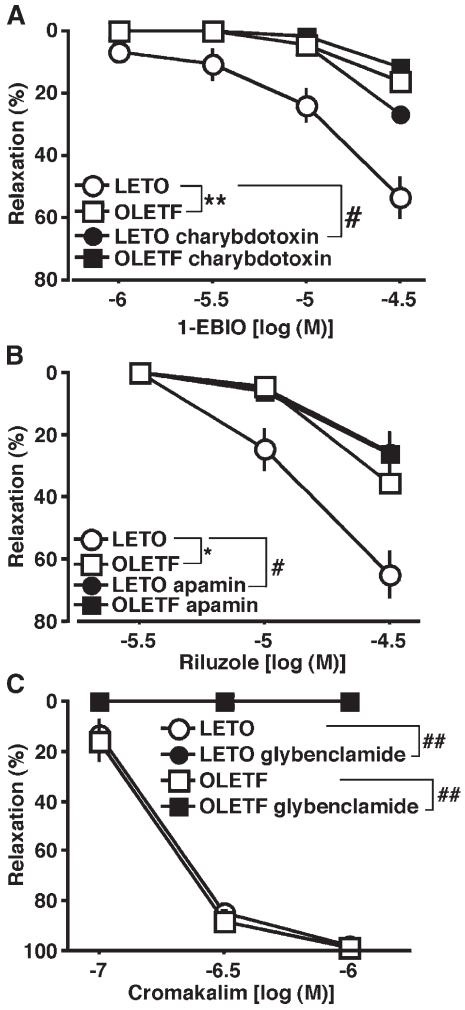

Fig. 4. Concentration–response curves for the effects of K^+ channel activation in mesenteric arteries from age-matched LETO and OLETF rats. Relaxations were induced by (A) an IK_{Ca} channel activator (1-EBIO, 10^{-6}–$10^{-4.5}$ M), (B) a SK_{Ca} channel activator (riluzole, $10^{-5.5}$–$10^{-4.5}$ M), or (C) a K_{ATP} channel activator (cromakalim, 10^{-7}–10^{-6} M), in each case in the presence of L-NNA (100 μM)+indomethacin (10 μM). Such concentration–response curves were also generated in the combined presence of L-NNA, indomethacin, and one of three K^+ channel inhibitors [charybdotoxin (70 nM) in (A), apamin (100 nM) in (B), and glybenclamide (1 μM) in (C)]. Ordinate shows relaxation of mesenteric arteries as a percentage of the contraction induced by phenylephrine (0% being defined as the stable tonic level of precontraction). Each data-point represents the mean±SEM from 3 to 7 experiments; the SEM is included only when it exceeds the dimension of the symbol used. *$P<0.05$, **$P<0.001$, LETO vs. OLETF. $^{\#}P<0.05$, $^{\#\#}P<0.001$, K^+ channel inhibitor-untreated vs. -treated group.

level of PKA activity in mesenteric arteries was significantly lower in the OLETF group than in the age-matched LETO group ($P<0.05$).

4. Discussion

OLETF rats are characterized by an early increase in serum insulin (see below), and also by late-onset hyperglycemia, mild obesity, and mild type 2 diabetes (Kawano et al., 1992). Although there are several reports of abnormalities of vascular function in this diabetic model (Kagota et al., 2000; Kim et al.,

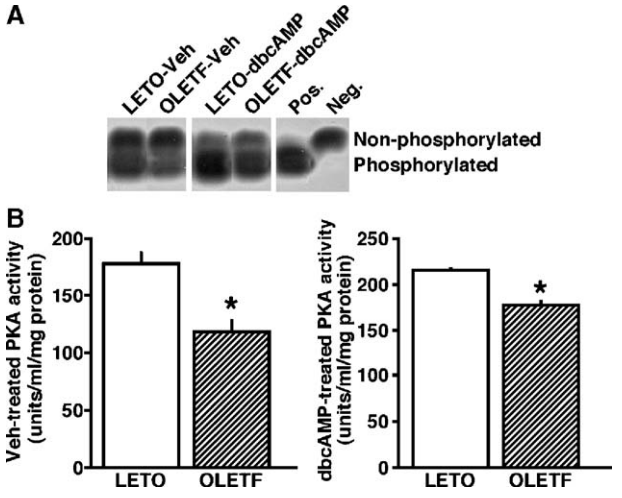

Fig. 5. Measurement of PKA activity in mesenteric arteries from LETO and OLETF rats. The mesenteric arteries were pretreated with 20 μ M IBMX for 30 min, and subsequently either 100 μM db-cAMP or Vehicle (Veh) was added for 30 min. Details are given under Materials and methods. (A) The PKA assay was performed using PepTag assay protein kinase kits according to the manufacturer's instructions. The phosphorylation state of the peptide was examined by agarose-gel electrophoresis. Pos, positive control (10 ng PKA; provided with the kit); Neg, negative control (deionized water). (B) Phosphorylated peptide bands were quantitated by spectrophotometry. Results are mean±SEM from 3 or 4 experiments. *$P<0.05$, vs. corresponding LETO group.

2002; Minami et al., 2002; Sakamoto et al., 1998), no previous study has investigated the mechanisms involved in EDHF signaling in this model. In the present study, we demonstrated that in OLETF rats, a model of type 2 diabetes, the EDHF-type relaxation in the isolated mesenteric artery is impaired (versus that in LETO rats), and that the mechanisms underlying this impairment may be related to reductions both in cAMP/PKA signaling and in the activities of certain endothelial K_{Ca} channels.

Despite numerous studies aimed at identifying a specific factor responsible for endothelium-dependent hyperpolarization, the putative mediator of the EDHF response may actually be one, or a combination, of a number of candidates (Ahluwalia and Hobbs, 2005; Busse et al., 2002; Edwards et al., 1998, 1999a; Feletou and Vanhoutte, 2004; Griffith, 2004; Sandow, 2004). There is evidence that EDHF-type relaxation involves the transfer of a mediator from the endothelium to the smooth muscle via myoendothelial gap junctions (Griffith, 2004; Sandow, 2004). Furthermore, it has recently been reported that cAMP facilitates EDHF-type relaxation in conduit arteries by enhancing electrotonic conduction via gap junctions (Chaytor et al., 2002; Griffith, 2004; Griffith et al., 2002). Endogenous formation of cAMP may therefore play an important role in the EDHF phenomenon, since agonists such as acetylcholine are capable of promoting the endothelial synthesis of cAMP through a mechanism that is independent of the formation of prostanoids (Kamata et al., 1996; Taylor et al., 2001). Furthermore, elevations in smooth muscle cAMP levels have been shown to facilitate electrotonic signaling within the vascular media, and thereby to amplify and prolong the transmission of acetylcholine-induced hyperpolarizations to smooth muscle cells remote from the endothelium (Griffith et al., 2002). Thus, cAMP would seem to play an important role in the regulation of EDHF responses. In a recent in vitro study in our laboratory (Matsumoto et al., 2003a) on mesenteric arteries from streptozotocin-induced diabetic rats, the acetylcholine-induced EDHF-type relaxation, which was sensitive to 18α-GA, was found to be impaired. Further investigation revealed increased PDE activity in this diabetic artery, specifically of PDE3, as well as reduced cAMP levels (Matsumoto et al., 2003a). In that study, PDE inhibitors enhanced the EDHF-type relaxations in mesenteric arteries from both control and diabetic rats, but the augmentation was greater in the diabetics, with the result that maximal relaxation amplitude was then similar to control. Under normal conditions, cAMP appears to facilitate the spread of current through gap junctions, thus enabling the potentiation and transmission of EDHF-mediated hyperpolarization to regions electrically distant from the endothelium (see above). Hence, it is possible that a reduction in cAMP activity (via an increase in PDE) contributes to the impairment of EDHF-type relaxation observed in the mesenteric arteries of diabetic rats. Moreover, in recent studies, we have demonstrated that the abnormal vascular relaxation responsiveness seen in mesenteric arteries from streptozotocin-diabetic rats may be attributable not only to increased PDE activity (Matsumoto et al., 2003a), but also to decreases in PKA activity (Matsumoto et al., 2004d) and AC activity (Matsumoto et al., 2005b). PKA, which is produced by a pleiotropic mechanism, plays key roles in the transduction of many external signals through the cAMP second messenger pathway (Haynes et al., 1992; Murray, 1990), and we recently demonstrated that the EDHF response in rat mesenteric arteries is partly mediated by this enzyme (Matsumoto et al., 2005a). Taken together, the above evidence suggest that the cAMP/PKA signaling pathway could be intimately involved in the specific alteration of the EDHF-mediated response in the mesenteric artery that is seen in diabetic rats. Indeed, our recent study demonstrated that a selective PDE3 inhibitor could improve the EDHF response in the rat diabetic mesenteric artery via increases in cAMP and PKA signaling (Matsumoto et al., 2005a). In the present study, we found that in rat mesenteric arteries: (a) the acetylcholine-induced EDHF-type relaxation was significantly decreased by a PKA inhibitor and by 18 α -GA in the LETO group, but not in the OLETF group; (b) the PDE3 inhibitor-induced relaxation was significantly weaker in the OLETF group; (c) the relaxation induced by a cAMP analog was significantly weaker in the OLETF group, and (d) cAMP analog-stimulated or -unstimulated PKA activity was decreased in the OLETF group. These results strongly suggest that the impairment of EDHF-type relaxation seen in the OLETF rat mesenteric artery is due to a decrease in cAMP/PKA signaling. It was noteworthy that although the EDHF-type relaxation was enhanced by IBMX in mesenteric arteries from both OLETF and LETO groups, the enhanced relaxation was weaker in OLETF rats than in LETO rats. Moreover, the PKA-and/or gap junction-resistant EDHF-type relaxation was

weaker in OLETF rats than in LETO rats. To judge from these results, the impairment of EDHF-type relaxation seen in OLETF rats is attributable not only to a defect in cAMP/PKA signaling, but also to defect(s) in other signaling pathway(s).

Activation of both SK_{Ca} and IK_{Ca} channels in the arterial endothelium is a key step leading to smooth muscle hyperpolarization and relaxation, independently of both NO and prostacyclin (Busse et al., 2002; Nilius and Droogmans, 2001; Walker et al., 2001). Since activation of these endothelial SK_{Ca} and IK_{Ca} channels is also of crucial importance in the initiation of the EDHF signal following agonist stimulation (Busse et al., 2002; Doughty et al., 1999), a defective EDHF-mediated relaxation as well as an impaired endothelial hyperpolarization-mediated relaxation would be expected to be caused by any significant loss of these hyperpolarizing K_{Ca} channels from the endothelium. In mice, an increased expression of endothelial SK3 channels affects the arterial tone of the isolated mesenteric artery, the diameter of these arteries in situ, and the blood pressure of the animal (Taylor et al., 2003). Decreasing the expression of the SK3 channel causes the converse effects (Feletou and Vanhoutte, 2004). Furthermore, Kohler et al. (2005) recently demonstrated that experimental chronic renal failure leads to a loss of EDHF-type relaxation that is caused at least in part by an impaired functional expression of endothelial hyperpolarizing K_{Ca}. In the present study, we found that (a) the relaxation induced by 1-EBIO–an agent without effect on vascular smooth muscle, but which opens charybdotoxin-sensitive IK_{Ca} channels in vascular endothelial cells (Edwards et al., 1999b)–was significantly impaired in OLETF rats, and that this relaxation was blocked by charybdotoxin in LETO rats, but not in OLETF rats, (b) the relaxation induced by riluzole, an opener of SK_{Ca} channels (Crane and Garland, 2004), was significantly impaired in OLETF rats, and that this relaxation was blocked by apamin in LETO rats, but not in OLETF rats, and (c) the relaxation induced by cromakalim, an activator of K_{ATP} channels (Matsumoto et al., 2004c), was not significantly different between OLETF and LETO rats, and that this relaxation was blocked by glybenclamide in both groups. These results suggest that in type 2 diabetic mesenteric arteries, the activation of endothelial K_{Ca} channels (SK_{Ca}, IK_{Ca}) is impaired, whereas the activity of smooth muscle K_{ATP} channels is preserved.

Several studies have suggested that impairments of EDHF-mediated responses are present in disease states, indicating the potential for therapeutic interventions (Feletou and Vanhoutte, 2004). For example, chronic treatment with an ACE inhibitor, with an angiotensin-receptor antagonist, or with a diuretic normalizes EDHF-mediated responses in spontaneously hypertensive rats (Feletou and Vanhoutte, 2004; Onaka et al., 1998). Moreover, treatment with folate, with an aldose-reductase inhibitor, or with calcium dobesilate (an angioprotective agent) restores impaired EDHF-mediated responses in diabetes (Angulo et al., 2003; De Vriese et al., 2000a; Feletou and Vanhoutte, 2004). In addition, dietary supplements and exercise have beneficial effects on EDHF responses (Feletou and Vanhoutte, 2004). However, the mechanisms underlying these drug-induced and adjutant-induced improvements in EDHF responses remain poorly understood. It is clear that EDHF-mediated responses are affected in a variety of pathological conditions, and that the above therapeutic or adjutant interventions can improve these EDHF-mediated responses, with anticipated beneficial effects for patients. Especially interesting is the possibility that an enhancement of EDHF-mediated responses might contribute to improvements in diabetic microvascular complications such as retinopathy, nephropathy, and neuropathy (because EDHF plays important roles in the microvasculature).

In experimental studies using streptozotocin-induced diabetic rats (a Type 1 diabetes model), an impaired EDHF-mediated response has been reported both by us (Matsumoto et al., 2003a, b, 2005a) and by others (Fukao et al., 1997; Wigg et al., 2001). However, there is little experimental data concerning such responses in Type 2 diabetes because a suitable model of Type 2 diabetes has not previously been available. OLETF rats manifest stable clinical and pathological features that resemble human Type 2 diabetes (Kawano et al., 1992). Briefly, OLETF rats are characterized by (1) increasing body weight just after weaning, in contrast to streptozotocin-induced diabetic rats, (2) a late onset of their hyperglycemia (after 18 weeks of age) and diagnosable diabetes after 24 weeks of age, (3) a hyperinsulinemia that is present at 24 weeks of age and declines after 55 weeks of age, and (4) increasing plasma cholesterol and triglyceride concentrations after 21 weeks of age (Kawano et al., 1992, 1994). Although hypertension is one of the major risk factors for cardiovascular diseases and is closely related to Type 2 diabetes (Epstein and Sowers, 1992), the mechanism underlying the hypertension seen in OLETF rats remains to be elucidated. In the present study, the blood pressure of the OLETF rats at 60 weeks of age was higher than that of the agematched LETO rats. Because the contribution made by EDHF-mediated responses appears significantly greater in small than in large arteries (Busse et al., 2002; Feletou and Vanhoutte, 2004; Griffith, 2004: Shimokawa et al., 1996), EDHF responses may be important for blood-pressure homeostasis. Indeed, impaired EDHF-mediated responses have been demonstrated in several hypertensive models (Feletou and Vanhoutte, 2004). Moreover, Taylor et al. (2003) demonstrated that an altered expression/activity of SK_{Ca} channels modulated arterial tone and blood pressure. Taken together, the above observations suggest to us that the increased blood pressure in OLETF rats may be attributable to impairment of the EDHF-mediated response due not only to a defect in cAMP/PKA signaling (as seen in streptozotocin-induced diabetic rats too; Matsumoto et al., 2003a, 2005a), but also to reduced endothelial K_{Ca} channel activity. These impairments could conceivably be secondary to the long-term presence of disease states such as hyperglycemia, hyperlipidemia, increased abdominal fat accumulation, and/or insulin resistance in Type 2 diabetes. This speculation is supported by two findings: (a) that EDHF-mediated relaxation and K_{Ca} channel activity are each impaired in a fructose-fed insulin-resistance model (Erdos et al., 2002; Katakam et al., 1999), and (b) that the acetylcholine-induced EDHF response is reduced by an elevated glucose level in the rat mesenteric artery (Ozkan and Uma, 2005). However, further study will be required

to establish causal relationships in the actual mechanism underlying the attenuation of the EDHF-mediated response seen in animal models of Type 2 diabetes.

In conclusion, we found that EDHF-type relaxation was impaired in type 2 diabetic mesenteric arteries (from OLETF rats), and that this impairment may be attributable to reduced cAMP/PKA signaling and/or reduced endothelial K_{Ca} activities.

We believe that our findings should stimulate further interest in the improvement or restoration of EDHF responses in the continuing efforts to reduce diabetes-associated vascular disease.

Acknowledgments

We thank E. Arai and Y. Nasu for technical help. We also thank Otsuka Pharmaceutical for providing LETO and OLETF rats. This study was supported in part by the Ministry of Education, Culture, Sports, Science and Technology, Japan and by the Promotion and Mutual Aid Cooperation for Private Schools of Japan.

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