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Two distinct pathways account for EDHF-dependent dilatation in the *gracilis* artery of dyslipidaemic hApoB$^{+/+}$ mice

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1 A universal endothelium-derived hyperpolarising factor (EDHF – non-NO/non-PGI_2) has not been identified. EDHF, however, is essential for the physiological control of resistance artery tone. The impact of dyslipidaemia (DL), a risk factor for cardiovascular diseases, on the nature and the efficacy of EDHF has not been evaluated yet.

2 Pressurised (80 mmHg) *gracilis* arterial segments isolated from mice expressing the human apoB-100 and C57Bl/6 wild-type (WT) mice were used. EDHF-dependent dilatations to acetylcholine (ACh) were measured in the presence of L-NNA (100 μM, NOS inhibitor) and indomethacin (10 μM, COX inhibitor).

3 Maximal EDHF-induced dilatations were increased in DL when compared to WT (95 ± 2 *versus* 86 ± 4% in WT; $P<0.05$). Combination of apamin and charybdotoxin strongly reduced ($P<0.05$) ACh-induced dilatation in WT (22 ± 4%) and DL (25 ± 5%).

4 Combined addition of barium (Ba^{2+}) and ouabain abolished EDHF-induced dilatations in WT arteries (13 ± 3%; $P<0.05$). In vessels isolated from DL mice, however, only the addition of 14,15-EEZE (a 14,15-EET antagonist) to Ba^{2+} and ouabain prevented EDHF-induced dilatations (5 ± 3% compared to 54 ± 11% in the presence of combined Ba^{2+} and ouabain; $P<0.05$).

5 Our data suggest that EDHF-mediated dilatation depends on the opening of endothelial SK_{Ca} and IK_{Ca} channels. This is associated with the opening of K_{ir} channels and activation of the Na^+/K^+-ATPase pump on smooth muscle cells leading to dilatation. In arteries from DL mice, a cytochrome *P*450 metabolite likely to be 14,15-EET equally contributes to the dilatory action of ACh. The early increased efficacy of EDHF in arteries isolated from DL mice may originate from the duplication of the EDHF pathways.
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Abbreviations: ACh, acetylcholine; Apa, apamin; Ba^{2+}, barium; Chtx, charybdotoxin; DL, dyslipidaemia; E_{max}, maximal dilatation; EDHF, endothelium-derived hyperpolarising factor; EETs, epoxyecosatrienoic acids; EEZE, 14,15-epoxyeicosa-5(Z)-enoic acid; 18α-GA, 18α-glycyrrhetinic acid; HC, hypercholesterolemia; indo, indomethacin; K_{Ca}, Ca^{2+}-sensitive K^+ channel; K_{ir}, inward rectifier potassium channels; L-NNA, N^{ω}-nitro-L-arginine; MT, myogenic tone; NO, nitric oxide; 17-ODYA, 17-octadecynoic acid; Oub, ouabain; PE, phenylephrine; PGI_2, prostacyclin; PSS, physiological salt solution; SNP, sodium nitroprusside; WT, wild type

Introduction

Three factors have been identified as being endothelium-derived relaxing factors (EDRFs). Of these, three EDRFs, only nitric oxide (NO) and prostacyclin (PGI_2) pathways are well characterised, while the endothelium-derived hyperpolarising factor (EDHF) is still the subject of debates as to its nature and its mechanisms of action. Studies have identified EDHF, a non-NO/non-PGI_2 factor, as being an augmentation of the extracellular concentration of potassium ions ($[K^+]_o$) between smooth muscle and endothelial cells (Edwards *et al*., 1998). The apamin (Apa)-sensitive small conductance calcium-dependent potassium channel (SK_{Ca}) (Adeagbo & Triggle, 1993; Parsons *et al*., 1996; Véquaud & Thorin, 2001), the charybdotoxin (Chtx)-sensitive intermediate conductance calcium-dependent potassium channel (IK_{Ca}) (Cowan *et al*., 1993; Lischke *et al*., 1995), or a combination of both channels have been shown to account for the accumulation of K^+ ions in the extracellular space (Zygmunt & Hogestatt, 1996; Edwards *et al*., 1998). The hyperpolarising action on the smooth muscle cells of this augmentation of the $[K^+]_o$ has been shown to be driven by the activation of barium (Ba^{2+})-sensitive smooth muscle inward-rectifier potassium (K_{ir}) channels (Knot *et al*., 1996; Edwards *et al*., 1998) and/or of the ouabain-sensitive smooth muscle sodium-potassium pump (Na^+/K^+-ATPase) (Edwards *et al*., 1998; Félétou & Vanhoutte, 1988). Among the many other potential EDHFs, cytochrome *P*450 metabolites of arachidonic acid, such as the epoxyecosatrienoic acids (EETs), have also been postulated in some cases as being responsible for the non-NO and non-PGI_2 smooth muscle hyperpolarisation induced by acetylcholine (ACh) or bradykinine (Campbell *et al*., 1996; Fisslthaler *et al*., 1999; Gauthier *et al*., 2002). In addition, gap junctions have been involved in

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the dilatation of resistance arteries (Chaytor *et al*., 2001; 2005; Dora *et al*., 2003).

The vascular endothelial function is sensitive to pathological conditions often resulting in its degradation. Hypercholesterolemia (HC) has been shown to have deleterious effects on the NO-dependent relaxation (Cohen, 1995). As of today, however, little is known on the effect of HC and more generally dyslipidaemia (DL) on EDHF-dependent dilatation. The present experiments were designed to characterise the nature of EDHF in the wild-type (WT) mouse *gracilis* resistance artery and to study the effects of clinically relevant DL on EDHF dilatation in mice expressing the human apolipoprotein B100 (hApoB$^{+/+}$; Sanan *et al*., 1998).

Methods

Vascular preparation

The procedures and protocols were in accordance with our institutional guidelines and the *Guide for the Care and Use of Laboratory Animals* of Canada. Experiments were conducted on isolated *gracilis* arteries of 3-month-old C57BL/6 (WT) mice (27 ± 1 g) (Charles River Laboratories, St-Constant, Quebec, Canada) and DL mice expressing the human apolipoprotein B-100 (31 ± 1 g, $P<0.05$) (Sanan *et al*., 1998) using a method described previously (Nguyen *et al*., 1999). DL mice were kindly provided by Dr Helen Hobbs (University of Texas Southwestern Medical Center, Dallas, TX, U.S.A.). The plasma concentration of cholesterol was 3.2 ± 0.3 mM in WT and 4.6 ± 0.3 mM in DL mice ($P<0.05$). Triglycerides were increased ($P<0.05$) from 1.3 ± 0.3 mM in WT to 3.0 ± 0.3 mM in DL mice. The mice were killed by CO_2 inhalation. The right or left *gracilis* artery was dissected and placed in ice-cold physiological salt solution (PSS) of the following composition (mM): NaCl 130, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.17, $NaHCO_3$ 14.9, $CaCl_2$ 1.6, EDTA 0.023 and glucose 10, aerated with 12% O_2/5% CO_2/83% N_2 (37°C, pH 7.4). Segments of the *gracilis* artery were cleaned up of surrounding tissue and fat. A 2–3 mm length arterial segment was isolated, cannulated at both ends and pressurised at 80 mmHg in no-flow condition in a pressure myograph as previously described (Véquaud & Thorin, 2001). Internal pressure was maintained constant and real time diameter changes were monitored using a pressure servo-control and a video dimension analyser, respectively (Living System, Burlington, Vermont, U.S.A.). All experiments were conducted on segments with an internal diameter of 175–210 μm when pressurised at 80 mmHg. An equilibration time of 45 min was allowed before starting the experiment.

Table 1 Myogenic tone of the *gracilis* artery isolated from wild type and dyslipidaemic (hApoB$^{+/+}$) mice

Experimental conditions	*Wild type* Tone (%)	n	*hApoB$^{+/+}$* Tone (%)	n
Control PSS	14 ± 6	6	6 ± 3	6
L-NNA + Indo	7 ± 1	7	6 ± 2	7
+ Apamin	7 ± 1	6	35 ± 7*,†	6
+ Chtx	7 ± 4	7	39 ± 14*,†	7
+ Apamin + Chtx	26 ± 7*	7	31 ± 12*	5
+ Ba^{2+}	17 ± 1*	6	60 ± 14*,†	6
+ Ouabain	55 ± 14*	4	72 ± 9*	7
+ Ba^{2+} + ouabain	39 ± 7*	4	100 ± 1*,†	6
+ 17-ODYA	23 ± 9	4	15 ± 10	6
+ EEZE	48 ± 15	3	34 ± 11*	5
+ 18α-GA	38 ± 17	3	28 ± 11*	3

Data are expressed as mean ± s.e.m. All solutions contained indomethacin (Indo, 10 μM) and L-NNA (100 μM), except in Control PSS. *$P<0.05$ compared to L-NNA + Indo. †$P<0.05$ compared to WT.

Experimental protocols

A single cumulative concentration–response curve to ACh (1 nM–30 μM) was obtained in vessels precontracted with phenylephrine (PE, 1–30 μM). Following precontraction, average diameters of arteries isolated from WT and DL mice were 53 ± 1 and 52 ± 3 μm, respectively. The pretreatment with some of the pharmacological tools used in this study constricted the vessels (reduction in resting diameter, see Table 1); in these conditions, the concentration of PE was reduced to 1 μM to reach a similar level of precontraction in all experimental conditions. In one experimental condition using Ba^{2+} and ouabain, the reduction in diameter of arterial segments isolated from DL mice was maximal (Table 1) and PE was not added.

To study EDHF-like-dependent dilatation to ACh, N^{ω}-nitro-L-arginine (L-NNA, 100 μM) and indomethacin (indo, 10 μM) were present in the bath chamber to prevent NO and prostanoid formation, respectively (Véquaud & Thorin, 2001). In one series of experiments, combined NO- and PGI_2-dependent dilatations to ACh were obtained in the presence of 40 mM KCl-PSS. Depending on the channel, enzyme, or pump targeted, Apa (1 μM; Véquaud & Thorin, 2001), Chtx (0.1 μM; Edwards *et al*., 1999), 14,15-epoxyeicosa-5(Z)-enoic acid (EEZE, 1 μM; Gauthier *et al*., 2002), iberiotoxin (0.1 μM; Edwards *et al*., 1999), 17-octadecynoic acid (17-ODYA, 10 μM; Brandes *et al*., 2000), Ba^{2+} (30 μM; Edwards *et al*., 1999), 18α-glycyrrhetinic acid (18α-GA, 50 μM; Chaytor *et al*., 2000) or ouabain (1 mM; Edwards *et al*., 1999) were added to the bath 30 min before the start of the protocol. At the end of the protocol, the maximal diameter (D_{max}) was determined by changing the PSS to a Ca^{2+}-free PSS containing sodium nitroprusside (SNP, 10 μM) and ethylene glycol-bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA, 1 mM; Véquaud & Thorin, 2001).

Statistical analysis

In every case, *n* refers to the number of animals used in each protocol. Half-maximum effective concentration (EC_{50}) of ACh was measured from each individual concentration–response curve using a logistic curve-fitting program (Microcal™Origin™ version 5.0). The pD_2 value, the negative log of the EC_{50}, was obtained. Continuous variables are expressed as mean ± s.e. of the mean (s.e.m.). For each protocol, basal diameter in no-flow condition was determined at the end of the 45 min equilibration period. Myogenic tone (MT), which is a reduction in diameter induced by an increase in luminal pressure, was measured and expressed as percentage of the D_{max}. ACh-induced dilatation is expressed as a percentage of the D_{max}. ANOVA were performed to compare concentration–response curves. Differences were considered to be statistically significant when the *P*-value was <0.05 (Scheffe's *F* test).

Drugs

ACh, Apa, indo, L-NNA, PE, Chtx, ouabain, 17-ODYA and 18α-GA were purchased from Sigma. Barium was purchased from Mallinckrodt. All drugs were prepared daily and diluted in water except for indo, EEZE and 17-ODYA, which were prepared as stock solutions and diluted in ethanol and 18α-glycyrrhetinic, which was dissolved in DMSO. All drugs were then directly added to the bath chamber (extraluminally) and the final concentration of ethanol and DMSO never exceeded 0.1%. Equimolar amounts of NaCl were replaced with KCl to prepare the 40 mM K^+-PSS.

Results

EDHF, NO and PGI_2 dilatations

In the presence of indo and L-NNA, ACh-induced EDHF-dependent dilatations were increased ($P<0.05$) in the *gracilis* artery isolated from DL mice compared to WT mice (Figure 1, Table 2). In contrast, no differences were observed in ACh-induced NO- and PGI_2-dependent dilatation, measured in the presence of high external K^+ (Table 2). Compared to EDHF-mediated dilatation, however, the NO- and PGI_2-dependent dilatation induced by ACh was significantly lower. Vascular sensitivity to ACh was not different between groups. All subsequent experiments were performed in the presence of L-NNA (100 μM) and indo (10 μM) to study the EDHF pathways.

Contribution of small (SK_{Ca}) and intermediate (IK_{Ca}) conductance calcium-dependent potassium channels in EDHF-dependent dilatation to ACh

Inhibition of SK_{Ca} channels by Apa (100 nM) reduced by 10% ($P<0.05$). ACh-induced maximal dilatation of arteries isolated from WT mice (Table 2). This effect, however, was more pronounced ($P<0.05$) in arteries isolated from DL mice, in which Apa reduced maximal dilatation to ACh by 40% (Table 2). Inhibition of IK_{Ca} channels with Chtx reduced ($P<0.05$) ACh-dependent maximal dilatation from 86 to 54% as well as potency in arteries isolated from WT mice (Table 2), whereas in DL mice, Chtx had no significant effects (Table 2). Combination of Apa and Chtx, however, blunted ACh-induced dilatation in both groups of arteries (Table 2, Figure 1).

Since Chtx has been reported to have inhibitory effects on big (BK_{Ca}) conductance calcium-dependent potassium channels, these experiments were repeated in the presence of iberiotoxin alone, a BK_{Ca} specific inhibitor. Iberiotoxin

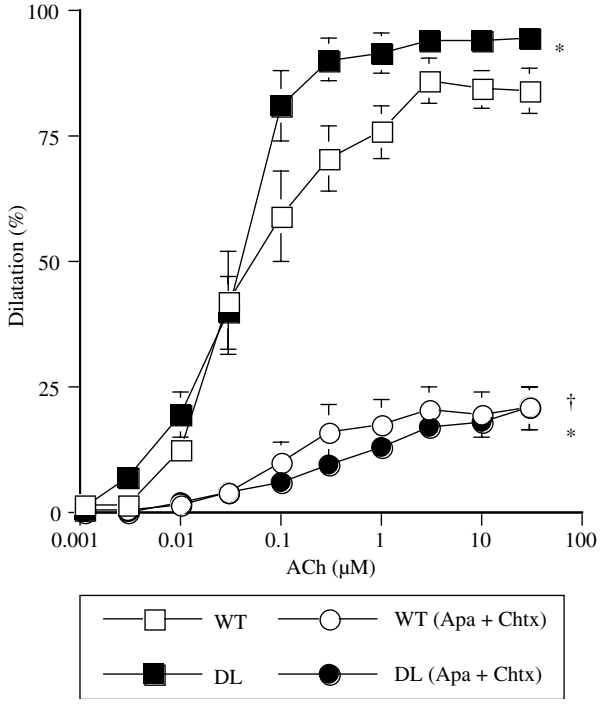

Figure 1 Effect of combined addition of apamin (Apa; 1 μM) and charybdotoxin (Chtx, 100 nM) on ACh-induced dilatation of *gracilis* arteries isolated from $hApoB^{+/+}$ (DL, $n=5$) and WT ($n=7$) mice. L-NNA (100 μM) and indomethacin (10 μM) were present in the bath. *$P<0.05$ compared to WT without Apa and Chtx. †$P<0.05$ compared to DL without Apa and Chtx.

Table 2 Efficacy (E_{max}) and sensitivity (pD_2) to ACh of the *gracilis* artery isolated from wild type and dyslipidaemic ($hApoB^{+/+}$) mice

Experimental conditions	*Wild type*			*$hApoB^{+/+}$*		
	E_{max}	pD_2	n	E_{max}	pD_2	n
High external K^+	48±4*	7.7±0.1	6	46±4*	7.6±0.3	6
L-NNA + Indo	86±4	7.4±0.2	7	95±2†	7.4±0.1	7
+ Apamin	73±4*	7.2±0.2	6	55±12*	7.3±0.4	6
+ Chtx	54±4*	6.7±0.2*	7	74±10†	7.0±0.2	7
+ Apamin + Chtx	22±4*	6.8±0.2	7	25±5*	6.0±0.4*	5
+ Ba^{2+}	72±4*	7.2±0.1	6	73±9*	7.4±0.2	6
+ Ouabain	19±7*	6.2±0.6*	4	75±10†	6.9±0.2	7
+ Ba^{2+} + ouabain	13±3*	6.2±0.5*	4	54±11*,†	7.0±0.2	6
+ 17-ODYA	89±5	7.6±0.4	4	42±11*,†	7.2±0.3	6
+ 17-ODYA + Ba^{2+} + ouabain	8±1*	Not measurable	3	5±3*	Not measurable	3
+ EEZE	82±2	7.2±0.2	3	32±4*,†	6.4±0.2*,†	5
+ EEZE + Ba^{2+} + ouabain	Not tested	Not tested		6±1*	Not measurable	3
+ 18α-GA	83±1%	7.6±0.24	3	92±1%	7.7±0.2	3

Data are expressed as mean±s.e.m. All solutions contained indomethacin (Indo, 10 μM) and L-NNA (100 μM), except in high external K^+ (vessels were precontracted with 40 mM KCl-PSS in this condition only). *$P<0.05$ compared to L-NNA + Indo. †$P<0.05$ compared to WT.

(0.1 μM) failed to affect EDHF-dependent dilatations. In segments isolated from DL mice ($n=3$), the maximal dilatation obtained in the presence of iberiotoxin was $95\pm2\%$ with a pD_2 value for ACh of 7.32 ± 0.35. These values are similar to those obtained in the presence of L-NNA + Indo (Table 2).

Involvement of the inward rectifier potassium channels (K_{ir}) and the Na^+/K^+-ATPase pump in EDHF-dependent dilatation to ACh

In the presence of L-NNA and indo, K_{ir} channels play a role in the dilatation induced by ACh in both groups of vessels. Ba^{2+} (30 μM) diminished the maximal dilatation to 72 ± 4 and $73\pm9\%$ without affecting potency in arteries isolated from WT and DL mice, respectively (Table 2). In contrast, ouabain (1 mM) blunted ($P<0.05$) ACh-induced dilatation in arteries isolated from WT mice, but had no significant effect in arteries isolated from DL mice (Table 2). In WT mice, a concentration of ouabain of 500 nM ($n=3$) also reduced ACh-induced maximal dilatation ($26\pm4\%$). This lower concentration, however, neither reduced the vascular sensitivity to ACh (pD_2 value of 7.23 ± 0.02) nor increased significantly MT ($24\pm10\%$).

In the presence of a combined blockade of K_{ir} channels and the Na^+/K^+-ATPase pump, ACh-induced EDHF-dependent dilatation of arteries isolated from WT mice was prevented; this inhibition, however, did not differ from the inhibition obtained in the presence of ouabain alone (Table 2, Figure 2). In contrast, combination of Ba^{2+} and ouabain, when compared to either drugs alone, resulted in a reduced ($P<0.05$) maximal dilatation without preventing EDHF-mediated dilatation in vessels isolated from DL mice (Table 2, Figure 2).

Cytochrome P450 and gap junction involvement in EDHF-dependent dilatation to ACh

In arterial segments isolated from WT mice, 17-ODYA (10 μM) did not impair the dilatation induced by ACh (Table 2). In contrast, 17-ODYA reduced ($P<0.05$) by half the maximal dilatation induced by ACh in vessels isolated from DL mice (Table 2). When 17-ODYA (10 μM) was applied in combination with Ba^{2+} (30 μM) and ouabain (1 mM), EDHF-dependent dilatation induced by ACh was abolished in arteries isolated from DL mice (Table 2).

EEZE (1 μM) reduced ($P<0.05$) by half the maximal dilatation induced by ACh in vessels isolated from DL mice (Table 2, Figure 3). When EEZE was applied in combination with Ba^{2+} (30 μM) and ouabain (1 mM), EDHF-dependent dilatation induced by ACh was abolished in arteries isolated from DL mice (Table 2, Figure 3). In arterial segments isolated from WT mice, EEZE did not impair the dilatation induced by ACh (Table 2, Figure 3).

In additional experiments, the effects of more specific cytochrome *P*450 inhibitors were tested in arterial segments isolated from DL mice. Sulphaphenazole (10 μM, 2C8 and 2C9 inhibitor), ketoconazole (10 μM, 3A4 inhibitor) and 2-(2-propynyloxy)benzenehexanoic acid (PPOH, 20 μM, 4A2 and 4A3 inhibitor) did not affect the dilatation induced by ACh (data not shown).

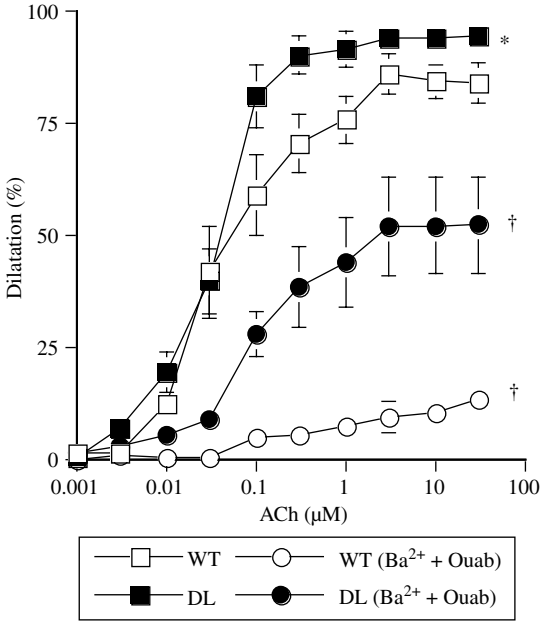

Figure 2 Effect of combined addition of Ba^{2+} (30 μM) and ouabain (Ouab, 1 mM) on ACh-induced dilatation of *gracilis* arteries isolated from $hApoB^{+/+}$ (DL, $n=6$) and WT ($n=4$) mice. L-NNA (100 μM) and indomethacin (10 μM) were present in the bath. $^*P<0.05$ compared to WT without Ba^{2+} and ouabain. $^\dagger P<0.05$ compared to DL without Ba^{2+} and ouabain.

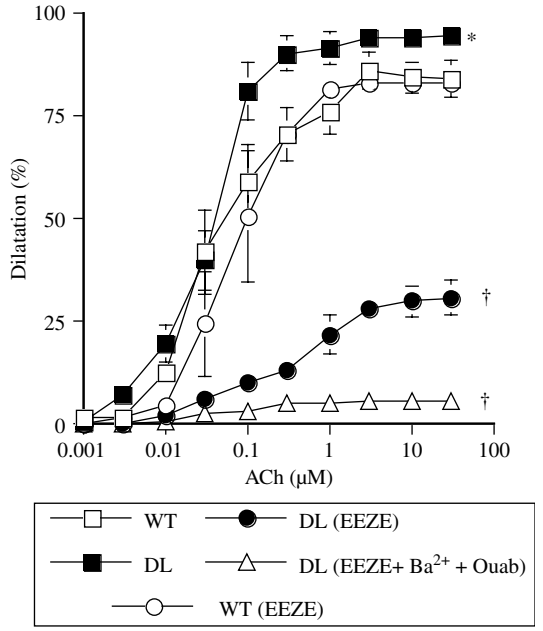

Figure 3 Effect of EEZE (1 μM) alone or with ($n=3$) the combination of Ba^{2+} (30 μM) and ouabain (Ouab, 1 mM) on ACh-induced dilatation of *gracilis* arteries isolated from $hApoB^{+/+}$ (DL, $n=6$) and WT ($n=4$) mice. L-NNA (100 μM) and indomethacin (10 μM) were present in the bath. $^*P<0.05$ compared to WT without EEZE. $^\dagger P<0.05$ compared to DL without EEZE.

EETs may act by increasing conduction of gap junction. In the presence of 18α-GA (50 μM), ACh-induced dilatation in the presence of Indo + L-NNA was affected neither in vessels isolated from WT nor in vessels isolated from DL mice (Table 2).

Discussion

The present study had two objectives: the first was to characterise the mechanisms involved in the dilatation resistant to L-NNA and indo of WT mouse *gracilis* resistance arteries induced by ACh and attributed to EDHF. The second objective was to investigate the impact of DL on this pathway. The results suggest that the nature and the mechanisms of action of EDHF differ greatly between WT and DL *gracilis* arteries. In both WT and DL arteries, activation of endothelial SK_{Ca} and IK_{Ca} channels mediate the effects of EDHF. In WT mice, activation of these channels fully dilates smooth muscle cells by activating the Na^+/K^+-ATPase pump, with a small contribution of K_{ir} channels. In *gracilis* arteries isolated from DL mice, this pathway contributes to half of the dilatory response to ACh. EETs, derived from the activity of a cytochrome *P*450, represent a secondary pathway contributing to the dilatation induced by ACh. Hence, DL leads to the duplication of the mechanisms responsible for EDHF-induced dilatation.

In this study, we used hApoB$^{+/+}$ mice. At 3 months of age, although the mice are dyslipidaemic, vessels are free of atherosclerotic lesions. In addition, NO- and PGI_2-dependent dilatation of isolated and pressurised *gracilis* arteries is similar in both groups, which demonstrate that endothelial function is not affected by DL at this age. The genetic background of the DL mice is 75% C57Bl6 and 25% SJl. As a control WT group, we used C57Bl6 mice. In preliminary control experiments ($n=3$), *gracilis* arteries isolated from SJl mice had an identical sensitivity to 1 mM ouabain ($16\pm1\%$) and were insensitive to 17-ODYA (E_{max}, $74\pm11\%$; pD_2, 7.5 ± 0.1) as vessels isolated from C57Bl6 mice. It is therefore the DL phenotype that is responsible for the changes in vascular function observed in this study.

In the presence of L-NNA and indo, the dilatation induced by ACh is attributed to EDHF (Adeagbo & Triggle, 1993; Bauersachs *et al*., 1996; Thorin *et al*., 1998; Brandes *et al*., 2000; Véquaud & Thorin, 2001). Our experimental conditions are favourable to reveal EDHF mechanisms since pressurised arteries are depolarised ($\approx$40 to 45 mV) and develop MT, as previously reported (Potocnik *et al*., 2000; Taylor *et al*., 2003). In arteries isolated from DL mice, the EDHF-dependent dilatation is significantly increased. This is in agreement with previous studies, which reported that DL augmented the EDHF-dependent relaxation of rabbit renal (Brandes *et al*., 1997) and carotid (Najibi *et al*., 1994) arteries. There are no clear reasons as why the efficacy of EDHF increases in the early stages of DL. Our data, however, demonstrate that in DL, EDHF is a multifaceted factor, whereas in arteries isolated from WT mice, only the activation of SK_{Ca} and IK_{Ca} accounts for EDHF. Hence, in the early stage of DL, several factors insensitive to NOS and COX inhibition may contribute more efficiently than endothelial SK_{Ca} and IK_{Ca} alone to the dilatation induced by ACh.

As others have demonstrated (Brandes *et al*., 2000; Dora *et al*., 2003), a combination of Apa and Chtx was required to prevent EDHF-dependent dilatation of arteries isolated from WT mice. Chtx also inhibits Kv1.2 and Kv1.3 (Garcia *et al*., 1995) but it is unlikely that these channels form the endothelial cell target for the toxin in vessels as clearly discussed by Edwards and co-workers (1999). It has been proposed that endothelial SK_{Ca} and IK_{Ca} are responsible for the rise in $[K^+]_o$ in the intercellular space (Edwards *et al*., 1998). This increases the activity of the smooth muscle Na^+/K^+-ATPase pump leading to hyperpolarisation and dilatation. Our data are in agreement with this concept since ouabain prevented ACh-induced dilatation of arterial segments isolated from WT mice. In addition, and as previously reported by Edwards and co-workers (1999), a lower concentration of ouabain (500 nM) also prevented the dilatation to ACh. This further confirms the concept first described by Edwards and co-workers in 1998.

In arteries isolated from DL mice, Apa combined with Chtx reduced EDHF-dependent dilatation to ACh, as in vessels isolated from WT mice. Ouabain, however, no longer prevented dilatation, suggesting a reduced contribution of the Na^+/K^+-ATPase. The origin of this loss of efficacy is unknown. It is possible that the basal activity of the Na^+/K^+-ATPase pump is increased in DL, and thus not further activable following endothelial SK_{Ca} and IK_{Ca} opening. Depolarisation of arteries isolated from DL mice could increase the activity of the Na^+/K^+-ATPase pump. K_{ir} activity, sensitive to Ba^{2+} ions, appears to be increased in arteries isolated from DL mice. The activity of this channel is linked to the resting membrane potential (Hirst & Edwards, 1989). The apparent increased activity of K_{ir} in vessels from DL mice may therefore be a consequence of a depolarised state, which would also contribute to an increased activation of the Na^+/K^+-ATPase pump. K_{ir} channel activity, however, may not be a target of any EDHF but rather a reflection of smooth muscle membrane potential. This hypothesis needs to be assessed by direct membrane potential recording in pressurised arteries. In addition, K_{ir} channel activity has been reported to be essential for the conduction of the hyperpolarisation wave along resistance arteries, but not for the initiation of the hyperpolarisation (Rivers *et al*., 2001).

This change in smooth muscle responsiveness led to an adaptive response from the endothelium. The reduced dilatory efficacy of endothelial SK_{Ca} and IK_{Ca} led to a compensatory endothelial release of an additional dilatory factor. This change in EDHF efficacy was not compensated by an upregulation of NO production and/or effect. In contrast, an arachidonic acid metabolite of cytochrome *P*450 activity, sensitive to both 17-ODYA and EEZE, accounted for the compensatory EDHF-dependent dilatation to ACh in arteries isolated from DL mice. Such factor, most likely an EET, has been proposed to be the EDHF in several species including human arteries (Fisslthaler *et al*., 1999; Brandes *et al*., 2000; Archer *et al*., 2003). As a consequence, a combination of ouabain, Ba^{2+} and 17-ODYA or EEZE were required to prevent EDHF-mediated dilatation induced by ACh in arteries isolated from DL mice, whereas ouabain alone is sufficient to prevent the dilatation of vessels isolated from WT mice.

The endothelial intracellular signal leading to EDHF production involves both SK_{Ca} and IK_{Ca}, as revealed by the inhibitory effects of a combination of Apa and Chtx in arteries isolated from both type of mice. As proposed previously (Véquaud & Thorin, 2001), activation of endothelial muscarinic receptors may directly activate these channels. The resultant endothelial hyperpolarisation leads to a rise in intracellular Ca^{2+} concentration (Edwards *et al*., 1998; Beny & Schaad, 2000), which could promote cytochrome *P*450 activation and EET release in arteries isolated from DL mice. This suggests, however, that DL *per se* may be responsible for

cytochrome expression. Cytochrome expression is known to be upregulated by numerous factors (Roman, 2002), and HC has been reported as one of these factors in the aorta of HC rabbit leading to augmented production of EETs (Pfister *et al*., 1991), which is in support of our current finding.

Although EETs account for EDHF in bovine coronary vessels, they usually hyperpolarise smooth muscle cells by activating BK_{Ca} (Baron *et al*., 1997). In our hands, however, iberiotoxin did not prevent ACh-induced dilatation (data not shown). EETs, however, have been shown to have other vascular effects such as an augmentation of gap junctional communication by a protein kinase C-dependent mechanism (Popp *et al*., 2002) and an augmentation of the open probability of endothelial calcium channels (Watanabe *et al*., 2003). These findings suggest that EETs could be implicated in the EDHF-dependent dilatation by acting as intracellular second messengers.

As mentioned above, EETs increase gap junction conductance (Popp *et al*., 2002). In our hands, the gap junction blocker 18α-GA did not reduce the dilatation induced by ACh in either group of vessels. It is clear, however, that gap junction are involved in the conduction of the hyperpolarisation (Brandes *et al*., 2000; Dora *et al*., 2003), but this is apparently not the case in the *gracilis* artery of the mouse. However, the rise in MT induced by 18α-GA, significant in vessels isolated from DL mice, suggest that gap junctions are involved in the maintenance of the basal vascular tone.

Finally, Apa had a stronger inhibitory effect on EDHF-dependent dilatation in arteries isolated from DL mice than WT mice. In contrast, Chtx reduced more dilatation in arteries from WT than from DL mice. This suggests that DL modifies the activated state of K_{Ca} channels. This hypothesis is strengthened by the impact of these toxins on MT. Used individually, Apa and Chtx, increased MT suggesting a lack of compensation of one conductance by the other, in contrast to what is observed in WT arteries. In combination, Apa and Chtx increased MT to a similar level in both groups. Hence, the 'cross-talk' of SK_{Ca} and IK_{Ca} channels observed in the WT is lacking in the DL mouse *gracilis* artery.

In conclusion, our results demonstrate that the nature and the mechanisms of action of EDHF differ between WT and DL mouse *gracilis* arteries. In both WT and DL arteries, activation of endothelial SK_{Ca} and IK_{Ca} channels is essential to induce dilatation. In the *gracilis* artery isolated from the DL mouse, this is no longer sufficient to induce a complete dilatation to ACh. A cytochrome *P*450 metabolite of the arachidonic acid, most likely EETs, contributes to the dilatory action of ACh. This early compensation takes place whereas the NO-dependent function is intact. It remains to demonstrate that EETs is essential for the maintenance of a normal endothelial function in DL mice.

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