

# ROS-sensitive cytochrome P450 activity maintains endothelial dilatation in ageing but is transitory in dyslipidaemic mice

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**1** Risk factors for cardiovascular diseases (CVD) have been proposed to accelerate the vascular endothelial dysfunction that develops during the normal ageing process. The objective of this work was to study the impact of dyslipidaemia (DL) on the dilatatory efficacy of the non-NO/non-PGI<sub>2</sub> endothelium-derived hyperpolarising factor (EDHF) through maturation and ageing.

**2** We isolated and pressurised (80 mmHg) *gracilis* arterial segments from 3, 12 and 20-month-old (m/o) DL mice expressing the human apolipoprotein B-100 and wild-type (WT) C57BL/6 mice. EDHF-dependent dilatations to acetylcholine (ACh) were measured in the presence of L-NNA (100 µM, NOS inhibitor) and indomethacin (INDO; 10 µM, COX inhibitor). Data are expressed as mean ± s.e.m.

**3** EDHF-mediated maximal dilatation of arteries isolated from WT mice declined by 44% with ageing, from 86 ± 3% at 3 months to 66 ± 8% at 12 and 48 ± 4% at 20 months of age ( $P < 0.05$ ). This decline was magnified by DL to 73%, characterised by an early increased efficacy at 3 m/o (95 ± 2%,  $P < 0.05$ ) and a worsening of the dysfunction at 20 m/o (26 ± 2%,  $P < 0.05$ ).

**4** 17-Octadecynoic acid (17-ODYA), a cytochrome P450/epoxygenase inhibitor, reduced by 56% ( $P < 0.05$ ) ACh-induced EDHF-dependent dilatation of arteries isolated from 3 m/o DL – but not WT – mice, an effect of 17-ODYA disappearing in older DL mice. 17-ODYA, however, reduced ( $P < 0.05$ ) ACh-induced EDHF-dependent dilatation in arteries isolated from 12 m/o WT mice by 35% and from 20 m/o WT mice by 31% ( $P < 0.05$ ).

**5** Reactive oxygen species production was increased in arteries isolated from 12 m/o DL mice. The antioxidant *N*-acetyl-L-cystein (NAC) restored the 17-ODYA-sensitive responses in arteries isolated from 12 – but not 20 – m/o DL mice (84 ± 3% from an  $E_{max}$  of 57 ± 8%;  $P < 0.05$ ). NAC did not affect the dilatation of arteries isolated from WT mice.

**6** Our data suggest that the decline in EDHF-dependent dilatation is hastened by DL despite the early expression of a 17-ODYA-sensitive pathway increasing the efficacy of the non-NO/non-PGI<sub>2</sub> endothelium-dependent dilatation. Acute free radical production contributes to the endothelial dysfunction in the presence of DL only, by abrogating this latter pathway. This 17-ODYA-sensitive pathway, however, appears in 12 m/o WT mice and remains active at 20 m/o.

*British Journal of Pharmacology* (2006) **147**, 897–904. doi:10.1038/sj.bjp.0706679; published online 6 February 2006

**Keywords:** Endothelium; *gracilis* artery; EDHF; hypercholesterolaemia; triglycerides; ageing; reactive oxygen species; mouse

**Abbreviations:** ACh, acetylcholine; Apa, apamin; Ba<sup>2+</sup>, 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; INDO, indomethacin;  $K_{Ca}$ , Ca<sup>2+</sup>-sensitive K<sup>+</sup> channel;  $K_{ir}$ , inward rectifier potassium channels; L-NNA, *N*<sup>o</sup>-nitro-L-arginine; MT, myogenic tone; NAC, *N*-acetyl-L-cysteine; NO, nitric oxide; Ouab, ouabain; PE, phenylephrine; PGI<sub>2</sub>, prostacyclin; PSS, physiological salt solution; SNP, sodium nitroprusside; 17-ODYA, 17-octadecynoic acid

## Introduction

The endothelium-derived hyperpolarising factor (EDHF) cannot be defined as a single factor. Different candidates have been proposed as potential EDHFs and over the years, K<sup>+</sup> ions, epoxyecosatrienoic acids (EETs) and direct electrical transfer through gap junctions have emerged as the most likely candidates (Edwards *et al.*, 1998; Brandes *et al.*, 2000; Dora *et al.*, 2003). Recently, we reported that in the C57BL/6 mouse *gracilis* artery, EDHF-dependent dilatations depend on the activation of endothelial small and intermediate conductance calcium-dependent potassium channels (SK<sub>Ca</sub> and IK<sub>Ca</sub>),

leading to the activation of the smooth muscle *via* Na<sup>+</sup>/K<sup>+</sup>-ATPase pump and, to a lesser extent, Ba<sup>2+</sup>-sensitive potassium channels ( $K_{ir}$ ) activation; neither gap junctions nor EETs contributed to acetylcholine (ACh)-induced dilatation of resistance arteries isolated from 3-month-old (m/o) C57BL/6 mice (Krummen *et al.*, 2005).

EDHF is the major contributor to endothelium-dependent dilatations induced by agonists such as ACh and bradykinin in small arteries (Nagao *et al.*, 1992; Véquaud & Thorin, 2001; Krummen *et al.*, 2005). The dilatatory function of the endothelium is known to decline with age (Marin, 1995; Gerhard *et al.*, 1996; Rodriguez-Martinez *et al.*, 1998; Csiszar *et al.*, 2002). In most cases, this diminished endothelium-

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dependent dilatation has been attributed to a reduced release, formation and/or increased degradation of NO or PGI<sub>2</sub> (van der Loo *et al.*, 2000; Woodman *et al.*, 2003), while it was reported that EDHF-dependent dilatation declines in some arteries (Fujii *et al.*, 1993; Urakami-Harasawa *et al.*, 1997) but not others (Muller-Delp *et al.*, 2002).

Risk factors for cardiovascular diseases (CVDs) are associated with an early expression of the endothelial dysfunction. Previous studies on diabetes, hypertension, atherosclerosis and hypercholesterolaemia likewise examined possible alterations in NO and PGI<sub>2</sub> responses (Creager *et al.*, 1990; Kolodgie *et al.*, 1990; Flavahan, 1992; Najibi *et al.*, 1994; Vanhoutte & Scott-Burden, 1994; Brandes *et al.*, 1997). In contrast, the impact of these risk factors on EDHF-mediated dilatation is not well characterised. It was reported that hypercholesterolaemia magnified the role of K<sup>+</sup> channels in the dilatation induced by ACh (Najibi *et al.*, 1994). In the same connexion, we recently reported that EDHF-mediated dilatation was magnified in dyslipidaemic (DL) 3 m/o mice due to a duplication of its pathways with the expression of an EET-dependent component (Krummen *et al.*, 2005). Similar results have been reported in spontaneously hypertensive rats (SHR) where cytochrome P450 metabolites contribute to the augmented dilatation dependent of EDHF (Bussemaker *et al.*, 2003). The knowledge on the impact of the combined expression of risk factors for CVDs and age on endothelial function is limited. Bussemaker and co-workers (2003) reported that aged SHR exhibited a selective loss of EDHF-mediated relaxation in the renal artery. The objective of the present study was therefore to examine the effects of age associated with DL on EDHF-induced dilatations of mouse *gracilis* arteries. Our results suggest that a clinically relevant DL leads to a selective and faster reduction in EDHF-mediated dilatation compared to the normal situation in WT despite the early expression of an EDHF alternative pathway sensitive to 17-octadecynoic acid (17-ODYA). Acute oxidative stress appears to be central to this process in DL.

## 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 isolated from 3, 12 and 20 m/o DL male mice expressing the human apolipoprotein B-100 (31 ± 1, 43 ± 2 and 41 ± 3 g body weight, respectively; Sanan *et al.*, 1998; Krummen *et al.*, 2005) and their WT C57Bl/6 control male mice (27 ± 1, 45 ± 1 and 53 ± 4 g body weight, respectively) using a method described previously (Nguyen *et al.*, 1999). Total cholesterol (mg dl<sup>-1</sup>) and triglycerides (mg dl<sup>-1</sup>) were significantly ( $P < 0.05$ ) elevated in DL at 3 and 12 months of age (3 m/o: 159 ± 14 and 204 ± 38, respectively;  $n = 5$ ; 12 m/o: 227 ± 17 and 198 ± 39, respectively;  $n = 6$ ) compared to WT mice (3 m/o: 107 ± 8 and 147 ± 24, respectively;  $n = 6$ ; 12 m/o: 102 ± 6 and 92 ± 7, respectively;  $n = 6$ ). At 20 months of age, cholesterol levels were similar in DL and WT mice (175 ± 31 and 147 ± 15 respectively;  $n = 4-6$ ), while triglycerides remained elevated ( $P < 0.05$ ) in DL (140 ± 47;  $n = 5$ ) compared to WT (64 ± 7;  $n = 6$ ). In a limited number of animals ( $n = 4$  per

group), blood pressure was measured under anaesthesia (pentobarbitone-Na, 65 mg kg<sup>-1</sup>) using a Millar catheter and revealed a similar systolic blood pressure (89 ± 3 and 87 ± 1 mmHg), heart rate (351 ± 16 and 395 ± 39 b.p.m.) and +dP/dt (4330 ± 198 and 4550 ± 166 mmHg s<sup>-1</sup>) in WT and DL mice, respectively.

Mice were killed by CO<sub>2</sub> 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<sub>2</sub>PO<sub>4</sub> 1.18, MgSO<sub>4</sub> 1.17, NaHCO<sub>3</sub> 14.9, CaCl<sub>2</sub> 1.6, EDTA 0.023 and glucose 10, aerated with 12% O<sub>2</sub>/5% CO<sub>2</sub>/83% N<sub>2</sub> (37°C, pH 7.4). All experiments were conducted on segments of 2–3 mm in length with an average internal diameter of 207 ± 2 μm (WT) and 210 ± 2 μm (DL) when pressurised at 80 mmHg. An equilibration time of 45 min was allowed before every experiment.

### Experimental protocols

Dilatations to ACh (1 nM–30 μM) were measured in vessels precontracted with phenylephrine (PE, 1–10 μM). Following precontraction, vessels isolated from 3, 12 and 20 m/o WT mice had average diameters of 54 ± 3, 53 ± 1 and 52 ± 1 μm, respectively, and 52 ± 3, 48 ± 1 and 47 ± 2 μm, respectively, for those isolated from DL mice. Some pharmacological tools induced a vasoconstriction *per se* (see Table 2). Thus, the concentration of PE was reduced to 1 μM to reach a similar level of precontraction in all experimental conditions.

When EDHF was studied, N<sup>o</sup>-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. Combined NO/PGI<sub>2</sub>-dependent dilatations to ACh were obtained in the presence of 40 mM KCl PSS and without the addition of INDO and L-NNA. Depending on the channel, enzyme or pump targeted, apamin (Apa, 1 μM), charybdotoxin (Chtx, 0.1 μM), iberiotoxin (0.1 μM), 17-ODYA, (10 μM), 14,15-epoxyeicosa-5(Z)-enoic acid (EEZE, 1 μM), barium (Ba<sup>2+</sup>, 30 μM), ouabain (1 mM), probucol (10 μM) or N-acetyl-L-cysteine (NAC, 1 μM) were added to the bath 30 min before the start of the protocol.

### Free radical measurement

Isolated and pressurised *gracilis* arteries were incubated in the presence of 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H<sub>2</sub>DCF-DA; Chaytor *et al.*, 2003; Miura *et al.*, 2003) added to the bath 30 min before the beginning of the experiment. Vessels were then washed three times, precontracted with angiotensin-II (10 μM) while recording changes in fluorescence intensity. Fluorescence intensity was collected with an Ionoptix Aquire system (IonOptix, Milton, MA, U.S.A.). Fluorescence intensities at 493 nm excitation were measured at 520 nm. Measurement of fluorescence intensity was assayed in the presence of L-NNA (100 μM). Before each experiment, basal fluorescence intensity was recorded. Results represent difference between recorded and basal intensity.

### Statistical analysis

In every case,  $n$  refers to the number of animals used in each protocol. Half-maximum effective concentration (EC<sub>50</sub>) 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  $\pm$  standard error 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, which is a reduction in diameter induced by an increase in luminal pressure, was measured and expressed as a percentage of the maximal diameter obtained at the end of the experiment using a  $Ca^{2+}$ -free PSS containing  $10\ \mu M$  of sodium nitroprusside. ACh-induced dilatation is expressed as a percentage of the precontraction. 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, apamin, INDO, L-NNA, phenylephrine, NAC, charybdotoxin, ouabain, probrucol and 17-ODYA were purchased from Sigma (Oakville, ON, Canada). CM-H<sub>2</sub>DCFDA was purchased from Molecular probe (Invitrogen, Burlington, ON, Canada) and was diluted daily in DMSO. Barium was purchased from Mallinckrodt. All drugs were prepared daily and diluted in water, except for INDO, EZEE, probrucol and 17-ODYA, which were prepared as stock solutions and diluted in ethanol. All drugs were then directly inserted in the bath chamber and the final concentration of ethanol never exceeded 0.1%. Equimolar amounts of NaCl were replaced with KCl to prepare the 40 mM K<sup>+</sup>-PSS.

## Results

### Myogenic tone

The myogenic tone increased with age both in vessels isolated from WT and from DL mice (Table 1). Whereas combined NOS and COX inhibition did not affect basal tone, combined inhibition of SK<sub>Ca</sub> and IK<sub>Ca</sub> induced a contraction. This contractile effect of apamin and charybdotoxin was lost in old WT mice but remained persistent and greater in 12 and 20 m/o DL mice. Finally, whereas ouabain constricted at all ages in both groups, 17-ODYA increased tone significantly in vessels isolated from 20 m/o DL mice only.

### EDHF in ageing DL mouse gracilis arteries

The endothelium-dependent dilatation to ACh independent of NO and PGI<sub>2</sub> gradually decreased with increasing age (Figure 1a, Table 2). This dysfunction was also present in DL mouse *gracilis* arteries but with differences compared to WT (Figure 1b, Table 2). First, in vessels isolated from 3 m/o DL mice, the efficacy of ACh was greater ( $P<0.05$ ) when compared to that measured in arteries isolated from WT mice. Second, the decrease in EDHF-dependent dilatation was greater ( $P<0.05$ ) at 20 m/o in arteries isolated from DL compared to WT mice.

### NO and PGI<sub>2</sub> dilatations

NO/PGI<sub>2</sub>-induced dilatations to ACh, revealed on a precontraction induced by a 40 mM KCl-PSS, were 50% less potent than EDHF-induced dilatations (Table 2). NO/PGI<sub>2</sub>-induced dilatations were also less affected by ageing and DL than EDHF-dependent dilatations. NO/PGI<sub>2</sub>-induced maximal dilatations were reduced ( $P<0.05$ ) to  $30\pm 5\%$  at 12 months of age but were then maintained in arteries of 20 m/o mice (Table 2). The vascular sensitivity to ACh-induced NO/PGI<sub>2</sub>-dependent dilatation was not affected by age in DL mice (Table 2). Hence, the following experiments focussed on the impact of age and DL on EDHF-dependent dilatation.

### Effect of ageing and DL on cytochrome P450 involvement in EDHF-dependent dilatation to ACh

In arteries isolated from 3 m/o WT mice, 17-ODYA, a cytochrome P450/epoxygenase enzyme inhibitor, had no impact on the dilatation triggered by ACh (Figure 1c, Table 2). In vessels isolated from 12 to 20 m/o WT mice, however, 17-ODYA reduced ( $P<0.05$ ) ACh-induced EDHF-dependent dilatation (Figure 1c, Table 2).

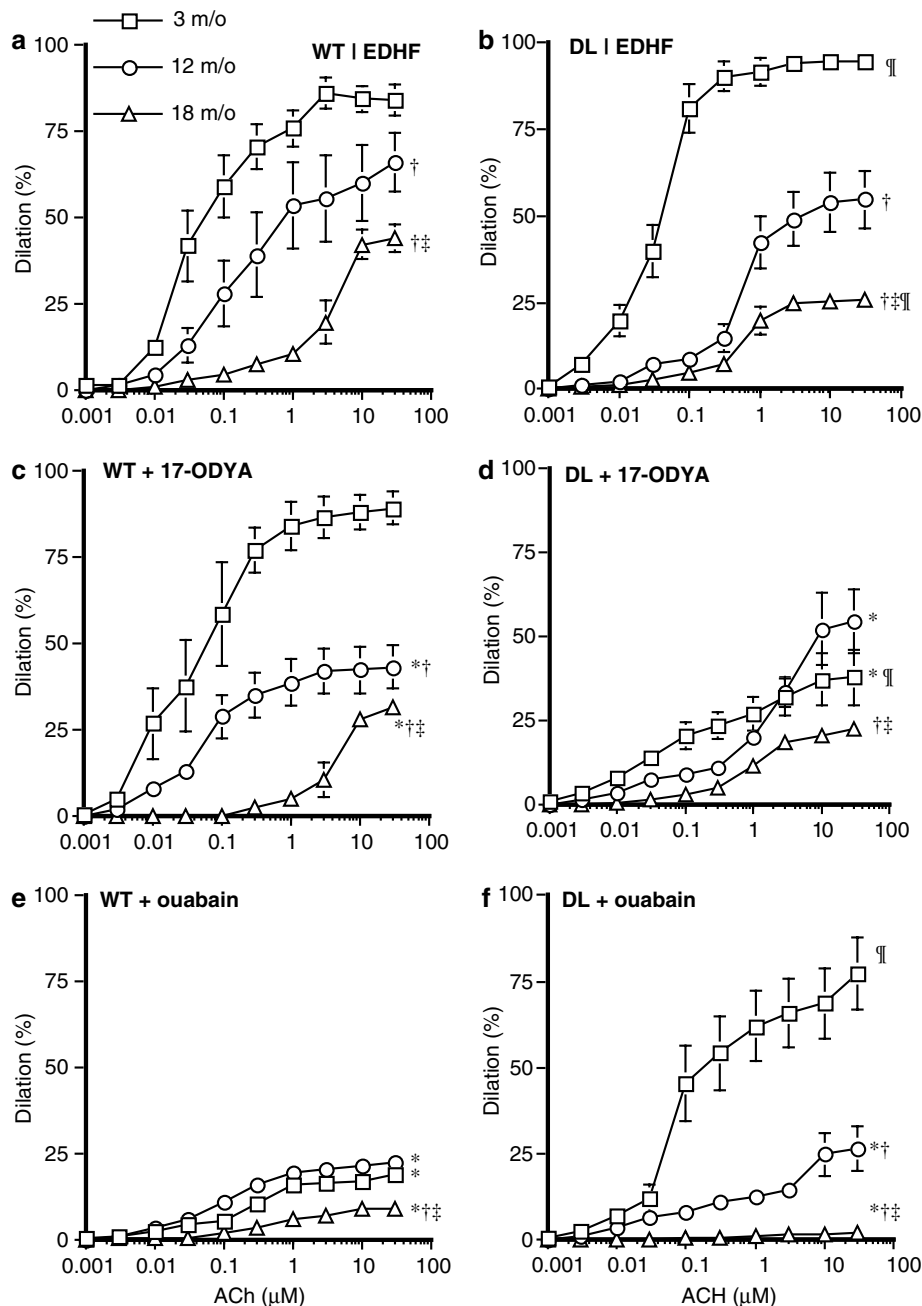
In contrast, 17-ODYA ( $10\ \mu M$ ) reduced ( $P<0.05$ ) EDHF-dependent maximal dilatations by 66% in arteries isolated from 3 m/o DL mice (Figure 1d, Table 2). The inhibitory effect of 17-ODYA was lost in arteries isolated from 12 to 20 m/o DL mice (Figure 1d, Table 2). Accordingly, while the EET receptor antagonist EZEE ( $1\ \mu M$ ) reduced ACh-induced dilatation in 3 m/o DL but not WT mice (Krummen *et al.*, 2005), it no longer reduced the dilatation induced by ACh in vessels isolated from 12 m/o DL mice (Figure 2).

**Table 1** Impact of the pharmacological agents on the myogenic tone of the *gracilis* artery isolated from 3, 12 and 20 m/o WT and DL mice

Conditions	WT			DL		
	3 m/o	12 m/o	20 m/o	3 m/o	12 m/o	20 m/o
Myogenic tone (control conditions)	14 $\pm$ 6 (6)	32 $\pm$ 6 <sup>†</sup> (6)	22 $\pm$ 7 (5)	6 $\pm$ 3 (6)	27 $\pm$ 8 (8)	30 $\pm$ 11 <sup>†</sup> (5)
INDO+L-NNA	7 $\pm$ 1 (7)	29 $\pm$ 6 <sup>†</sup> (6)	19 $\pm$ 6 (5)	6 $\pm$ 2 (7)	28 $\pm$ 8 <sup>†</sup> (6)	31 $\pm$ 10 <sup>†</sup> (5)
+ Apamin + Chtx	26 $\pm$ 7* (7)	57 $\pm$ 6* <sup>†</sup> (6)	36 $\pm$ 16 (3)	30 $\pm$ 12* (5)	74 $\pm$ 10* <sup>†</sup> (4)	77 $\pm$ 6* <sup>†</sup> (4)
+ 17-ODYA	23 $\pm$ 9 (4)	28 $\pm$ 6 (7)	33 $\pm$ 16 (3)	15 $\pm$ 10 (6)	45 $\pm$ 11 (6)	55 $\pm$ 8 <sup>†</sup> (5)
+ Ouabain	55 $\pm$ 14* (4)	55 $\pm$ 11* (6)	36 $\pm$ 28 (3)	72 $\pm$ 9* (7)	70 $\pm$ 5* (6)	63 $\pm$ 3* (4)

Data are expressed as mean  $\pm$  s.e.m. All solutions contained INDO ( $10\ \mu M$ ) and L-NNA ( $100\ \mu M$ ), except in control conditions.

\* $P<0.05$  compared to INDO+L-NNA. <sup>†</sup> $P<0.05$  compared to 3 m/o.



**Figure 1** ACh-induced dilatation of *gracilis* arteries isolated from 3, 12 and 20 m/o WT (left panels) and DL (right panels) mice. (a and b) In control conditions ( $n=7, 8$  and  $5$ , respectively), (c and d) in the presence of 17-ODYA ( $10\ \mu\text{M}$ ;  $n=6, 6$  and  $5$ , respectively) and (e and f) in the presence of ouabain ( $1\ \text{mM}$ ;  $n=7, 6$  and  $4$ , respectively). L-NNA ( $100\ \mu\text{M}$ ) and INDO ( $10\ \mu\text{M}$ ) were present in the bath. \* $P < 0.05$  compared to EDHF (a or b); † $P < 0.05$  compared to 3 m/o; ‡ $P < 0.05$  compared to 12 m/o; § $P < 0.05$  compared to WT, same age and experimental conditions.

#### Effect of ageing and DL on the involvement of the $\text{Na}^+/\text{K}^+/\text{ATPase}$ pump and $\text{K}_{\text{ir}}$ channels in EDHF-dependent dilatation to ACh

Ouabain alone ( $1\ \text{mM}$ ), a  $\text{Na}^+/\text{K}^+/\text{ATPase}$  pump inhibitor, strongly reduced ( $P < 0.05$ ) ACh-induced dilatation in arteries isolated from WT mice at all ages (Figure 1e, Table 2). The addition of  $\text{Ba}^{2+}$  ( $30\ \mu\text{M}$ ), a  $\text{K}_{\text{ir}}$  channel inhibitor, to ouabain had no impact in arteries isolated from WT mice (data not shown).

In contrast, EDHF-induced dilatation of *gracilis* arterial segments isolated from 3 m/o DL mice was insensitive to ouabain (Figure 1f, Table 2). At 12 and 20 months of age, however, ouabain strongly reduced ( $P < 0.05$ ) ACh-induced EDHF-dependent dilatation ( $26 \pm 6$  and  $2 \pm 1\%$ , respectively) (Figure 1f, Table 2). The addition of  $\text{Ba}^{2+}$  ( $30\ \mu\text{M}$ ) to ouabain reduced ( $P < 0.05$ ) EDHF-dependent maximal dilatation from  $95 \pm 2$  to  $54 \pm 11\%$  in arteries from 3 m/o DL mice but did not further reduce dilatations induced by EDHF in arteries from 12 m/o mice ( $26 \pm 9\%$ ).  $\text{Ba}^{2+}$ /ouabain experiments were not

**Table 2** Efficacy ( $E_{max}$ ) and sensibility ( $pD_2$ ) to ACh of the *gracilis* artery isolated from 3, 12 and 20 m/o WT and DL mice

Conditions	3 m/o WT		12 m/o WT		20 m/o WT		3 m/o DL		12 m/o DL		20 m/o DL	
	$E_{max}$	$pD_2$	$E_{max}$	$pD_2$	$E_{max}$	$pD_2$	$E_{max}$	$pD_2$	$E_{max}$	$pD_2$	$E_{max}$	$pD_2$
40 mM KCL-PSS	48 ± 4* (6)	7.7 ± 0.1	30 ± 5*† (6)	7.9 ± 0.2*	26 ± 2*† (5)	5.2 ± 0.1*††	46 ± 4* (6)	7.6 ± 0.3	30 ± 5† (8)	7.8 ± 0.1*	24 ± 5* (4)	6.9 ± 0.5
INDO + L-NNA	86 ± 3 (7)	7.4 ± 0.2	66 ± 8† (6)	6.9 ± 0.2	48 ± 4†† (5)	5.7 ± 0.1††	95 ± 2* (7)	7.4 ± 0.1	57 ± 8† (8)	6.3 ± 0.1†	26 ± 2*† (5)	6.3 ± 0.1†
+ 17-ODYA	89 ± 5 (4)	7.6 ± 0.4	43 ± 6*† (7)	7.2 ± 0.2	33 ± 2*†† (3)	5.4 ± 0.2††	42 ± 11* (6)	7.2 ± 0.3	57 ± 9 (6)	5.7 ± 0.2*†	23 ± 3*†† (5)	5.4 ± 0.5††
+ Ouabain	19 ± 7* (4)	6.2 ± 0.6*	22 ± 5* (6)	7.2 ± 0.2	10 ± 1*†† (3)	6.3 ± 0.3†	75 ± 10† (7)	6.9 ± 0.2	26 ± 6*† (4)	5.9 ± 1.0	2 ± 1*††† (7)	NM
+ Apamin + Chtx	22 ± 4* (7)	6.8 ± 0.2	46 ± 2*† (6)	7.2 ± 0.1	12 ± 3*† (3)	NM	25 ± 5* (5)	6.0 ± 0.4*	8 ± 2*†† (4)	NM	2 ± 1*††† (5)	NM
+ NAC	NT	NT	66 ± 10 (6)	7.0 ± 0.3	42 ± 8 (3)	5.4 ± 0.1	88 ± 5 (3)	8.1 ± 0.5	84 ± 3*† (6)	7.1 ± 0.2*	27 ± 5*† (4)	6.4 ± 0.3

Data are expressed as mean ± s.e.m. Vessels were preconstricted by 40 mM KCl-PSS or by PE in the presence of INDO (10  $\mu$ M) and L-NNA (100  $\mu$ M) combined with apamin and charybdotoxin, 17-ODYA, ouabain or NAC. \* $P$  < 0.05 compared to 3 m/o. † $P$  < 0.05 compared to 12 m/o. †† $P$  < 0.05 compared to 20 m/o. ††† $P$  < 0.05 compared to 12 m/o. †††† $P$  < 0.05 compared to 20 m/o. NT = not tested. NM = not measurable.

conducted in arteries from 20 m/o mice since ouabain alone completely prevented ACh-induced EDHF-dependent dilations (Figure 1f, Table 1).

#### Effect of ageing and DL on the contribution of small ( $SK_{Ca}$ ) and intermediate ( $IK_{Ca}$ ) conductance calcium-dependant potassium channels in EDHF-dependent dilatation to ACh

Apamin (1  $\mu$ M), an  $SK_{Ca}$  channel inhibitor, combined with charybdotoxin (0.1  $\mu$ M), an  $IK_{Ca}$  channel inhibitor, reduced by  $\approx$  75% the non-NO/non-PGI<sub>2</sub> dilatation induced by ACh in arteries isolated from 3 m/o WT and DL mice (Table 2). In vessels from 12 m/o WT mice only, the inhibitory effect of apamin and charybdotoxin was reduced (inhibition of the dilatation by 30%), whereas at 20 months of age, the dilatation was prevented by 75%. In contrast, in DL mice, the combination of the two toxins prevented EDHF-dependent dilatation induced by ACh at 12 and 20 m/o (Table 2).

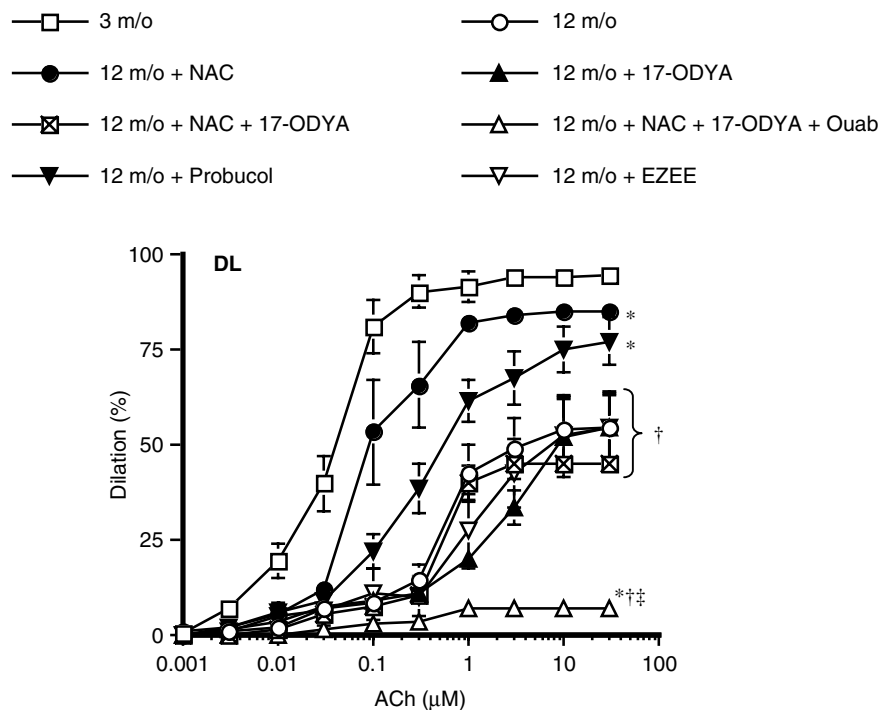
#### Effect of ROS on EDHF-dependent dilatation to ACh in ageing and DL

Reactive oxygen species production was increased in arteries isolated from 12 m/o DL mice compared to 12 m/o WT mice (Figure 3). This increased ROS generation was demonstrated in stimulated conditions using angiotensin II (10  $\mu$ M). NAC (1  $\mu$ M), a free radical scavenger, did not affect EDHF-induced dilations of vessels isolated from WT mice (Table 2). Similarly, NAC did not affect the dilatation to ACh of arterial segments isolated from 3 m/o DL mice. At 12 m/o, however, NAC restored ( $P$  < 0.05) ACh-induced EDHF-dependent dilatation of the DL mouse *gracilis* artery (increased the dilatation by 47%; Figure 2, Table 2). The beneficial effect of NAC was confirmed using probucol, another free radical scavenger (Figure 2). 17-ODYA (10  $\mu$ M) abolished ( $P$  < 0.05) the beneficial effect of NAC on EDHF-dependent dilations (Figure 2). No effect of NAC could be observed at 20 months of age (Table 1).

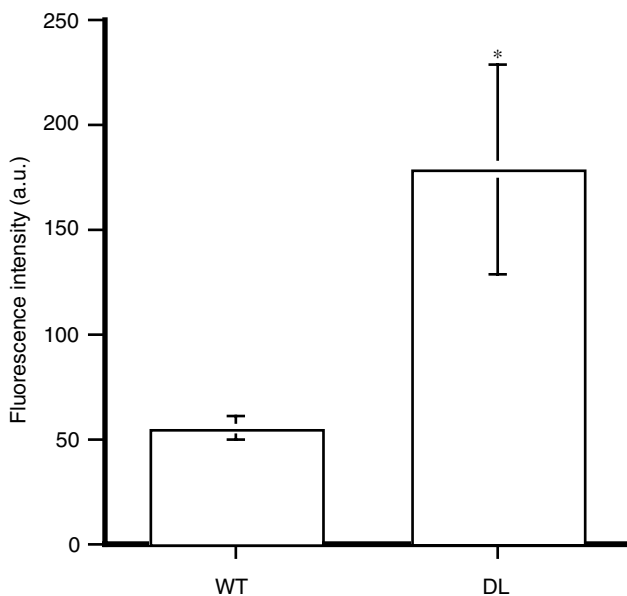
## Discussion

This study was designed to investigate the effects of DL associated with the ageing process on the nature, efficacy and mechanisms of EDHF-dependent dilatation to ACh of the mouse *gracilis* artery. EDHF-dependent dilatory responses were observed in the presence of NOS and COX inhibitors (Adeagbo & Triggle, 1993; Brandes *et al.*, 2000; Véquaud & Thorin, 2001).

We previously reported that in the *gracilis* artery, a resistance vessel, activation of endothelial  $SK_{Ca}$  and  $IK_{Ca}$  channels mediate the effects of EDHF. In 3 m/o WT mice, activation of these channels fully dilates smooth muscle cells by activating the  $Na^+/K^+$ -ATPase pump (Krummen *et al.*, 2005). In vessels isolated from DL mice, this pathway contributes to half of the dilatory response to ACh. EETs, derived from the activity of a cytochrome P450 (Krummen *et al.*, 2005), represent a secondary pathway contributing to the dilatation induced by ACh as reported by others (Campbell *et al.*, 1996; Baron *et al.*, 1997; Fisslthaler *et al.*, 1999; Brandes *et al.*, 2000; Archer *et al.*, 2003). The results of the present



**Figure 2** ACh-induced dilatation of *gracilis* arteries isolated of 3 and 12 m/o DL mice ( $n=7$  and  $8$ , respectively). In arteries isolated from 12 m/o mice, the effects of NAC ( $1 \mu\text{M}$ ,  $n=6$ ), Probucol ( $10 \mu\text{M}$ ;  $n=9$ ), EZEE ( $1 \mu\text{M}$ ), NAC plus 17-ODYA ( $10 \mu\text{M}$ ,  $n=6$ ) and NAC plus 17-ODYA and ouabain ( $1 \text{mM}$ ,  $n=6$ ) are reported. L-NNA ( $100 \mu\text{M}$ ) and INDO ( $10 \mu\text{M}$ ) were present in the bath. \* $P < 0.05$  compared to 3 m/o; † $P < 0.05$  compared to 12 m/o NAC. ‡ $P < 0.05$  compared to 12 m/o 17-ODYA.



**Figure 3** Angiotensin II ( $10 \mu\text{M}$ )-induced free radical production of pressurised segments of the *gracilis* artery isolated from 12 m/o WT and DL mice ( $n=4$ ). \* $P < 0.05$  compared to WT.

study demonstrate that over the course of ageing, the functional activity of the cytochrome *P450* is inhibited in DL mice due to a rise in oxidative stress, while this pathway becomes expressed in WT mice. At 20 m/o, the activity of the cytochrome *P450* remains in WT mice but is irreversibly blunted in DL mice. Hence, the decline in EDHF-dependent

dilatation occurring during the normal course of ageing is accompanied by a compensatory duplication of the EDHF pathways; DL hastens the expression of this compensatory process.

Ageing has been reported to be associated with a reduced EDHF-dependent dilatation (Fujii *et al.*, 1993; Urakami-Harasawa *et al.*, 1997; Froese *et al.*, 1999). Our data collected in the *gracilis* artery are in agreement with these earlier reports. One important finding, however, is the demonstration that the nature of the EDHF released upon muscarinic stimulation is dynamic with age, that is, it is not a single factor that accounts for the dilatation through life. As we reported previously (Krummen *et al.*, 2005), activation of endothelial  $\text{SK}_{\text{Ca}}$  and  $\text{IK}_{\text{Ca}}$  channels leads to dilatation of the *gracilis* artery isolated from 3 m/o WT mice. It has been proposed that endothelial  $\text{SK}_{\text{Ca}}$  and  $\text{IK}_{\text{Ca}}$  are responsible for the rise in  $[\text{K}^+]_{\text{o}}$  in the intercellular space (Edwards *et al.*, 1998). This increases the activity of the smooth muscle ouabain-sensitive  $\text{Na}^+/\text{K}^+$ -ATPase pump leading to hyperpolarisation and dilatation. After 9 months, however, apamin and charybdotoxin only partially reduced the dilatation induced by ACh, suggestive of a change in the endothelial pathways leading to EDHF-mediated dilatation. Since 17-ODYA, a cytochrome *P450*/epoxygenase inhibitor, reduced the dilatation induced by ACh, it suggests that this pathway is upregulated during the normal ageing process in addition to being activated by DL in young animals.

An important observation is the greater decline in EDHF-mediated dilatation of vessels isolated from DL mice between the age of 3 and 20 months compared to WT. In arteries

isolated from 12 m/o DL mice, the activity of the cytochrome *P450* pathway no longer contributes to ACh-induced dilatation. The regression of the activity of this pathway is certainly responsible for the sharp drop in EDHF efficacy observed at 12 m/o and further exacerbated at 20 m/o. This *P450* pathway, however, has been reported to be sensitive to oxidative stress (Miura & Gutterman, 2004). As demonstrated in Figure 3, the production of ROS is augmented in arteries isolated from 12 m/o DL mice. To validate the potential inactivating role of ROS on the activity of the *P450* pathway, we tested the acute effect of NAC and probucol on ACh-induced dilatation of vessels isolated from 12 m/o DL mice (Figure 2). The antioxidants restored the dilatation induced by ACh in these vessels, which was identical to the dilatation induced by ACh in arteries isolated from 12 m/o WT mice (Table 2). This strongly suggests that oxidative stress, which is increased at 12 months in DL (Figure 3), contributes to the decline of the efficacy of EDHF associated with DL. Importantly, addition of 17-ODYA cancelled the protective effect of NAC. Thus, DL-associated ROS production reduces EDHF-dependent dilatation by inhibiting the cytochrome *P450* function and EET production. Hence, DL favours the early expression of a secondary EDHF pathway, a pathway that only appears at 12 months of age in WT mice. DL, however, is associated with a rise in oxidative stress, which is responsible for the functional inactivation of this early compensatory pathway.

The signals leading to the duplication of the EDHF pathways are unknown. NAC only improved dilatation of vessels isolated from DL mice to normal WT mice levels. In contrast, NAC did not magnify the dilatation in WT mice. It suggests that the NAC-sensitive oxidative stress *per se* is not responsible for the upregulation of this cytochrome *P450* pathway. It is also unlikely that maturation alone is responsible for the late expression of the cytochrome *P450* pathway in WT mice since this expression is hastened by DL. Hence, the expression of this pathway must be sensitive either to the functional status of the endothelium or to the extracellular matrix environment.

The mechanisms underlying EDHF-induced dilatation are indeed different at 3 m/o in DL and WT mice (Krummen *et al.*, 2005). Whereas the sensitivity to apamin and charybdotoxin is identical, ouabain did not prevent dilatation of arteries isolated from DL mice. At 12 m/o, however, the contribution of these signalling proteins changed: the inhibitory effects of apamin and charybdotoxin on the dilatation induced by ACh in WT mice is reduced while the combination of the toxins prevented the dilatatory response in DL mice. In both groups, however, the toxins augmented the myogenic tone (Table 1), suggesting that the  $K_{Ca}$  channels are contributing to the baseline regulation of smooth muscle contractility. Similarly, ouabain reduced the dilatation induced by ACh in both groups of 12 m/o animals, while myogenic tone was augmented by ouabain at all ages for both groups. Hence, the basal activity of the endothelial  $K_{Ca}$  channels and the smooth muscle  $Na^+/K^+$ -ATPase are similar in arteries isolated from WT and DL mice, if one uses the myogenic tone (baseline diameter) as an index, whereas upon muscarinic receptor stimulation discrepancies appear. This, therefore, is more suggestive of an impaired receptor/effector

coupling rather than a fundamental change in the endothelial function.

This does not eliminate the possibility that the extracellular environment is altered by the ageing process and exacerbated by DL. It is known that ageing is associated with intimal thickening of the vessels (Stary, 1990). In this condition, coupling between endothelial and smooth muscle cells is expected to be impaired, and the hyperpolarising diffusion of  $K^+$  ions from the endothelium to the smooth muscle cells, as proposed by our data and Edwards and co-workers (1998) would be reduced. This is, however, unlikely because the histological analysis did not reveal changes in the structure of the *gracilis* arteries (data not shown). In addition, blockade of  $K_{Ca}$  channels by the toxins or the  $Na^+/K^+$ -ATPase with ouabain reduced the resting diameter at all ages (Table 1). These mechanisms remain therefore effective. In addition, we previously reported that gap junction were not involved in the mediation of the effects of EDHF at 3 months (Krummen *et al.*, 2005).

Finally, ageing affected NO/ $PGI_2$ -dependent dilatation similarly whether arteries were isolated from WT or DL mice. This age-dependent endothelial dysfunction is well established (Gerhard *et al.*, 1996; Rodriguez-Martinez *et al.*, 1998; van der Loo *et al.*, 2000; Woodman *et al.*, 2003). In a recent study, it was reported that the NO-dependent dysfunction could be restored by acute exposure to a SOD mimetic (Csiszar *et al.*, 2002). It is, therefore, likely that in our model too the decline in this function is concomitant with a chronic rise in oxidative stress.

In conclusion, a 17-ODYA-sensitive metabolite of the cytochrome *P450*/epoxygenase pathway, most likely EETs, and  $K_{Ca}$  channels are responsible for ACh-induced EDHF-dependent dilatation of the DL mouse *gracilis* artery. At 12 m/o, DL targets the 17-ODYA-sensitive component of the dilatation mediated by ACh, as revealed by the rescue of this dilatation by antioxidants, whereas this 17-ODYA-sensitive mechanism appears in 12 m/o WT mice. Free radical production was therefore responsible for this early – but reversible – endothelial dysfunction in DL mice. At 20 months of age, NAC no longer rescued the dilatation suggestive of an irreversible damage to the endothelium. Hence, the 17-ODYA-dependent dilatation is a secondary endothelium-dependent dilatatory mechanism recruited early in the presence of a risk factor for CVDs such as DL and later in life during the normal ageing process. This time-dependent evolution of the endothelial function suggests that there is a therapeutic window for the prevention of the endothelial dysfunction associated with DL. Because of the sensitivity of EDHF to oxidative stress, antioxidant therapeutic agents may be of use in the early phases of DL and later in life to prevent the deterioration of the endothelial dysfunction, although the impact of oxidative stress to age-associated endothelial dysfunction needs further exploration.

This work has been supported in part by the Foundation of the Montreal Heart Institute, the Heart and Stroke Foundation of Quebec and the Canadian Institute for Health Research (MOP14496), the NIH (GM31278, JRF) and the Robert A. Welch Foundation (JRF). E. Thorin is a senior scholar of the *Fonds de la Recherche en Santé du Québec*.

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(Received November 16, 2005)

Accepted January 6, 2006)

Published online 6 February 2006)