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Disruption of endothelial caveolae is associated with impairment of both NO- as well as EDHF in acetylcholine-induced relaxation depending on their relative contribution in different vascular beds

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Abstract

Caveolae represent an important structural element involved in endothelial signal-transduction. The present study was designed to investigate the role of caveolae in endothelium-dependent relaxation of different vascular beds. Caveolae were disrupted by cholesterol depletion with filipin (4×10^{-6} g L^{-1}) or methyl-β-cyclodextrin (MCD; 1×10^{-3} mol L^{-1}) and the effect on endothelium-dependent relaxation was studied in rat aorta, small renal arteries and mesenteric arteries in the absence and presence of L-NMMA. The contribution of NO and EDHF, respectively, to total relaxation in response to acetylcholine (ACh) gradually changed from aorta (71.2±6.1% and 28.8±6.1%), to renal arteries (48.6±6.4% and 51.4±6.4%) and to mesenteric arteries (9.1±4.0% and 90.9±4.1%). Electron microscopy confirmed filipin to decrease the number of endothelial caveolae in all vessels studied. Incubation with filipin inhibited endothelium-dependent relaxation induced by cumulative doses of ACh (3×10^{-9}–10^{-4} mol L^{-1}) in all three vascular beds. In aorta, treatment with either filipin or MCD only inhibited the NO component, whereas in renal artery both NO and EDHF formation were affected. In contrast, in mesenteric arteries, filipin treatment only reduced EDHF formation. Disruption of endothelial caveolae is associated with the impairment of both NO and EDHF in acetylcholine-induced relaxation.

Keywords: Disruption; Caveolae; Endothelial dysfunction; NO; EDHF; Vascular beds

Introduction

Caveolae are specific cholesterol-rich microdomains in the plasma membrane and their importance in normal endothelial function has been increasingly recognized (Darblade et al., 2001; Feron et al., 1998; Yuhanna et al., 2001). They are considered to be a subcategory of lipid rafts in which, amongst others, receptor activated signal-transduction appears to be organized (Frank et al., 2003). Caveolae are particularly abundant in endothelial cells, where they regulate nitric oxide synthase (eNOS) localization and NO production via interaction with caveolin-1, the structural protein of caveolae (Bucci et al., 2000; Cohen et al., 2004; Gratton et al., 2004). The role of caveolae in endothelial NO production has been studied intensively using cultured endothelial cells and caveolin-1 knockout mice. Results of these studies demonstrate that the absence of this organelle impairs NO and calcium signaling in the cardiovascular system, causing aberrations in endothelium-dependent relaxation, contractility, and maintenance of myogenic tone (Blair et al., 1999; Darblade et al., 2001; Je et al., 2004). Conversely, very little is known about the role of caveolae in the regulation of other endothelial mediators of relaxation. Only one other study reported that caveolae may also be implicated in EDHF-mediated relaxation in pig coronary artery (Graziani et al., 2004). Furthermore, functional studies providing direct evidence that intact caveolae are essential for

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NO production are limited mainly to the aorta, either from cav-1 knockout mice or pathological models, only (Darblade et al., 2001; Razani et al., 2001).

It may be derived from the above that caveolae could provide a platform not only for control of NO but for the generation of endothelial relaxing factors in general. Given the importance of endothelial function in cardiovascular health and disease, it seems of interest and potential relevance to further clarify the involvement of caveolae in NO- as well as EDHF-mediated relaxation in different vascular beds. To this end, we hypothesized that caveolae disruption in normal artery preparations mimics endothelial dysfunction in all vascular beds by affecting NO- and/or EDHF-mediated relaxation, depending on their relative importance in a particular bed. To address this hypothesis, acetylcholine-induced endothelium-dependent relaxations – as well as the contribution of NO and EDHF herein – were studied in isolated preparations of normal rat aorta, small renal arteries and superior mesenteric arteries, in the absence and presence of a cholesterol depleting agent to disrupt caveolae.

Materials and methods

Animal

Male Wistar rats (260–280 g, Harlan, Zeist, The Netherlands) were housed at the animal facility of the University Medical Center Groningen and were studied in compliance with institutional and legislative regulations. Rats were anesthetized with 1.5% isoflurane in N_2O/O_2 and thoracic aorta, kidney and intestine were removed and kept in cold Krebs solution to isolate aorta, renal arteries and mesenteric arteries for electron microscopy samples and functional studies. Animal studies were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Transmission electron microscopy

Artery segments were isolated and treated with filipin (4×10^{-6} g L^{-1}) or vehicle for 20 min at 37 °C. Then, the artery segments were fixed in 1% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.6 for 24 h. Following postfixation in 1% osmium tetroxide plus 1.5% potassium ferrocyanide in 0.1 M cacodylate buffer for 15 min, artery segments were dehydrated in a graded alcohol series and embedded in Epon-812 resin. Ultrathin sections were made perpendicular to the substrate and contrasted with uranyl acetate and lead citrate. Images were taken on a Philips electron microscopy CM100 operated at 60 kV and digitized. The photos were randomly taken and quantitatively analyzed with analySIS image software (Munster, Germany). To assess the number of caveolae per micron of membrane, a total of 15 random fields of 3 different sections (5 fields per section) per artery were photographed, for each of above indicated three different artery types per animal ($n=3$ animals in total). Caveolae were defined as uncoated surface invaginations with a diameter of 50 to 100 nm with clear connections to the plasma membrane on endothelial cells (Bruns and Palade, 1968a,b). Samples were scored by an investigator blinded for the treatment of the samples. The average number of caveolae per micrometer of sectioned plasma membrane is expressed as mean ± S.E.M.

Studies with isolated aortic, renal and mesenteric artery preparations

Aortic rings (2 mm in length) were mounted for studies of isotonic contraction in an organ bath setup, as described previously (Buikema et al., 2000). Rings were subjected to a preload of 14 mN and allowed to stabilize for 60 min before they were checked for viability by evoking a contraction with 10^{-6} mol L^{-1} phenylephrine (PE). In some cases, denuded rings were prepared by rubbing the intimal surface of the rings with a stainless steel wire of appropriate size. Removal of endothelium was confirmed by the absence of relaxation to 10^{-5} mol L^{-1} ACh in PE precontracted rings, and these rings were used to study responses to sodium nitroprusside (SNP).

Second- to third-order branches of mesenteric arteries and interlobular renal arteries were isolated from surrounding perivascular tissue and cut into 2–3 mm long vascular rings. Arterial rings were connected to force transducers in individual organ bath chambers for isometric tension recordings (MYO-2, EMKA Technologies, Paris, France). After mounting, the vessel was equilibrated for 30 min in Krebs solution. Then, the calculated length of the vessel at 100 mm Hg was determined as described by Delaey et al. (2002). Briefly, the vessel was stretched by stepwise increasing the distance between two stainless steel wires in steps of 10–20 μm until the calculated transmural pressure exceeded 100 mm Hg. Vessels were held at each length for 1 min and the generated force and internal circumference were used to calculate the wall tension. The internal circumference and corresponding wall tension for each point could thus be fitted on an exponential curve for determination of L100 (i.e. calculated length of the vessel at 100 mm Hg), as previously described (Delaey et al., 2002). Arteries were allowed to equilibrate for 30 min in Krebs solution at an internal circumference of 0.9 L100 before being preconstricted with 10^{-6} mol L^{-1} U46619.

All organ bathes were filled with Krebs solution bubbled with $95\%O_2/5\%CO_2$ at 37 °C. Preconstricted rings were studied for endothelium-dependent and endothelium-independent relaxation by applying cumulative doses of ACh (3×10^{-9}–10^{-4} mol L^{-1}) and SNP (10^{-10} mol L^{-1}–3×10^{-6} mol L^{-1}), respectively, to the organ bath in the absence or presence of the caveolae-disrupting agents filipin (4×10^{-6} g L^{-1} for 20 min) or methyl-beta-cyclodextrin (MCD, 10^{-3} mol L^{-1} for 60 min). To determine the contribution of NO and EDHF in endothelium-dependent relaxation, the response to ACh was additionally studied in the presence of various inhibitors added to the bath 20 min prior to addition of ACh. To this end, N^G-mono-methyl-arginine (L-MMA, 10^{-4} mol L^{-1}) was used to inhibit NO production, and a combination of apamin (apa, 0.5×10^{-6} mol L^{-1}) and charybdotoxin (chtx, 10^{-7} mol L^{-1}) was used to inhibit EDHF, as described previously (Gschwend et al., 2002a,b, 2003; Ozkan and Uma, 2005). Indomethacin (10^{-5} mol L^{-1}) was

always present in the bath and used to inhibit cyclooxygenase-derived prostanoid production.

Drugs

Vascular studies were performed using a Krebs bicarbonate solution with the following composition (mmol L^{-1}): NaCl 120.4, KCl 5.9, $CaCl_2$ 2.5, $MgCl_2$ 1.2, NaH_2PO_4 1.2, glucose 11.5, and $NaHCO_3$ 25.0, freshly prepared daily. Stock solution (10 mmol L^{-1}) for indomethacin was prepared in 1% $NaHCO_3$ solution. Filipin and U46619 were prepared in 96% ethanol. MCD, L-NMMA, apamin and charybdotoxin were dissolved in saline solution. All other drugs were dissolved in deionized water and diluted with Krebs solution. All compounds were purchased from Sigma (St. Louis, MO, USA).

Calculations and statistical analysis

Concentration-response curves to ACh and SNP are expressed as percentage relaxation of preconstriction to PE or U46619. The Area Under the Curve (AUC) of the concentration-response curve of individual rats was determined (Sigma Plot, Jandell Scientific) and expressed in arbitrary units. The AUC was used to represent total (individual) ACh relaxation (in the presence of indomethacin) and for subsequent analysis of differences in ACh relaxation with and without L-NMMA to estimate the contribution of NO and EDHF. The number of caveolae was counted in each preparation and the average number of caveolae of all control preparations (i.e. those not treated with filipin) was calculated per artery type, and this average number of caveolae in control preparations was set to 100% per artery type. Subsequently, for each preparation (hence, including control preparations themselves) the number of caveolae was recalculated as a percentage of the (absolute) average number of caveolae (according to the appropriate artery type). Data are expressed as mean ± S.E.M. Comparisons were performed using Student's *t*-statistics, or repeated measures ANOVA combined with Bonferroni for post-hoc testing in case of full concentration-response curves. Differences were considered significant at $P<0.05$.

Results

Ultrastructure and number of caveolae

It has been demonstrated that caveolae-like structures may be disrupted by cholesterol depletion using filipin or MCD in cultured cells (Schnitzer et al., 1994), and for MCD in isolated rabbit aorta (Darblade et al., 2001). To confirm the latter observation for filipin, we examined its effects on the ultrastructure and number of caveolae on endothelial cells in our isolated artery preparations. The caveolae profiles were clearly visible as revealed by electron microscopy in endothelial cells of control-treated vascular beds used in this study (Fig. 1A). Filipin treatment disrupted the structural integrity of caveolae in the plasma membrane as demonstrated by the reduction of caveolar invaginations at the membrane surface in all vascular beds (Fig. 1B). As a result, the number of morphologically recognizable caveolae was significantly lower in aorta, renal and mesenteric arteries, the reduction

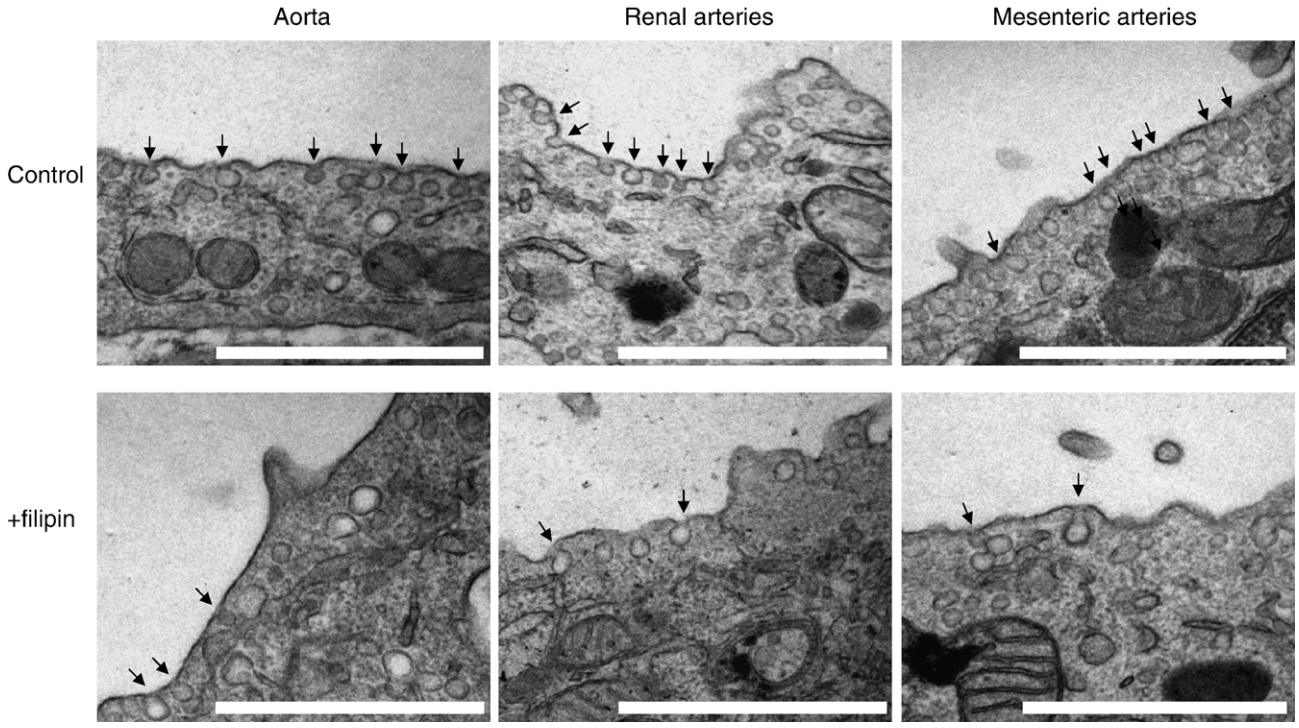

Fig. 1. Visualization of endothelial caveolae using transmission electron microscopy on thin sections of rat aorta, small renal and mesenteric arteries, (A) before, and (B) after treatment with filipin. Caveolae appear as the uncoated surface invaginations with a diameter of 50 to 100 nm with clear connections to the plasma membrane (arrowheads). Bar, 1 μm in each case.

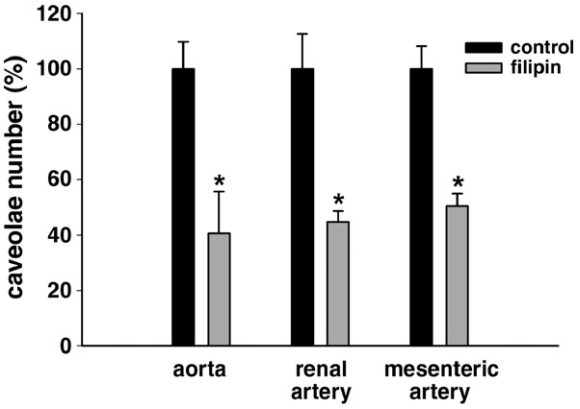

Fig. 2. Caveolae number is significantly decreased at the luminal plasma membrane of endothelial cells from aorta, renal arteries and mesenteric arteries following filipin treatment. Caveolae number % is expressed as mean±S.E.M. *$P<0.05$ filipin treatment versus control.

averaging 59%, 55% and 50% respectively (Fig. 2). These data demonstrate that filipin treatment destroyed the caveolar structure and decreased the number of visible caveolae on the endothelial surface by the same extent in all three vascular beds.

Relaxation studies in isolated artery preparations

NO- and EDHF-contribution to ACh-induced relaxation

Full concentration-response curves for ACh-induced relaxation and the effects of various inhibitors are presented in Fig. 3 (on the left side). Consistent with previous findings from our lab, the ACh relaxation that persisted in the presence of indomethacin and L-NMMA was totally blocked by the additional presence of apa/chtx in all artery types, and considered to represent EDHF-mediated relaxation (Gschwend et al., 2002a,b, 2003; Ozkan and Uma, 2005). Similarly, the part of the total ACh relaxation sensitive to inhibition with L-NMMA was considered to represent NO (Gschwend et al., 2002a,b, 2003; Ozkan and Uma, 2005). Using the AUC to calculate the differences between the curves as a means to estimate the contribution of NO and EDHF (Fig. 3 on the right side), the relative contribution of both mediators in total ACh-induced relaxation in each artery type is summarized in Fig. 5. The relative NO-contribution was greatest in the aorta > renal artery > mesenteric artery, and the reverse was true for the relative EDHF-contribution, which was highest in the mesenteric artery.

Effect of caveolar disruption

Contractions elicited by PE in aorta and U46619 in renal and mesenteric arteries were similar in the filipin-treated and control rings (data not shown). In contrast, presence of filipin to disrupt caveolae significantly attenuated total ACh-induced relaxations in all three vascular beds (Fig. 3). We also tested the effect of another caveolae-disrupting agent (namely MCD) so as to rule out potential drug-related effects: filipin and MCD similarly reduced ACh-induced relaxations in aorta preparations (Fig. 4A). Moreover, endothelium-independent relaxation to SNP in endothelium-denuded aorta rings remained unaffected by both filipin and MCD (Fig. 4B).

Although filipin attenuated total ACh-induced relaxation in all artery types (31.9%, 47.6% and 23.8% reduction on average in aorta, renal and mesenteric artery, respectively), the extent to which the two different endothelial mediators studied here were attenuated, differed among the three vascular beds. In the aorta, ACh-induced relaxation did not differ between rings incubated with L-NMMA plus filipin versus L-NMMA only, suggesting that the NO-, but not the EDHF-component was sensitive to caveolar disruption (Fig. 3). Vice versa, in mesenteric artery it appeared that the EDHF-, but not the NO-component was sensitive to caveolar disruption. Intermediate results were obtained with renal arteries in which both the NO- as well as the EDHF-components were affected. These findings have been summarized in Fig. 5, showing that caveolar disruption attenuates both NO- and/or EDHF-mediated relaxation, depending on the relative importance of these mediators in a particular bed.

Discussion

Caveolar disruption mimicked endothelial dysfunction in all arteries investigated in the present study (i.e. aorta, small intrarenal and mesenteric artery) by decreasing the contribution of NO and/or EDHF in ACh-induced relaxation, the latter apparently depending on their relative contribution in a particular artery type. This extends previous findings (Darblade et al., 2001; Linder et al., 2005) about the importance of caveolae in (stimulated) NO production in aorta, to the concept that caveolae may serve as an integration center on endothelial cells to control (stimulated) production of different endothelium derived relaxing factors, including EDHF.

Caveolae are increasingly considered as important signaling platforms, which serve to compartmentalize and integrate signals. Caveolins, a family of highly conserved integral membrane proteins, interact specifically with signaling molecules and many of their binding partners, apparently providing a scaffold that places members of a signal-transduction in close proximity with one another (Engelman et al., 1998). Because of their high abundance in vascular endothelial cells, caveolae and their related signal-transduction pathways have also been implicated in vascular/endothelial functions, including vasomotor regulation, angiogenesis (Griffoni et al., 2000; Liu et al., 2002; Woodman et al., 2003) and atheroprotection (Frank and Lisanti, 2004; Frank et al., 2004). An important role of caveolae and caveolins in pathogenesis and possible prevention of human disease has been particularly implicated in cancer, diabetes, Alzheimer disease, and muscular dystrophy, whereas studies of its potential role in cardiovascular disease (although emerging) have been rather limited so far. In atherosclerosis, aortic preparations of hypercholesterolemic rabbit in which the luminal side was covered by fatty streaks, the impaired ACh-induced relaxation is associated with a reduction of plasmalemmal caveolae (Blair et al., 1999; Darblade et al., 2001).

In the present study, reduction of endothelial caveolae after cholesterol depletion also resulted in impaired ACh-induced

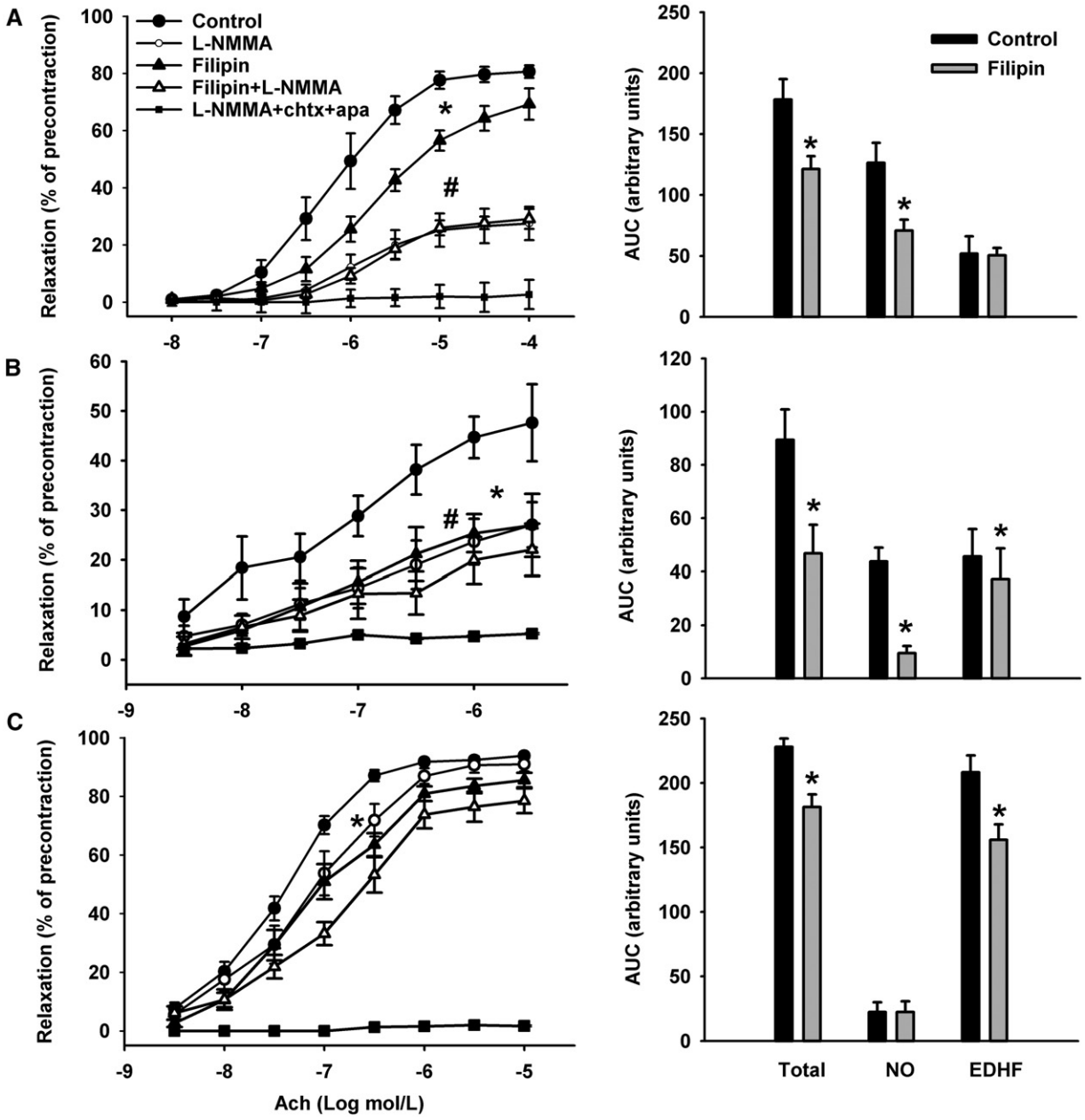

Fig. 3. Effects of filipin on ACh-induced relaxation. The responses to cumulative doses of ACh were examined in rings from rat aorta (the left panel A), renal arteries (the left panel B) and mesenteric arteries (the left panel C). Values are expressed as percentage of relaxation relative to precontraction. Relative contribution of NO and EDHF are expressed as Area Under the Curve (AUC) in arbitrary units in absence (closed bars) or presence of filipin (open bars) in rat aorta (the right panel A), renal arteries (the right panel B) and mesenteric arteries (the right panel C). Data are expressed as mean ± S.E.M. *$P<0.05$ filipin treatment versus control; #$P<0.05$ L-NMMA treatment versus control.

relaxation, without affecting endothelium-independent relaxation to SNP. Although some agonists causing contraction of vascular smooth muscle act on receptors that are localized in caveolae or aggregate in caveolae upon ligand binding (Chun et al., 1994; de Weerd and Leeb-Lundberg, 1997; Ishizaka et al., 1998), filipin and MCD did not change the contractile response to PE or U46619 in the present study, indicating that both α-adrenergic receptor and TXA_2 receptor were not involved in the alteration of ACh-induced relaxation in these three vascular beds. Similar observations were reported in tail arteries (Dreja et al., 2002) and rat aorta (Darblade et al., 2001; Linder et al., 2005).

Based on the results of various studies, there is little doubt about endothelial caveolae acting as integrating centers for endothelial NO production, and about the relationship of eNOS with the principle protein constituting caveolae, caveolin-1. Direct interaction of caveolin-1 with NO synthase inhibiting its enzymatic activity has been demonstrated in biochemical studies (Garcia-Cardena et al., 1997; Ghosh et al., 1998; Ju et al., 1997). Additional evidence for the role of caveolae in NO

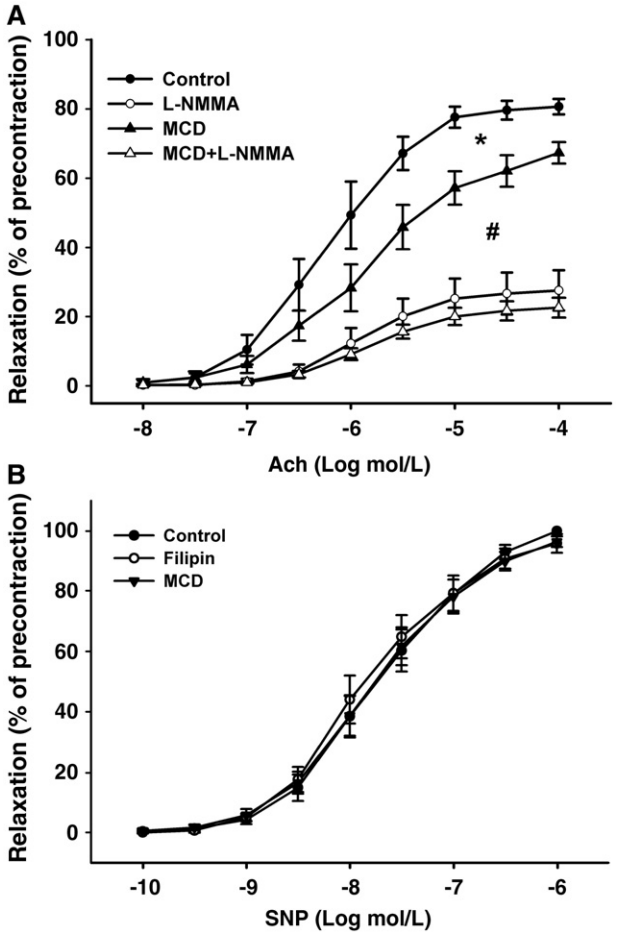

Fig. 4. Effects of MCD and/or filipin on the relaxation of rat aorta rings. Panel A: Aortic rings were exposed to cumulative dose of ACh in the absence or presence of L-NMMA after MCD treatment. Panel B: Aortic rings without endothelium were exposed to cumulative dose of SNP after MCD or filipin treatment. Values are expressed as percentage of relaxation relative to precontraction. Data are expressed as mean±S.E.M. *$P<0.05$ MCD treatment versus control; #$P<0.05$ L-NMMA treatment versus control.

metabolism in the cardiovascular system may be derived from observations in caveolin-1 knockout mice, who feature the absence of caveolae on the endothelium. These mutant mice displayed a five-fold increase in plasma NO levels (Zhao et al., 2002) and marked ACh-induced NO-mediated relaxation of isolated aortic rings (Drab et al., 2001). Consequently, our present observations and those of others employing cholesterol depletion (Blair et al., 1999; Darblade et al., 2001) in which caveolar disruption decreased NO production, are opposite to the above observations from caveolin-1 knockout mice. Most likely, this is explained by the differences in the amount of caveolin-1 present in endothelial cells. The low levels of caveolin-1 in knockout mice result in reduced inhibition of eNOS activity, whereas levels of the protein are unchanged when caveolae are disrupted by cholesterol depletion. Indeed, we observed that filipin treatment did not affect levels of caveolin-1 in cultured BAEC cells (data not shown). Thus, one explanation for the decreased NO mediated relaxation following disruption of caveolae may be an enhanced "scavenging" of eNOS by caveolin-1 freed from caveolae, possibly accompanied by internalization (Michel, 1999). Alternatively, co-localization of eNOS with caveolin-1 in caveolae could contribute in optimizing its activation following binding of agonists (such as bradykinin and ACh) to their receptors. When eNOS and caveolin-1 translocation from plasmalemmal caveolae to the endoplasmic reticulum or Golgi apparatus occurs in cholesterol depleted cells (Blair et al., 1999), eNOS internalizes in association with caveolin without the usual agonist-induced activation events occurring (Michel, 1999). These data fit with our findings in the aorta and suggest that alteration in the plasmalemmal cholesterol may alter the caveolae–eNOS activity and lead to endothelial dysfunction. Therefore, the results from knockout caveolin-1 mice and acute disruption of caveolae model both indicate the importance of caveolae in maintaining appropriate regulation of eNOS activity.

Of notice, disruption of caveolae also decreased endothelium-dependent relaxation in small renal and mesenteric arteries, in which NO-contribution in ACh-induced relaxation is less prominent and even marginal. Our results demonstrate that in addition to NO, caveolae also play a major role in organizing EDHF signaling, particularly in small sized arteries. To our knowledge, the only prior evidence for caveolae playing a role in EDHF signaling is limited to pig coronary artery in a previous study by Graziani and co-workers (2004). These authors showed that EDHF signaling in pig coronary arteries involves Ca^{2+}-dependent phospholipase A_2, an enzyme which produces arachidonic acid, a putative precursor molecule in EDHF signaling (Hecker et al., 1994; Fisslthaler et al., 1999). Phospholipase A_2 is able to form a complex with caveolin-1

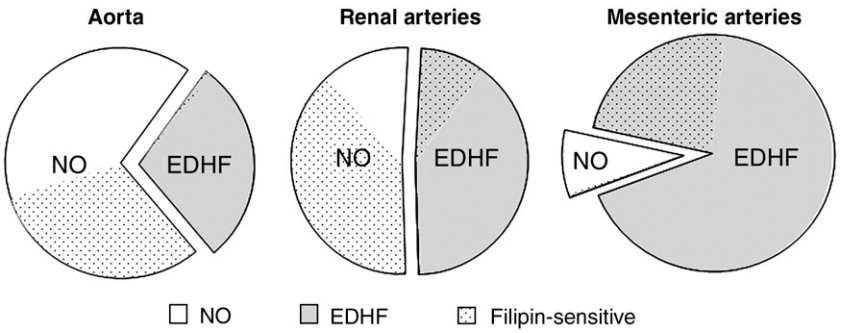

Fig. 5. Relative contribution of NO (open area) and EDHF (grey area) in aorta, renal and mesenteric arteries. Dotted area indicates the part sensitive to treatment with filipin.

and resides in low-density, caveolin-1 containing membranes, i.e. caveolae. Disintegration of caveolae with MCD was associated with suppression of EDHF signaling, an effect that was blunted by supplementation of arachidonate in that study. The gap in current knowledge regarding the role of caveolae in NO versus its role in EDHF is probably due to the relatively unknown and proposedly differential nature of EDHF in different vascular beds (Campbell et al., 1996; Edwards et al., 1998; Fisslthaler et al., 1999; Hutcheson et al., 1999; Randall et al., 1996). Also in the present study, we assessed its contribution by blocking the K_{Ca} channels involved in the EDHF-mediated relaxation rather than investigating its nature. Nevertheless, the role of caveolae was independent of specific endothelial mediators in the present study, thereby suggesting the existence of a common pathway governed by caveolae for endothelium-dependent relaxation regardless of the different mediators involved. Previously, Darblade et al. (2001) showed that in contrast to ACh, receptor-independent stimulation for endothelium-dependent relaxation elicited by the calcium-ionophore A23187 was not affected by caveolae disruption. Such findings are very suggestive for impaired receptor signaling to stimulate production of endothelial relaxing factors rather than per se impairment of the enzymes producing these EDRFs. At present it is still unclear whether this may involve the endothelial muscarinic receptor. In cardiac myocytes, translocation of muscarinic receptors to caveolae may be necessary to initiate specific downstream signaling cascades (Feron et al., 1997) such as eNOS activity (Dessy et al., 2000). However, various other mechanisms may also be implicated in endothelial cells, such as mechanisms involved in Ca^{2+} entry (Frank et al., 2003), enzyme redistribution or sequestration (Blair et al., 1999), the functioning of G-proteins and receptor tyrosine kinases (Foster et al., 2003).

Interestingly in the above context, we and others previously reported endothelium-dependent relaxation in isolated aortic rings of myocardial infarcted rats with chronic heart failure (CHF) to be impaired after stimulation with ACh while at the same time to be intact after stimulation with A23187, again pointing at selective impairment of receptor-mediated endothelial dysfunction (Buikema et al., 2000; Ontkean et al., 1991). In addition to that, preliminary (unpublished) data from our lab indicate that the number of endothelial caveolae may be decreased in aortas of rats with experimental CHF. Clearly these findings have to be substantiated and confirmed by others, but they do imply a possible role for caveolae in impaired (receptor-mediated) endothelial dysfunction in cardiovascular disease that may be worthwhile pursuing. Our present finding that caveolae may be involved in organizing appropriate EDHF as well as NO signaling further implies that the relevance of caveolar impairment for endothelial dysfunction is not restricted to a particular artery type or vascular bed. It would be interesting, therefore, to investigate whether alterations in caveolar number (and function?) may underlie endothelial dysfunction in cardiovascular disease, and whether such alterations may be local or generalized as a means to explain heterogeneity in the occurrence of endothelial dysfunction among different vascular beds. It should be noted, for that matter, that our present study was performed with one endothelial agonist only. Therefore, future studies may also employ endothelial agonists other than acetylcholine, and preferably in combination with localization techniques demonstrating NO and EDHF signaling and expression in caveolae as well as the variability hereof in different vascular beds.

In conclusion, disruption of caveolae induced an impairment of (ACh-induced) receptor-mediated endothelial function in isolated artery preparations by affecting NO and/or EDHF, the latter depending on the principal endothelial component that mediates relaxation in a particular artery type. Additional studies are needed to confirm these findings and make linkages to endothelial dysfunction in conditions of cardiovascular disease.

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