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RESEARCH PAPER

Oleanolic acid induces relaxation and calcium-independent release of endothelium-derived nitric oxide

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Background and purpose: The present study investigated the mechanisms by which oleanolic acid, a component of olive oil, increases release of nitric oxide (NO).
Experimental approach: Measurements of isometric tension, NO concentration, or endothelial cell calcium were made in rat isolated mesenteric arteries. Immunoblotting for endothelial NOS (eNOS) and Akt kinase were performed in primary cultures of human umbilical vein endothelial cells (HUVECs).
Key results: Oleanolic acid (3–30 μM) evoked endothelium-dependent relaxations in noradrenaline-contracted rat superior and small mesenteric arteries. In rat superior mesenteric arteries, oleanolic acid induced simultaneous increases in NO concentration and relaxation, and these responses were inhibited by an inhibitor of NOS, asymmetric dimethyl-L-arginine (300 μM) and by the NO scavenger, oxyhaemoglobin (10 μM). Oleanolic acid-evoked NO increases were not reduced in Ca^{2+}-free solution and in the presence of an inhibitor of endoplasmic reticulum calcium-ATPase, thapsigargin (1 μM). Oleanolic acid evoked relaxation without changes in endothelial cell calcium, but decreased smooth muscle calcium in arterial segments. Oleanolic acid failed to increase calcium in HUVECs, but increased time-dependently phosphorylation of Akt kinase at $Serine^{473}$ (Akt-Ser^{473}) and eNOS at $Serine^{1177}$ (eNOS-Ser^{1177}), which was attenuated by inhibitors of phosphoinositide-3-kinase.
Conclusions and implications: This study provides direct evidence that a component of olive oil, oleanolic acid, activated endothelium-dependent release of NO and decreased smooth muscle cell calcium followed by relaxation. The oleanolic acid-evoked endothelium-derived NO release was independent of endothelial cell calcium and involved phosphoinositide-3-kinase-dependent phosphorylation of Akt-Ser^{473} followed by phosphorylation of eNOS-Ser^{1177}.
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Keywords: ACh; calcium; endothelium; nitric oxide; NO microsensor; oleanolic acid

Abbreviations: ADMA, asymmetric dimethyl-L-arginine; Akt-Ser^{473}, phosphorylation of Akt kinase at $Serine^{473}$; AMPK, AMP-activated PK; EDHF, endothelium-derived hyperpolarizing factor; eNOS, endothelial NOS; eNOS-Ser^{1177}, phosphorylation of eNOS at $Serine^{1177}$; HUVEC, human umbilical vein endothelial cell; $[Ca2+]_i$, intracellular calcium level; NO, nitric oxide; PI3K, phosphoinositide-3-kinase; PSS, physiological salt solution; Rp-CAMPS, Rp-adenosine-3′,5′-cyclic monophosphorothioate

Introduction

During the last few decades, the Mediterranean dietary pattern has been consistently associated with a lower incidence of cardiovascular diseases and cancer (Trichopoulou *et al.*, 2003; Estruch *et al.*, 2006). One of the best known and most important characteristics of the Mediterranean diet is the presence of virgin olive oil as the main source of energy from fat. It is, therefore, of particular interest to clarify whether the bioactive components in olive oil may explain a beneficial effect on cardiovascular disease of Mediterranean diet (Estruch *et al.*, 2006). In recent studies, natural triterpenoids present in olive oil and pomace olive oil (oleanolic acid and erythrodiol) were found to have vasorelaxant activity in isolated artery of both normotensive and spontaneously hypertensive rats (Rodriguez-Rodriguez *et al.*, 2004; Rodriguez-Rodriguez *et al.*,

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2006). In addition, the ingestion of pomace olive oil with a high proportion of oleanolic acid attenuated the endothelial dysfunction associated with hypertension (Rodriguez-Rodriguez *et al*., 2007). These findings are further supported by earlier studies revealing significant antioxidant and anti-inflammatory properties attributed to oleanolic acid and structurally related natural triterpenoids (see Herrera *et al*., 2006). Nevertheless, the mechanism underlying these vasoprotective properties of oleanolic acid remains poorly understood, although oleanolic acid relaxation seems to be mediated by endothelium-derived factors, mainly nitric oxide (NO) (Rodriguez-Rodriguez *et al*., 2004; Rodriguez-Rodriguez *et al*., 2006).

Endothelial NO is produced by endothelial NOS (eNOS). This key enzyme in cardiovascular homeostasis is classically activated by agonists such as ACh, histamine and bradykinin that increase intracellular calcium levels ($[Ca^{2+}]_i$) by means of calcium/calmodulin-dependent modulation (Moncada, 1997). Over the past 5–10 years, however, a far more complex model of eNOS regulation has emerged involving post-translational mechanisms such as protein–protein interactions and tightly regulated multisite phosphorylation. Overall, Ser^{1177} is the most extensively studied and appears to be the most important of the regulatory eNOS phosphorylation sites (Fleming and Busse, 2003). Phosphorylation of eNOS at $Serine^{1177}$ (eNOS-Ser^{1177}) is associated with an increase in activation and NO production in response to a growing list of stimuli including mechanical factors, such as shear stress (Boo and Jo, 2003), and humoral factors, such as insulin (Montagnani *et al*., 2001), even at resting levels of calcium (Montagnani *et al*., 2001; McCabe *et al*., 2000). Oestrogen, mechanical shear stress and inhibitors of tyrosine phosphatase such as vanadate have all been demonstrated to cause eNOS-Ser^{1177} activation and NO release by calcium-independent mechanisms (Corson *et al*., 1996; Russell *et al*., 2000; Papapetropoulos *et al*., 2004). eNOS-Ser^{1177} can be phosphorylated by several PKs including Akt/PKB, PKA and AMP-activated PK (AMPK) (Dimmeler *et al*., 1999; Fulton *et al*., 1999; Boo and Jo, 2003).

In this study, we have examined the involvement of NO in oleanolic acid-evoked relaxation in different-sized arteries from the rat mesenteric vascular bed. The role of endothelial cell calcium and phosphorylation of eNOS in the formation of NO elicited by this natural triterpenoid was evaluated. The results derived from this investigation revealed that oleanolic acid induces vasorelaxation in rat superior and small mesenteric arteries through increases in NO release. In addition, we established that the mechanisms underlying these responses are strongly related to eNOS-Ser^{1177} and Akt kinase at $Serine^{473}$ (Akt-Ser^{473}) phosphorylation in human endothelium in response to oleanolic acid in a calcium-independent manner.

Materials and methods

Vasorelaxation studies in rat superior and small mesenteric arteries

All animal procedures conformed to the Guide for the Care and Use of Laboratory Animals (US National Institutes of Health, Publications No. 85–23, 1996). Twelve- to sixteen-week-old male Wistar rats were killed by cervical dislocation followed by exsanguination. Segments from the proximal part between the aorta and the first branch of the superior mesenteric artery and third-order small mesenteric resistance arteries (internal diameter: 900–1100 and 150–250 μm, respectively) were dissected in cold physiological salt solution (PSS) (composition in mM: 119 NaCl, 25 $NaHCO_3$, 4.7 KCl, 1.18 KH_2PO_4, 1.17 $MgSO_4$, 1.6 $CaCl_2$, 0.026 EDTA and 5.5 glucose). Segments (length 2 mm) were mounted in a wire myograph (model 410A, JP-Trading I/S, Denmark) for isometric tension recording as described previously (Simonsen *et al*., 1999). The solution was gassed with 5% CO2 in air to maintain pH at 7.4. Drugs were added to the myograph bath and mixed by steady and continuous bubbling. In some segments, the endothelium was removed by passing a human scalp hair through the vessel lumen (Simonsen *et al*., 1999). After equilibration, contractility and endothelial function was tested by the addition of noradrenaline (0.5–5 μM), and when the contraction was stable, ACh (10 μM) was added. Thereafter, concentration–response curves for oleanolic acid (0.01–100 μM) were constructed by cumulative addition of the triterpenoid in both superior and small mesenteric arteries contracted with noradrenaline (0.5–5 μM). Cumulative addition of the vehicle, dimethyl sulphoxide, had no significant effect on vascular tone. To investigate the contribution of endothelium-derived factors, the same experiment was conducted after 30 min incubation with the NOS inhibitor, N^G-asymmetric dimethyl-L-arginine (ADMA, 300 μM), the cyclooxygenase inhibitor, indomethacin (3 μM), ADMA plus indomethacin or ADMA plus indomethacin and a combination of blockers of calcium-activated K channels, charybdotoxin (0.1 μM) and apamin (0.3 μM).

Simultaneous measurements of relaxation and NO release in rat superior mesenteric artery

Arterial NO release was detected by a NO-sensitive microsensor with an outer diameter of 30–60 μm (ISO-NOP30; World Precision Instruments, Stevenage, UK). After the calibration by using NO solution in PSS, the microelectrode was introduced into the lumen of the superior mesenteric arterial segment mounted in a wire myograph, as described previously (Simonsen *et al*., 1999). A working potential of 865 mV was applied to the electrode. To test selectivity of the electrodes, a lack of response to sodium nitrite ($NaNO_2$) up to 3 μM was regarded as evidence for an intact coating of the sensor (Simonsen *et al*., 1999; Wadsworth *et al*., 2006). Electrodes were discarded if the experimental concentrations of noradrenaline, oleanolic acid or vehicle in the absence of vascular tissue increased electrode current. The microelectrodes measure changes in small currents (pA), and therefore only microelectrodes with drift below 3 nM over 10 min were accepted for this study. In noradrenaline (0.5 μM)-contracted arteries, NO release and isometric tension were measured in response to oleanolic acid (3–30 μM) and ACh (0.1–10 μM) in the absence and presence of the NOS inhibitor ADMA (300 μM, 30 min) and/or the NO scavenger oxyhaemoglobin (10 μM, 5 min). In previous studies, we have observed that the NOS inhibitor, N^G-nitro-L-arginine, releases NO, which

can interfere with the measurements of NO concentration (Simonsen *et al*., 1999), but ADMA does not have the inconvenience of increasing basal NO levels (Stankevicius *et al*., 2006).

To investigate the role of calcium, a first response in NO detection was obtained to oleanolic acid and ACh in non-contracted vessels in normal PSS (1.6 mM Ca^{2+}). The bath solution was changed for Ca^{2+}-free PSS containing EGTA (0.1 mM), and after 5 min increasing concentrations of oleanolic acid or ACh were added. Also, in EGTA containing Ca^{2+}-free PSS, NO responses were recorded after incubation for 15 min with an inhibitor of endoplasmic reticulum Ca^{2+}-ATPase thapsigargin (1 μM).

Simultaneous measurements of $[Ca^{2+}]_i$ and relaxation in rat superior mesenteric artery

For measurements of endothelial cell calcium, a segment of rat superior mesenteric artery was everted (intimal surface outside lumen), as described earlier (Stankevicius *et al*., 2006), and mounted in a myograph bath, which was placed on an inverted microscope equipped for dual excitation wavelength fluorescence microfluorimetry (Carl Zeiss, Gottingen, Germany). During arterial contraction movement, artefacts may affect fluorescence measurements, and this is avoided by use of a ratiometric fluorophore such as fura-2. The everted vessel was loaded with fura-2 acetoxymethyl ester (6.5 μM) for 2 h. To load the endothelial layer selectively, the experiments were performed at room temperature (Stankevicius *et al*., 2006).

For measurements of smooth muscle cell calcium, vessel segments were mounted in normal configuration and loaded with fura-2 acetoxymethyl ester (8 μM) and 0.1% Cremophor EL for 3 h at 37 °C, as described previously (Stankevicius *et al*., 2006). The non-ionic detergent Cremophor EL was used to increase solubility of the dye in smooth muscle cells (Nilsson *et al*., 1998).

After loading with fura-2, the bath solution was changed and the vessel was contracted by noradrenaline (0.5 μM), followed by the addition of ACh (10 μM), oleanolic acid (30 μM) or vehicle. At 10 s intervals, the vessel was excited through 340 ± 5 and 380 ± 5 nm filters, and emitted light was collected by a photomultiplier through a 500–530 nm band-pass filter. Emission intensities at the two wavelengths (F_{340} and F_{380}) and force were sampled at intervals of 0.1–10 s as required by computer running software (Felix, Photon Technology International, USA) (Stankevicius *et al*., 2006). Changes in fluorescence were expressed as the ratio F_{340}/F_{380}.

Measurements of calcium and phosphorylation of eNOS and Akt in human umbilical vein endothelial cells

This part of the investigation conformed to the principles outlined in the Declaration of Helsinki and was approved by the local Ethics Committee of Aarhus (permission 20040154). Pregnant women attending routine antenatal care in the period November 2004 to April 2006 at the Department of Obstetrics and Gynaecology, Aarhus University Hospital, Skejby, were invited to participate in the study. Endothelial cells were isolated from segments of human umbilical cord veins ($n = 20$) by collagenase digestion as described previously (Hansen *et al*., 2004) and cultured in endothelial cell basal medium-2 (PromoCell, Heidelberg, Germany) with 2% fetal calf serum and growth supplement (PromoCell, Heidelberg, Germany). Cells were plated out in gelatine-coated flasks or dishes and allowed to adhere overnight. Growth medium was changed every second day, and cells were passaged with 0.25% trypsin when confluent. In all experiments, cells were used at passages 2 or 3. The endothelial cell phenotype was confirmed using phase-contrast microscopy (cobblestone-appearing monolayer of cells) and positive immunofluorescence staining with antibodies against von Willebrand factor.

To measure endothelial cell calcium, HUVECs were loaded with fura-2 acetoxymethyl ester (4 μM) in endothelial cell basal medium-2 for 30 min at 37 °C. Cells were mounted on a thermostated stage (37 °C) (Warner Instruments, MA, USA) of a Zeiss Axiovert microscope and perfused with PSS. Cells were excited by a Deltaram lamp and images were captured (emission intensities F_{340} and F_{380}) by an intensified CCD camera operated using the IC300 digital imaging system (Photon Technology International, Birmingham, NJ, USA). Changes in $[Ca^{2+}]_i$ were expressed as the ratio F_{340}/F_{380}. Endothelial cells were perfused with PSS containing increasing concentrations of oleanolic acid (3, 10 and 30 μM) or vehicle. Autofluorescence was estimated by the addition of 1 μM histamine and 2 mM Mn^{2+}.

For immunoblotting, HUVECs (2–3 passages) were treated at 37 °C with vehicle, wortmannin (100 nM, 30 min; a phosphoinositide-3-kinase (PI3K) inhibitor), LY-294,002 (10 μM, 30 min; a PI3K inhibitor), iodotubercidin (30 μM, 30 min; an AMPK inhibitor) or Rp-adenosine-3′,5′-cyclic monophosphorothioate (Rp-cAMPS, 10 μM, 30 min; a PKA inhibitor), and then stimulated for 30 s to 10 min with oleanolic acid (30 μM), using histamine (1 μM, 5 min) as a positive control. Dimethyl sulphoxide (vehicle) was routinely used as a control. After treatment, cells were washed with ice-cold phosphate-buffered saline and lysed with lysis buffer containing ortho-vanadate (2 mM), phenylmethylsulphonyl fluoride (40 μg mL^{-1}) and protease inhibitors (104 mM 4-(2-aminoethyl)-benzenesulphonylfluoride hydrochloride, 1.5 mM pepstatin A, 1.4 mM E-64, 4 mM bestatin, 2 mM leupeptin and 0.08 aprotinin) (Sigma, St Louis, MO, USA). Proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes and then probed with specific antibodies: anti-eNOS (1:2500, Abcam, Cambridge, UK), anti-phospho-eNOS-Ser^{1177} (1:500), anti-phospho-Akt-Ser^{473} (1:500), anti-Akt (1:1000) (Cell Signaling Technology, MA, USA) and then with the secondary anti-rabbit antibody (1:4000, Zymed Laboratories, San Francisco, CA, USA) conjugated to horseradish peroxidase. Blots were detected by enhanced chemiluminescence system (ECL Plus, Amersham International plc, Little Chalford, Buckinghamshire, UK). Differences in protein abundance were determined by densitometry.

To reprobe western blots with alternative primary antibody, the polyvinylidene difluoride membranes were incubated at 50 °C for 30 min in a buffer containing: Tris-HCl (67.5 mM, pH 6.8), β-mercaptoethanol (100 mM) and SDS (2%). After extensive washing, the membranes were

incubated in blocking buffer and subsequently with the primary antibody.

To investigate phosphorylation of eNOS-Ser^{1177} in rat superior mesenteric artery, preparations were mounted for isometric tension recordings, and after adding oleanolic acid (30 μM, 5 min) to noradrenaline (0.5 μM)-contracted vessels, the arteries were kept at −70 °C until being processed for immunoblotting. Arterial homogenization and immunoblotting for Ser^{1177}-eNOS phosphorylation was performed and the rest of the procedure was carried out as described above for HUVECs.

Data analysis

Results were expressed as means ± s.e.mean of *n* number of rats. Differences between means were analysed using ANOVA followed by Bonferroni's *t*-test or unpaired *t*-test, where appropriate. Relaxations evoked in noradrenaline-contracted preparations are expressed as percentage of the contraction just before addition of the agonist. A value of $P<0.05$ was considered statistically significant.

Drugs

The following drugs were used: oleanolic acid and caulophyllogenin (Extrasynthese, Genay, France), ACh hydrochloride, noradrenaline, indomethacin, ADMA, thapsigargin, histamine, iodotubercidin, LY-294,002, oxyhaemoglobin, Cremophor EL (Sigma, St Louis, MO, USA), fura-2/acetoxymethyl ester (Molecular Probes, Eugene, OR, USA), wortmannin (Tocris, Bristol, UK), Rp-cAMPS (Calbiochem, San Diego, CA, USA). Oleanolic acid, caulophyllogenin and wortmannin were dissolved in dimethyl sulphoxide, whereas thapsigargin was dissolved in ethanol and indomethacin in $NaHCO_3$ (0.5% w/v). All other drugs were prepared in double-distilled water and kept at −20 °C until use. Oxyhaemoglobin and NO solutions were prepared as described previously (Simonsen *et al.*, 1999).

Results

Oleanolic acid-induced vasorelaxation in rat superior and small mesenteric arteries

Noradrenaline (0.5 and 5 μM) evoked sustained contractions in superior and small mesenteric arteries, respectively, and addition of oleanolic acid (0.01–100 μM) induced concentration-dependent relaxations in both preparations (Figures 1a and b). Thus, oleanolic acid-evoked maximal relaxations were, respectively, 54 ± 7% ($n=6$) and 52 ± 5% ($n=8$) in superior and small mesenteric arteries with endothelium. The structurally related compound caulophyllogenin (0.01–100 μM) did not change tone in noradrenaline-contracted superior mesenteric arteries, suggesting a specific action of oleanolic acid ($n=5$, results not shown). Oleanolic acid relaxation was significantly reduced in arterial segments without endothelium (Figures 1a and c). Incubation with the cyclooxygenase inhibitor, indomethacin (3 μM), failed to modify oleanolic acid relaxation in superior and mesenteric small arteries (Figures 1a and c). In the presence of the NOS inhibitor, ADMA (300 μM), relaxation induced by oleanolic acid in both superior and mesenteric small arteries with

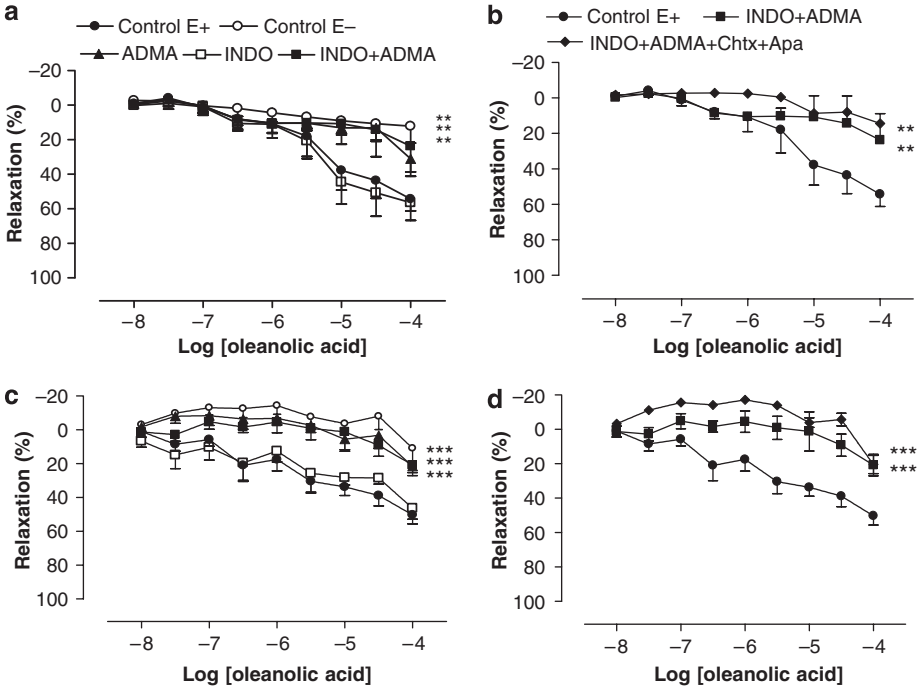

Figure 1 Concentration–response curves for oleanolic acid in rat superior (**a**, **b**) and small mesenteric arterial segments (**c**, **d**) contracted with noradrenaline (0.5–5 μM). Curves were obtained in arteries with endothelium in the absence (Control E+) or the presence of ADMA (300 μM), indomethacin (INDO, 3 μM), ADMA plus indomethacin, ADMA and indomethacin plus blockers of calcium-activated K channels, charybdotoxin (Chtx; 0.1 μM) and apamin (Apa; 0.3 μM), or in arteries without endothelium (Control E−). The values shown are means ± s.e.mean of 6–8 experiments. $^{**}P<0.01$, $^{***}P<0.001$ versus Control E+ (ANOVA and *post hoc t*-test with Bonferroni correction).

endothelium was reduced to the same extent as in arteries without endothelium (Figures 1a and c). Simultaneous incubation of the vessels with ADMA plus indomethacin yielded identical results as in the presence of ADMA alone (Figures 1a and c). In the presence of ADMA plus indomethacin, additional incubation with blockers of calcium-activated K channels, charybdotoxin (0.1 μM) and apamin (0.3 μM), had no additional effects on relaxation caused by adding oleanolic acid in rat superior and small mesenteric arteries (Figures 1b and d). These data indicate that relaxation of both rat superior and mesenteric small arteries caused by oleanolic acid are mediated by endothelium-derived NO. The following experiments were designed to further evaluate this observation in both arterial preparations and endothelial cell cultures.

Oleanolic acid evokes simultaneous increases in NO and vasorelaxation in rat superior mesenteric artery

To further investigate whether oleanolic acid leads to vasodilatation of superior mesenteric artery by simultaneous increments in endothelial NO release, a NO microsensor was introduced into the lumen of the arterial segment allowing real-time recording of variations in NO concentration and force. In noradrenaline-contracted intact superior mesenteric arteries, oleanolic acid (3–30 μM) evoked concentration-dependent relaxations and increases in NO concentration (Figures 2a and c). In segments without endothelium, oleanolic acid failed to induce any effect on relaxation and NO concentration (Figures 2a and c). In paired experiments, ACh (0.1–10 μM) elicited simultaneous dilatation and NO increases in preparations with endothelium (Figures 2b and d), whereas both effects were inhibited by endothelial cell removal (Figures 2b and d). Even though oleanolic acid induced relaxation lesser than ACh in intact arteries, the increases in endothelial NO release were virtually similar (Figure 2). In the presence of an inhibitor of NOS, ADMA, increases in NO elicited by both oleanolic acid and ACh were significantly attenuated (Figure 2). However, transient increases in NO concentrations were still observed and a residual ACh-evoked relaxation was evident after NOS inhibition with ADMA (Figure 2). Oleanolic acid (30 μM) increased NO by 10.4 ± 1.8 nM (range 5.5–15.5 nM; $n = 6$), and addition of the NO scavenger oxyhaemoglobin (10 μM) abolished the response and even lowered NO concentration to −6.2 ± 0.9 nM ($P < 0.05$, $n = 6$), that is, below baseline levels. ACh (0.3 μM) increased the NO concentration, respectively, 6.4 ± 2.2 and 2.0 ± 0.7 nM in the absence and presence of ADMA. Oxyhaemoglobin also reversed and lowered the NO concentration, respectively, −11 ± 2.9 ($P < 0.05$, $n = 6$) and −7.2 ± 1.9 nM ($P < 0.05$, $n = 6$), in the absence and presence of ADMA.

Calcium and oleanolic acid-evoked increases in NO concentration

As NO synthesis in endothelial cells is classically dependent on calcium, we determined whether NO increases elicited by oleanolic acid were equally affected by the removal of extracellular calcium. Data revealed that arterial NO release

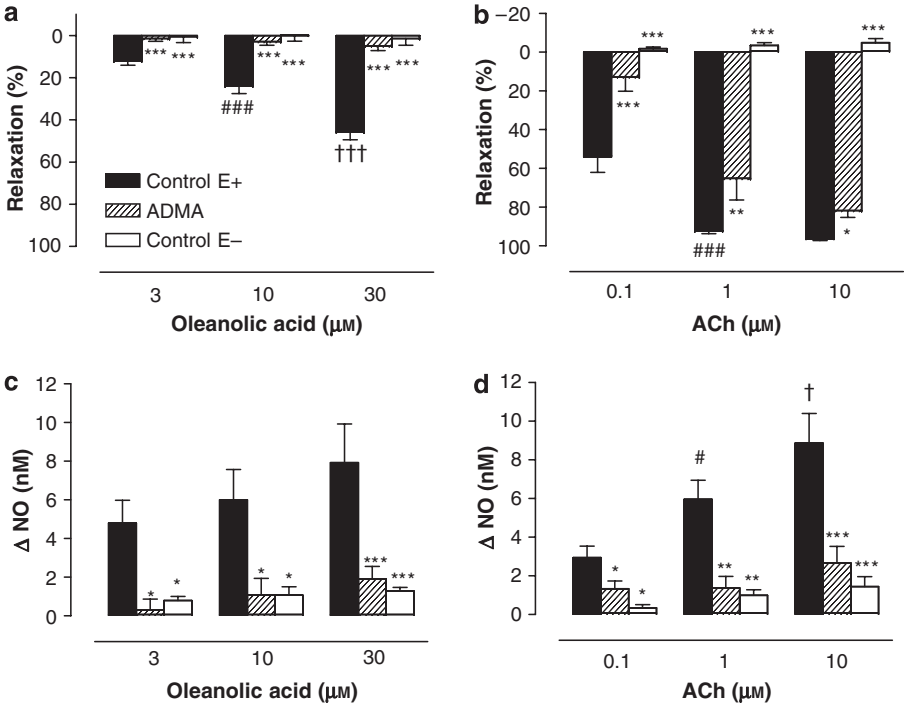

Figure 2 Average simultaneous relaxation (**a**, **b**) and increases in NO concentration (Δ[NO], nM) (**c**, **d**) induced by oleanolic acid (left panels) or Acetylcholine (ACh, 10μM) (right panels) in rat superior mesenteric artery in the absence (control E+) or presence of the NOS inhibitor, ADMA (300 μM), and in segments without endothelium (control E−). Relaxations are expressed as percentage of noradrenaline contraction. The columns are means ± s.e.mean of 5–10 experiments. Differences in responses were evaluated by two-way ANOVA: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus control E+; $^{\dagger}P < 0.05$, $^{\dagger\dagger\dagger}P < 0.001$ versus 10 μM oleanolic acid (**a**, **c**) or 1 μM ACh (**b**, **d**); $^{\#}P < 0.05$, $^{\#\#\#}P < 0.05$ versus 3 μM oleanolic acid (**a**, **c**) or 10 μM ACh (**b**, **d**).

induced by oleanolic acid reached similar levels either in the presence of calcium or in calcium-free PSS medium in non-contracted arterial segments. After incubation with thapsigargin (1 μM) in a calcium-free medium, increments in NO evoked by 30 μM oleanolic acid were even higher than those under control conditions in normal PSS (Figure 3). In contrast, ACh (10 μM) did not alter NO concentration in calcium-free PSS medium and in thapsigargin-treated preparations without extracellular calcium (Figure 3).

In another set of experiments, ACh (10 μM) evoked simultaneous relaxation and endothelial cell calcium increases in noradrenaline (0.5 μM)-contracted everted superior mesenteric artery kept at room temperature, whereas oleanolic acid (30 μM) elicited relaxation without changes in endothelial cell calcium (Figures 4a and b). Addition of noradrenaline induced a minor change in endothelial cell calcium (increase ratio: 0.02 ± 0.003, $n = 8$) (Figure 4b). Similar responses to ACh and oleanolic acid in endothelial cell calcium were observed at resting tension (data not shown). In segments with endothelium mounted in normal configuration and loaded at 37 °C, noradrenaline evoked a pronounced increase in smooth muscle calcium, which was lowered by ACh and oleanolic acid (Figure 4c).

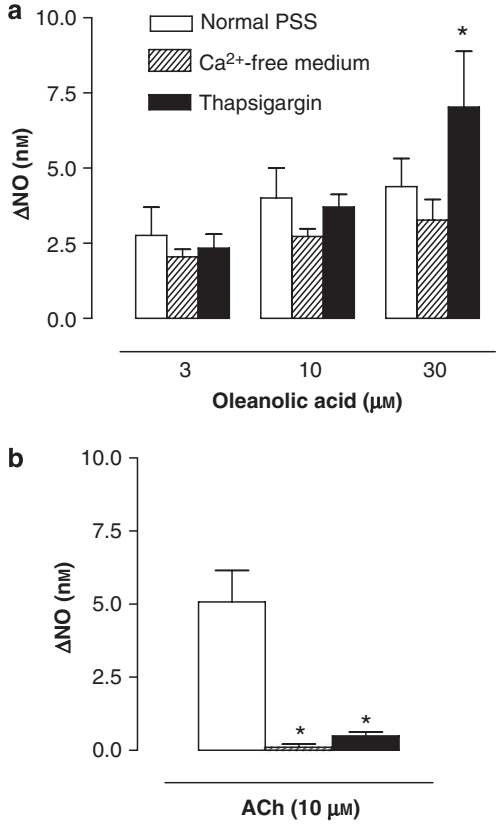

Figure 3 Average increases in NO concentration measured in rat superior mesenteric arterial segments at baseline tension after application of (**a**) increasing concentrations of oleanolic acid and (**b**) Acetylcholine (ACh, 10μM) in normal PSS (1.6 mM Ca^{2+}, $n = 6$), in Ca^{2+}-free medium containing EGTA (0.1 mM) ($n = 6$) and after treatment with 1 μM thapsigargin in Ca^{2+}-free medium ($n = 4$). *$P < 0.05$ versus normal PSS (ANOVA and *post hoc t*-test with Bonferroni correction).

Finally, increasing concentrations of oleanolic acid (3–30 μM) in HUVECs failed to evoke any increase in $[Ca^{2+}]_i$ (data not shown), whereas the subsequent application of histamine (1 μM) raised the fluorescence intensity (Δratio: 0.59 ± 0.02, $n = 6$).

Effect of oleanolic acid on eNOS phosphorylation

Phosphorylation of eNOS at Ser^{1177} plays a key role in the regulation of eNOS, and activation of eNOS-Ser^{1177} has been associated with calcium-independent responses to several stimuli. Having shown the lack of participation of calcium in arterial relaxation and NO releases induced by oleanolic acid, as well as the inability of the triterpenoid to evoke calcium increases in HUVECs, we evaluated for the first time the phosphorylation of eNOS-Ser^{1177} caused by adding oleanolic acid in arterial segments and endothelial cell cultures. In noradrenaline-contracted rat superior mesenteric arterial rings, oleanolic acid (30 μM) increased eNOS-Ser^{1177} phosphorylation (Figure 5a). In another set of experiments, stimulation of HUVECs with oleanolic acid (30 μM) also elicited a transient increase in phosphorylation of eNOS-Ser^{1177}, which peaked within 1 min and remained elevated at 5 min (Figure 5b). An identical result was obtained after 5 min incubation with histamine (1 μM) (Figure 5b). To detect possible kinases involved in eNOS-Ser^{1177} activation, HUVECs were incubated with different inhibitors before addition of oleanolic acid (30 μM). In the presence of an inhibitor of PI3K (the upstream mediator for Akt activation), wortmannin, oleanolic acid-evoked eNOS-Ser^{1177} phosphorylation at 5 min was reduced by $46 \pm 13\%$ (% of maximal response to oleanolic acid in the absence of inhibitor) ($n = 5$) (Figure 6a), whereas an inhibitor of AMPK, iodotubercidin, reduced oleanolic acid-evoked eNOS-Ser^{1177} phosphorylation by $40 \pm 12\%$ (% of maximal response to oleanolic acid in the absence of inhibitor) ($n = 5$) (Figure 6a). In contrast, PKA inhibition by Rp-cAMPS was unable to modify oleanolic acid-stimulation in eNOS-Ser^{1177} (Figure 6a). To further support the evidence that oleanolic acid induced phosphorylation of eNOS-Ser^{1177} through PI3K/Akt, the effect of oleanolic acid on Akt-Ser^{473} was investigated. Oleanolic acid caused a rapid phosphorylation of Akt-Ser^{473}, which was detected after 0.5–1 min (Figure 6b). A lack of phosphorylation on Akt-Ser^{473} following oleanolic acid was shown in the presence of the PI3K inhibitors, wortmannin and LY-294,002 (Figure 6b).

To address whether PI3K was also involved in NO releases elicited by oleanolic acid in intact segments from the rat superior mesenteric artery, preparations were incubated with wortmannin (100 nM), which markedly reduced oleanolic acid-evoked NO release and relaxation (Figures 7a and c). The presence of the PI3K inhibitor inhibited arterial NO concentration and relaxations induced by low ACh concentrations (Figures 7b and d), whereas *S*-nitro-*N*-acetylpenicillamine relaxations were unaltered in the presence of wortmannin ($n = 4$, results not shown).

Discussion

Cardioprotection associated with olive oil intake has been well documented by clinical and epidemiological studies

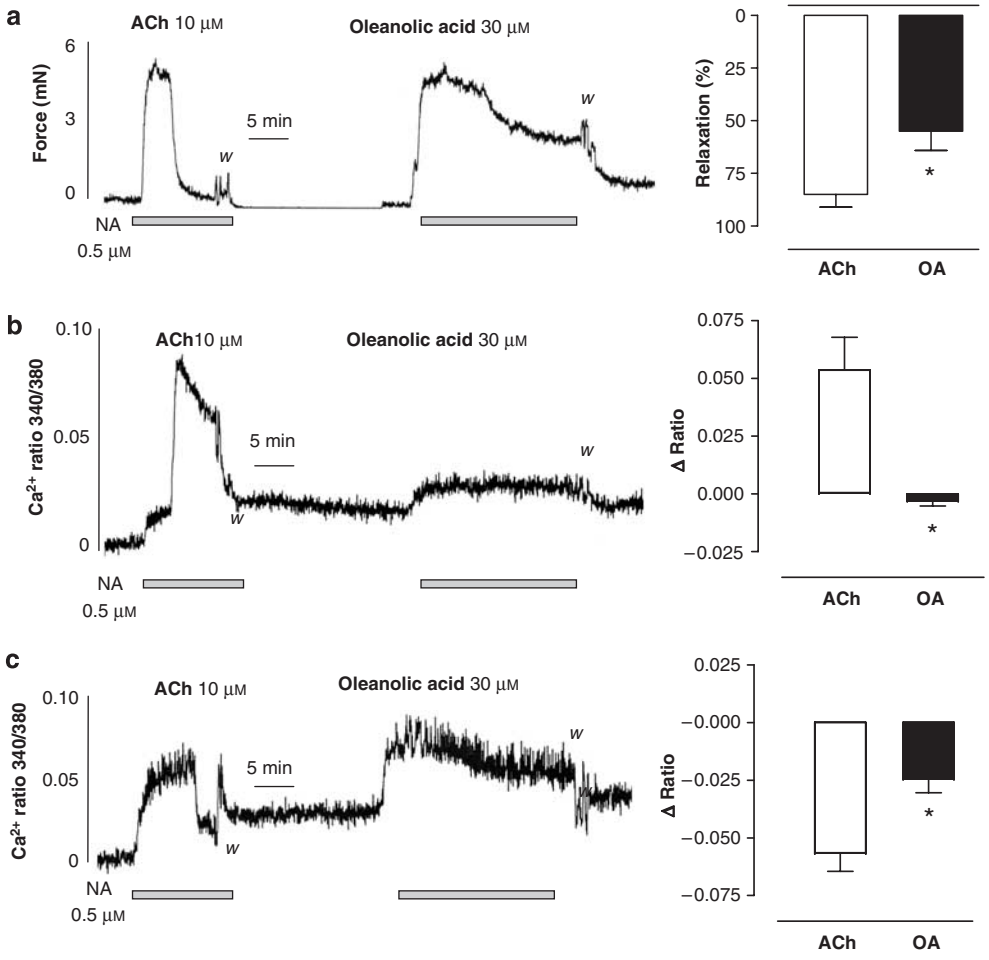

Figure 4 Simultaneous measurements of endothelial cell calcium and force in rat superior mesenteric artery. Original trace recordings (left panels) and average (right panels) relaxation and changes in Fura-2 fluorescence expressed as increases in ratio (Δ ratio) of emission intensities at the two excitation wavelengths (340 versus 380 nm). Vasorelaxation (**a**) and increases in endothelial cell calcium (**b**) recorded for acetylcholine (ACh, 10 μM) and oleanolic acid (30 μM) in everted intact superior mesenteric arterial segments contracted by noradrenaline (NA, 0.5 μM) ($n=8$). (**c**) Smooth muscle calcium measurements recorded for ACh (ACh, 10 μM) and oleanolic acid (30 μM) in intact superior mesenteric arterial segment mounted in normal configuration and contracted by noradrenaline (NA, 0.5 μM) ($n=4$). *$P<0.05$ versus ACh response (unpaired *t*-test).

(Serra-Majem *et al*., 2006), but it is still unclear which mechanisms and specific olive oil constituents contribute to this protective effect. In previous reports, we have found that the main triterpenoid in olive oil and olive residues, oleanolic acid, elicits endothelium-dependent vasorelaxation in isolated aorta of both normal and hypertensive rats (Rodriguez-Rodriguez *et al*., 2004; Rodriguez-Rodriguez *et al*., 2006). In the present investigation, we report for the first time that: (1) oleanolic acid evokes endothelium-dependent vasorelaxation in different-sized arteries of the rat mesenteric arterial bed; (2) the profile of vasorelaxation to oleanolic acid was similar in both superior and small mesenteric arteries, suggesting that NO is involved in these relaxations; (3) oleanolic acid increased NO release through calcium-independent mechanisms; (4) oleanolic acid induced a rapid phosphorylation of eNOS-Ser^{1177} and Akt-Ser^{473} in HUVECs. These findings led us to suggest that oleanolic acid evokes endothelium-dependent release of NO and relaxation by activation of pathways involving PI3K/Akt followed by phosphorylation of eNOS-Ser^{1177}.

Vasodilator effect of oleanolic acid in rat mesenteric arteries

The relative contribution of prostanoids, NO and endothelium-derived hyperpolarizing factor (EDHF) to endothelium-dependent vasodilatation varies among species, vascular bed and vessel size (Feletou & Vanhoutte, 2006). In rat mesenteric arteries, ACh relaxation is mainly mediated by NO in the superior mesenteric artery, whereas ACh EDHF-type relaxation is most pronounced in small mesenteric arteries (Hwa *et al*., 1994; Shimokawa *et al*., 1996). Incubation with the inhibitor of cyclooxygenase, indomethacin, did not modify relaxation to oleanolic acid in this study, a finding similar to our previous observations in rat aorta (Rodriguez-Rodriguez *et al*., 2004). These findings suggest that prostanoids are not involved in oleanolic acid relaxation. In contrast, incubation with an inhibitor of NOS, ADMA, caused pronounced inhibition of oleanolic acid-induced relaxation. The latter findings suggest that NO is involved in relaxation to oleanolic acid in both rat superior and small mesenteric arteries.

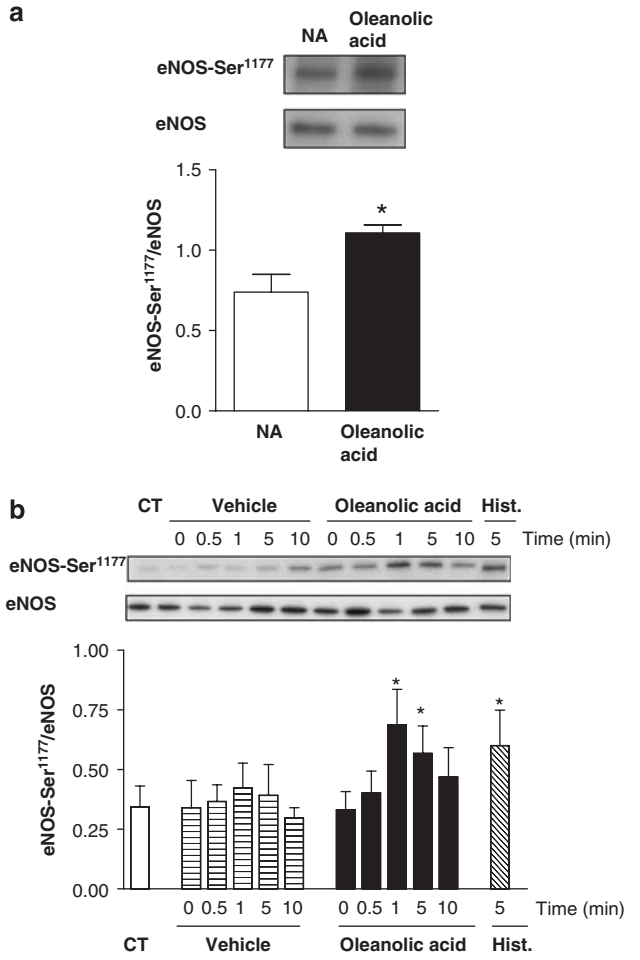

Figure 5 Representative western blots showing phosphorylation of Ser^{1177}-eNOS in (**a**) rat mesenteric arterial segments (of six animals) and (**b**) the time course of the oleanolic acid-induced phosphorylation of Ser^{1177}-eNOS and total eNOS in the presence of vehicle, oleanolic acid (30 μM, 0.5–10 min) and histamine (Hist., 1 μM, 5 min) in HUVECs. Phosphorylation was quantified densitometrically as the ratio of eNOS-Ser^{1177}/ eNOS ($n=3$ separate experiments). In the time course for oleanolic acid-evoked phosphorylation, differences in responses were evaluated by two-way ANOVA. *$P<0.05$ versus control (vehicle 0 min).

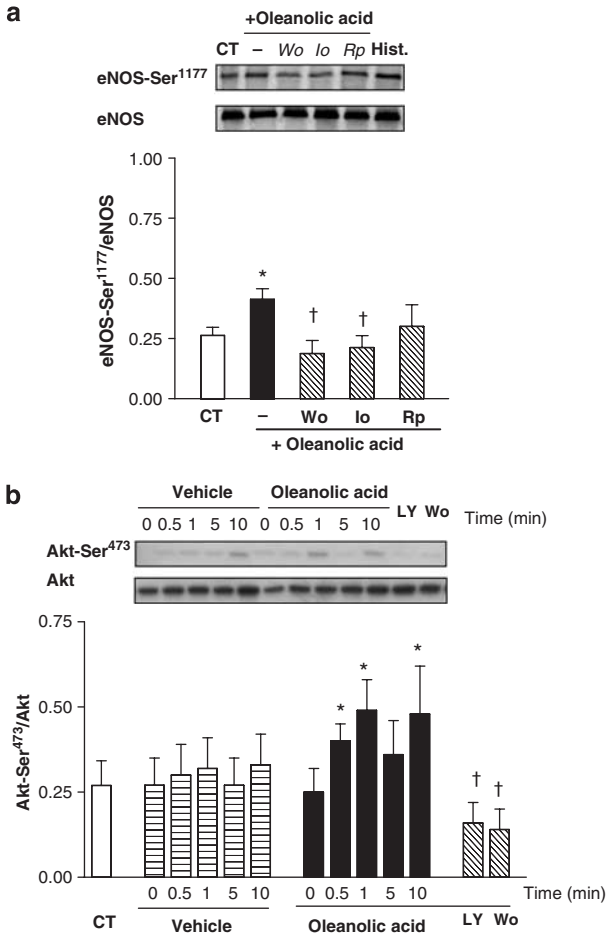

Figure 6 Effects of kinase inhibitors on oleanolic acid-evoked phosphorylation of eNOS-Ser^{1177} and Akt-Ser^{473} in HUVECs. (**a**) Blots and average phosphorylation of eNOS-Ser^{1177} obtained after incubation with an inhibitor of phosphoinositide-3 kinase (PI3K) wortmannin (Wo, 100 nM); an inhibitor of AMPK, iodotubercidin (Io, 30 μM); an inhibitor of PKA, Rp-cAMPS (Rp, 10 μM); or vehicle followed by stimulation with oleanolic acid (30 μM) for 5 min. (**b**) Time course of the oleanolic acid-evoked phosphorylation of Akt-Ser^{473} in HUVECs. Representative western blots showing time-dependent phosphorylation of Akt-Ser^{473} and total Akt in the presence of vehicle or oleanolic acid (30 μM, 0.5–10 min) in the presence and absence of the PI3K inhibitors, LY-294,002 (LY) (10 μM, 30 min) and wortmannin (100 nM, 30 min). Phosphorylation was quantified densitometrically as the ratio of Akt-Ser^{473}/Akt ($n=5$ separate experiments). The effects of inhibitors of PI3K, LY294002 (LY) or wortmannin (Wo) were evaluated by one-way ANOVA followed by *t*-test with Bonferroni correction. *$P<0.05$ versus control (vehicle 0 min); $^{\dagger}P<0.05$ versus oleanolic acid without inhibitors.

In previous studies, we have found that in the presence of ADMA and indomethacin, the addition of a combination of blockers of calcium-activated K channels, charybdotoxin and apamin, inhibits ACh-evoked vasodilatation in rat small mesenteric arteries, suggesting that EDHF-type relaxation is involved in these responses (Thorsgaard *et al*., 2003). Moreover, these latter findings exclude short-term incubation with ADMA affects EDHF-type relaxation, as was recently proposed on the basis of the observation that prolonged incubation (48 h) of endothelial cell cultures with ADMA downregulates small conductance calcium-activated K channels (Li *et al*., 2007). However, not all kinds of endothelial cell activation lead to EDHF-type relaxation in small mesenteric arteries, as we have previously found that flow-induced vasodilatation is mainly NO-mediated (Thorsgaard *et al*., 2003; Christensen *et al*., 2007). In this study and in the presence of ADMA and indomethacin, the combination of charybdotoxin and apamin did not modify oleanolic acid relaxation. These findings indirectly suggest that, rather than EDHF, NO mediates the relaxation caused by oleanolic acid in rat superior and small mesenteric arteries, and that the endothelial signalling pathways for ACh and oleanolic acid leading to endothelium-dependent relaxations are different.

Oleanolic acid-evoked NO release

Simultaneous measurements of NO and relaxation caused by oleanolic acid in superior mesenteric arterial segments

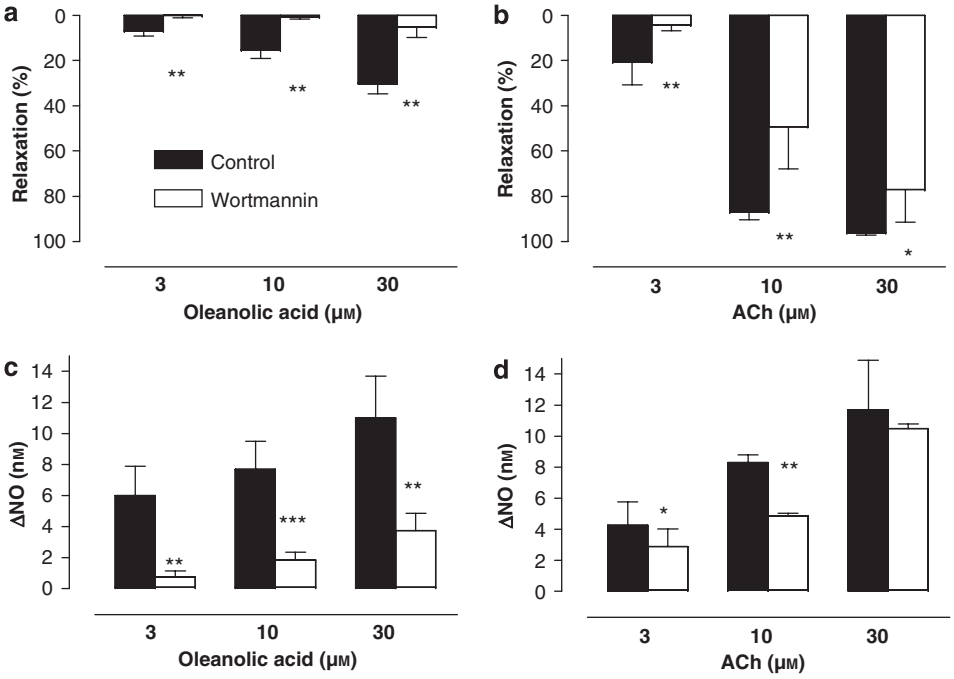

Figure 7 Average simultaneous relaxation (**a**, **b**) and increases in NO concentration (Δ[NO], nM) (**c**, **d**) induced by oleanolic acid (left panels) or acetylcholine (ACh, 10μM) (right panels) in rat superior mesenteric artery in the absence and presence of an inhibitor of phosphoinositide-3 kinase (PI3K), wortmannin (100 nM). Statistics as for Figure 2.

provided direct evidence that oleanolic acid increases the release of NO from the endothelial cell layer, substantiating previous data. This was further supported by the observation that in the presence of the NOS inhibitor, ADMA, and a NO scavenger, oxyhaemoglobin, oleanolic acid did not evoke increases in NO concentration and relaxation. ADMA caused a mild inhibition of relaxation caused by ACh compared with that caused by oleanolic acid, and these findings may suggest that relaxation caused by oleanolic acid and ACh is mediated by different endothelium-derived factors. However, in line with previous studies (Stankevicius *et al*., 2006), oxyhaemoglobin abolished the residual relaxation and increase in NO concentration induced by ACh in the presence of ADMA, hence suggesting that relaxations evoked by both oleanolic acid and ACh are NO mediated.

The magnitude of relaxation induced by oleanolic acid was markedly less than the relaxations induced by ACh even for equivalent increases in NO. NO can react with free radicals, such as superoxide, but it is an unlikely explanation for the different magnitude of relaxation induced by oleanolic acid and ACh in our model, as previous studies have revealed that formation of free radicals affects both increase in NO concentration and relaxation (Christensen *et al*., 2007), and that the microsensors do not detect peroxynitrite and superoxide (Wadsworth *et al*., 2006). NO concentration is measured with the microsensor placed in the lumen of the vascular preparation, whereas it is abluminal NO that causes relaxation. The present NO measurements do not permit us to draw a distinction between the fact that oleanolic acid and ACh are preferentially increasing luminal versus abluminal release of NO. However, the activation of eNOS at different subcellular locations has been related to different levels of calcium dependence (Fulton *et al*., 2002; Fulton *et al*., 2004), and an alternative explanation for less oleanolic acid-evoked relaxation for equivalent NO concentrations is that oleanolic acid and ACh activate different signal transduction pathways and eNOS pools.

Role of calcium in oleanolic acid-evoked NO release

Agonists, such as ACh and histamine, that activate G-protein coupled receptors increase endothelial cell calcium and lead to the release of endothelium-derived factors (Nilius and Droogmans, 2001; Stankevicius *et al*., 2006). In this study, ACh increased endothelial cell calcium, and ACh-evoked release of NO was abolished in the absence of extracellular calcium. Thapsigargin, which is an inhibitor of endoplasmic Ca^{2+}-ATPase, also inhibited increases in NO to ACh in extracellular Ca^{2+}-free medium. These results are consistent with previous studies revealing that both release of calcium from the endoplasmic reticulum and calcium influx through a calcium channel in the membrane play a role in ACh-induced increase in $[Ca^{2+}]_i$ and NO formation (Stankevicius *et al*., 2006). In contrast, in this study, oleanolic acid did not increase endothelial cell calcium in the everted superior mesenteric artery or in HUVECs, and oleanolic acid still increased NO in the absence of extracellular calcium and in the presence of thapsigargin. These findings suggest that oleanolic acid and ACh increase vascular NO concentration by different signal transduction pathways. Therefore, in contrast to ACh, oleanolic acid induces NO release involving mechanisms independent of both calcium influx and release of calcium from endoplasmic reticulum.

eNOS is localized in caveolae, and oleanolic acid is lipid soluble, and therefore it may be that oleanolic acid, through alterations in membrane fluidity, can alter activity of eNOS

and hence release of NO, without changing intracellular calcium. However, several observations suggest that the effect of oleanolic acid on cell function is specific. Thus, structurally modified analogues of oleanolic acid bind with different potency to Kelch-like-ECH-associated protein 1, a cytoplasmic repressor of the transcription factor, NRF2 (Dinkova-Kostova *et al*., 2005). The structurally related compounds, oleanolic acid, erythrodiol, maslinic acid and uvaol, which differ only in substitution of one chemical group, cause relaxations with different potency and magnitude in rat aorta (Rodriguez-Rodriguez *et al*., 2006), and caulophyllogenin, which is structurally related to oleanolic acid and differs only by two hydroxyl substitutions, failed to induce relaxation in rat mesenteric arteries. Although these findings suggest that the effects of oleanolic acid on endothelial cell function are specific, further studies are required to clarify the role of structural modifications of oleanolic acid for the release of NO and endothelium-dependent relaxation.

Role of eNOS phosphorylation in oleanolic acid-evoked NO release
eNOS is regulated by a complex series of regulatory mechanisms, including phosphorylation at key sites in response to several stimuli (Fleming and Busse, 2003). The most thoroughly studied phosphorylation site is eNOS-Ser^{1177}. A number of kinases have been reported to phosphorylate eNOS-Ser^{1177} and increase eNOS activity and NO production. Some of them, such as PKA, are activated by an increase in endothelial $[Ca^{2+}]_i$, whereas kinases such as Akt and AMPK can be activated independently of an increase in $[Ca^{2+}]_i$ and are responsible for the phenomenon previously referred to as the 'Ca^{2+}-independent activation of eNOS' (Fleming *et al*., 1998). This study suggests that calcium does not contribute to NO release induced by oleanolic acid and is further supported by the inability of the triterpenoid to evoke calcium increases in HUVECs.

Therefore, our findings of increased NO concentrations in the absence of associated increase in intracellular calcium led us to investigate whether oleanolic acid caused phosphorylation of NOS-Ser^{1177} in arterial segments and cultured endothelial cells. We found for the first time that oleanolic acid elicited a rapid phosphorylation of eNOS-Ser^{1177} in segments of the rat superior mesenteric artery and in HUVECs. Treatment of the cells with PK inhibitors established a potential role of PI3K/Akt and AMPK in eNOS phosphorylation. The lack of effect of Rp-CAMPS, an inhibitor of PKA, suggests that activation of PKA is unlikely to be involved. In contrast, when we examined the role of PI3K, an upstream mediator for Akt activation, in oleanolic acid-evoked responses, we found that the presence of the PI3K inhibitor, wortmannin, markedly decreased NO increases in rat superior mesenteric artery and eNOS-Ser^{1177} phosphorylation in HUVECs induced by oleanolic acid. Moreover, both wortmannin and LY-294,002 clearly inhibited oleanolic acid-stimulated Akt-Ser^{473} phosphorylation in endothelial cells. It is important to emphasize that, in HUVECs, oleanolic acid evoked a time-dependent phosphorylation of Akt-Ser^{473} peaking at 0.5–1 min, whereas eNOS-Ser^{1177} phosphorylation elicited by the triterpenoid peaked after 1–5 min. These findings suggest that Akt-Ser^{473} phosphorylation precedes oleanolic acid-evoked eNOS-Ser^{1177} stimulation. The inhibition of the response by iodotubercidin, however, suggests a possible involvement of AMPK, as had been demonstrated for eNOS phosphorylation caused by different stimuli (Fleming *et al*., 2003; Thors *et al*., 2004). Nevertheless, iodotubercidin is not a specific AMPK inhibitor and had been reported to inhibit other PKs including ERK2 (Bollag *et al*., 2005). Therefore, additional experiments would be necessary to establish the involvement of AMPK in oleanolic acid-evoked eNOS activation. Moreover, several recent reports demonstrated the importance of a cross-talk between AMPK and Akt for eNOS phosphorylation in response to different stimuli (Kovacic *et al*., 2003; Ouchi *et al*., 2004). In contrast, Thors *et al*. (2004) reported that eNOS phosphorylation caused by histamine was mediated by AMPK-dependent and PI3K/Akt-independent pathways. The cross-talk between Akt and AMPK is indeed relevant but controversial. Therefore, it is important to design future studies to further determine the importance of Akt and AMPK on eNOS stimulation elicited by oleanolic acid. Altogether, our present findings imply that the increase in NO concentration evoked by oleanolic acid occurs through rapid phosphorylation of eNOS-Ser^{1177} by PI3K/Akt and probably AMPK-dependent pathways.

Polyphenolic compounds from red wine were found to induce endothelium-dependent relaxation by redox-sensitive activation of the PI3K/Akt pathway in porcine coronary arteries (Ndiaye *et al*., 2004). Red wine polyphenolic compounds and oleanolic acid both lead to the activation of endothelial PI3K/Akt pathway, but in contrast to oleanolic acid, red wine polyphenolic compounds increase endothelial cell calcium, probably followed by activation of calcium-activated K channels (Martin *et al*., 2002; Ndiaye *et al*., 2004). Although we cannot exclude that the downstream signal transduction mechanisms for the PI3K/Akt pathway are different in bovine and porcine coronary endothelial cells versus rat mesenteric and HUVECs, the increase in endothelial cell calcium may explain why red wine polyphenolic compounds cause both NO- and EDHF-type relaxation, whereas oleanolic acid leads only to the release of NO.

Perspectives and conclusions
Although oleanolic acid has a moderate vasorelaxing activity compared with ACh, the clinical relevance of the NO-releasing and relaxing activity of oleanolic acid depends on systemic availability. In olive oil, the content of oleanolic acid is approximately 56 mg kg^{-1}, whereas in orujo olive oil it is 416 mg kg^{-1} (Rodriguez-Rodriguez *et al*., 2007). Recent studies reveal that after ingestion of 40 mg of oleanolic acid, plasma concentrations reached 12 ng mL^{-1} (Song *et al*., 2006), which is below the oleanolic acid concentrations causing release of NO and relaxation. However, both the anti-hypertensive effect of the substance (Somova *et al*., 2003) and the anti-atherosclerotic effect of the compound in ApoE knockout mice (Hansson and Simonsen, unpublished) suggest that the mechanisms revealed in this study are relevant for further clinical studies. Moreover, the substance

is hydrophobic and, therefore, the concentrations of oleanolic acid in intracellular compartments are probably higher.

In summary, this study provides direct evidence that a component of olive oil, oleanolic acid, caused endothelium-dependent release of NO and decreased smooth muscle cell calcium followed by relaxation. The oleanolic acid-evoked endothelium-derived NO release was independent of endothelial cell calcium and involved PI3K-dependent phosphorylation of Akt-Ser^{473} followed by phosphorylation of eNOS at Ser^{1177}.

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Conflict of interest

The authors state no conflict of interest.

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