Endothelin-1 Stimulates Small Artery VCAM-1 Expression through p38MAPK-Dependent Neutral Sphingomyelinase

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Key Words

Vascular remodelling • Inflammation • Signal transduction • Endothelial cells • Smooth muscle

Abstract

Endothelin-1 (ET-1) stimulates vascular cell adhesion molecule (VCAM-1) expression, a process associated with arterial remodelling. However, the pathways activated by ET-1 that lead to VCAM-1 expression are not fully understood. It is reported that sphingomyelinases are necessary for VCAM-1 expression in response to cytokines. Our aim was to investigate the role of sphingomyelinases in ET-1-induced VCAM-1 expression. Acid and neutral sphingomyelinase activities were measured in extracts from rat mesenteric small arteries (RMSA). ET-1 (1–100 nmol/l) stimulated neutral but not acid sphingomyelinase. The activation was rapid, peaking within 5 min and transient, returning towards baseline by 10 min and inhibited by BQ-788, GW4869 and SB203580, which are inhibitors of ET_B receptor, neutral sphingomyelinase and p38MAPK, respectively. Both GW4869 and SB203580 are reported to inhibit activation of neutral sphingomyelinase 2 implicating it in the response to ET-1. Accordingly we investigated the expression of this isoform and found it was present in RMSA, predominantly in endothelial cells. Treatment

of RMSA with ET-1 (1–100 nmol/l) for 16 h increased VCAM-1 expression, which was inhibited by GW4869 and SB203580. These results indicate that ET-1 stimulates arterial VCAM-1 expression through p38MAPK-dependent activation of neutral sphingomyelinases. This suggests a role for sphingolipids in ET-1-induced vascular inflammation in cardiovascular disease.

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Introduction

Vascular inflammation is associated with the remodelling of small arteries [1], a process involved in the development of hypertension, atherosclerosis and vascular complications of diabetes [1, 2], and there is now considerable evidence linking endothelin-1 (ET-1) to resistance artery inflammation and remodelling [3–6]. ET-1 is a potent vasoconstrictor [7], but it is also pro-inflammatory [6], inducing vascular cell adhesion molecule (VCAM-1) expression, leukocyte adherence to endothelial cells, smooth muscle cell proliferation and arterial remodelling in vitro [8–11] and in vivo [4, 5, 12, 13]. Furthermore, high plasma ET-1 levels correlate with raised soluble VCAM-1 levels in the blood of hypertensive patients and are asso-

ciated with end organ damage [14, 15]. Endothelin-1 mediates its effects through two G protein-coupled receptors, ET_A and ET_B. ET_A receptors on smooth muscle cells mediate contraction and proliferation, whilst ET_B receptors on endothelial cells exert opposing effects – antiproliferation of smooth muscle cells and vasodilation. The ET_B receptor is also expressed on smooth muscle cells and plays an important role in the clearance of endothelins, further counteracting the vasoconstrictor actions of these peptides [reviewed in 2]. ET-1 receptor stimulation leads to the activation of multiple signalling pathways including mitogen-activated protein kinases (MAPK) [16] that are implicated in inflammation [17].

MAPKs are serine/threonine kinases that regulate many cellular functions including adhesion, proliferation and collagen deposition [18]. This suggests they would play a role in vascular remodelling and indeed their activation is linked to hypertension [19, 20]. p38MAPKs are a family of four isoforms (α , β , δ , γ) activated by cellular stress, growth factors, cytokines and G protein-coupled receptor agonists. In cells they are implicated in the regulation of diverse processes including nuclear and cytosolic events and their *in vivo* functions include maintenance of tissue homeostasis and inflammation [21]. Pathologically, p38MAPKs are implicated in cancer, cardiac, neurodegenerative and inflammatory diseases [21]. Increased p38MAPK activity is reported in arteries and vascular smooth muscle cells from animal models of cardiovascular disease [22, 23] and activation of p38MAPKs in vascular tissue induces production of inflammatory markers in the arterial wall [24]. Although ET-1 is a potent activator of p38MAPK in vascular cells and tissues [25], the involvement of this pathway in pro-inflammatory effects is not clear.

Recently, p38MAPK was shown to be upstream of neutral sphingomyelinase 2 (nSMase2) in epithelial cells [26, 27]. Sphingomyelinases (SMase; classed as acid, alkali and neutral) hydrolyse sphingomyelin (SM) to form ceramide, the precursor of sphingosine and sphingosine-1-phosphate (S1P). These are the main bioactive sphingolipids [28] with multiple roles in the cardiovascular system including maintenance of vascular tone, vascular inflammation and atherosclerosis [29, 30]. Neutral sphingomyelinases (N-SMase) are redox sensitive enzymes activated during oxidative stress and by pro-inflammatory cytokines such as tumour necrosis factor- α (TNF α) [31]. nSMase2 is expressed in endothelial and smooth muscle cells and regulates contractility [32], TNF α -induced endothelial nitric oxide synthase (eNOS) activity [33] and VCAM-1 expression [26, 34]. Furthermore, ET-1 aug-

ments TNF α -induced VCAM-1 expression in endothelial cells [9] and stimulates sphingomyelinase activity in neural tissue [35]. Collectively, these studies suggest that SMase may be involved in the signalling pathway leading to vascular inflammation in response to ET-1.

In this study we have investigated whether N-SMase activation is required for ET-1-induced VCAM-1 expression in rat mesenteric small arteries (RMSA) and the role of p38MAPK in its regulation. We show that ET-1 activates N-SMase but not acid SMase, that an ET_B receptor antagonist inhibited N-SMase activation and that nSMase2 is expressed in both endothelial cells and within the artery wall. Inhibition of p38MAPK blocked ET-1 N-SMase activation and VCAM-1 expression, as did N-SMase activation alone. These findings indicate that activation of N-SMase is important for ET-1-induced inflammation in vascular tissue and suggests a role for bioactive sphingolipids in small artery remodelling in cardiovascular disease.

Methods

The investigation was carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), The University of Manchester Animal Experimentation Guidelines and the UK Animals (Scientific Procedures) Act 1986. Experiments were performed with the approval of the Review Board of the University of Manchester and the Home Office.

Animals and Incubation Conditions

Adult Sprague-Dawley rats (8–10 weeks of age, body weight 180–220 g) were used for all experiments. The mesentery was excised and placed in ice-cold physiological salt solution [36]. Mesenteric small arteries (internal diameter <400 μ m) were cleaned of adherent fat and connective tissue, and dissected from the mesenteric bed. Unless stated otherwise, arteries were equilibrated in 1 ml of tissue culture media M199 (Invitrogen, UK) for 1 h at 37°C before stimulation with ET-1 (1–100 nmol/l) for various time points. For inhibitor studies GW4869 10 μ mol/l (N-SMase inhibitor [37]), BQ-123 1 μ mol/l (ET_A receptor inhibitor [38]), BQ-788 2 μ mol/l (ET_B receptor inhibitor [39]), SB203580 1 μ mol/l and SB202474 1 μ mol/l (p38MAPK inhibitor and negative control, respectively [40]), or vehicle (DMSO 0.01%) were added 30 min before ET-1 stimulation. The inhibitors were used at concentrations known to be effective in rat small arteries [32, 36].

Tissue Homogenate Preparation

Arteries were homogenized in Tris-TX-100 buffer (25 mmol/l Tris pH 7.4, 5 mmol/l EDTA, 0.2% Triton X-100, protease inhibitors (Complete mini-tab, Roche), 1 mmol/l sodium orthovanadate, 200 μ mol/l sodium pyrophosphate) on ice. The homogenate was incubated at 4°C for 30 min, centrifuged at 800 g for 10 min at 4°C to pellet nuclei and cell debris, and the protein concentra-

tion of the supernatant was determined by Bradford assay. The sample volume was adjusted with Tris-TX-100 buffer to a final concentration of 1 mg/ml. An aliquot was removed for sphingomyelinase assay and to the remainder Laemmli sample buffer was added and the sample stored at -20°C .

In vitro Sphingomyelinase Assays

Sphingomyelinase activity was measured in freshly prepared homogenates using NBD-C6-SM [41]. For neutral sphingomyelinase activity, tissue homogenate (25 μg protein) was added to 100 μl of reaction mixture containing: 100 mmol/l Tris pH 7.4, 10 mmol/l MgCl_2 , 0.2% Triton X-100, 10 mmol/l dithiothreitol, 100 $\mu\text{mol/l}$ NBD-C6-SM and 100 $\mu\text{mol/l}$ phosphatidylserine [26]. For acid sphingomyelinase activity, tissue homogenate (25 μg protein) was added to 100 μl of reaction mix containing: 0.25 mol/l sodium-acetate pH 5.0, 1 mmol/l EDTA, 0.1% Triton X-100 and 100 $\mu\text{mol/l}$ NBD-C6-SM [41]. Following 30 min incubation at 37°C , reactions were terminated by the addition of 1 ml chloroform:methanol (2:1 v:v) and 200 μl dH_2O for phase separation. The upper aqueous phase was discarded and the lower organic phase dried under O_2 -free N_2 gas and resuspended in 15 μl chloroform:methanol (2:1 v:v). Samples and NBD-C6-ceramide standard were spotted onto heat-activated silica gel 60 thin-layer chromatography plates and developed in chloroform:methanol/10% NH_4OH (7/3/0.5 v/v/v). The plates were air-dried and the fluorescent lipid detected using an Alpha-Innotech Imager. NBD-C6-ceramide was identified by the co-chromatographed standard and quantified by densitometry using AlphaEaseFC software.

Immunoblotting

Equivalent amounts of tissue homogenates were subjected to SDS-PAGE and processed for Western blot analysis with the appropriate primary antibody. Signals were developed by horseradish peroxidase-conjugated secondary antibody and chemiluminescence (Pierce, UK). Where phospho-heat shock protein 27 (Hsp27) antibody was used, membranes were stripped and re-probed with Hsp27 antibody. Membranes for probing with VCAM-1 antibody were sliced horizontally at 50 kDa, the upper portion was probed with anti-VCAM-1 and the lower portion with anti-GAPDH as a loading control. Band intensities were measured using densitometry (BioRad GS800 Densitometer) and the phospho-antigen signal was corrected for total antigen. Images showing saturation were not used in analysis.

Immunohistochemistry and Immunofluorescence

Immunohistochemistry was performed on paraffin-embedded 10- μm sections of RMSA. The sections were incubated with nSMase2 antibody (1:50), washed and incubated with biotin-conjugated secondary antibody followed by streptavidin horseradish peroxidase (HRP). The signal was developed with nickel-enhanced 3,3'-diaminobenzidine (Ni-DAB). The negative controls were incubated with rabbit IgG (Santa Cruz) in place of the primary antibody. Immunofluorescence was performed on 5- μm sections of RMSA. The sections were co-incubated with nSMase2 (H-195) 1:50 and α -smooth muscle actin (Clone 1A4) 1:800 or nSMase2 (I-16) 1:50 and von Willebrand factor 1:800 antibodies overnight at 4°C . Negative controls were co-incubated with rabbit and mouse IgG or goat and mouse IgG (Santa Cruz) at the same concentrations as the corresponding primary antibody. The sections were then incubated with the appropriate Alexa Fluor-488-

or Rhodamine Red X-conjugated secondary antibodies and Hoechst nuclear stain. The sections were observed and photographed using a Leica CTR5000 microscope.

VCAM-1 Expression

Dissected arteries were placed in 3-ml L15 medium containing vehicle 0.01% DMSO or ET-1 1, 10 or 100 nmol/l and incubated for 16 h at 37°C . Following incubation the tissue was removed and processed for immunoblot analysis as described above. For experiments with N-SMase or p38MAPK inhibition, GW4869 10 $\mu\text{mol/l}$, SB203580 1 $\mu\text{mol/l}$, SB202474 1 $\mu\text{mol/l}$ or vehicle 0.01% DMSO were added 30 min before incubation with ET-1 and maintained in the solution throughout the 16-hour incubation period.

Data Analysis

Data are expressed as means $\pm$ SEM and the number of individual animals used for each experiment is given in the figure legends. Statistical analysis was performed by one-way ANOVA followed by Bonferroni's post hoc test using GraphPad Prism software and $p < 0.05$ was considered statistically significant.

Materials

GW4869 and Ni-DAB were purchased from Sigma (UK), BQ-788, BQ-123, SB203580, SB202474 and ET-1 were from Calbiochem (UK). The antibodies used were: rabbit polyclonal anti-phospho-Hsp27(Ser82) and anti-Hsp27 polyclonal (Upstate Biotechnology, Lake Placid, N.Y., USA), nSMase2 (H-195) rabbit polyclonal, nSMase2 (I-16) goat polyclonal, VCAM-1 (H-276; Santa Cruz Biotechnology, Santa Cruz, Calif., USA), GAPDH polyclonal (AbCam, UK), α -smooth muscle Actin Clone 1A4 (Sigma) and von Willebrand factor (DAKO, Denmark). HRP-, biotin- and fluorescent-conjugated secondary antibodies and streptavidin HRP were from Jackson Laboratories (West Grove, Pa., USA). NBD-C6-SM and NBD-C6-ceramide were from Molecular Probes (Invitrogen) and salts and chemicals were from Sigma.

Results

Effect of ET-1 on Sphingomyelinase Activity in RMSA

Both neutral and acid sphingomyelinase activity was detected in RMSA lysates, with N-SMase activity approximately 8-fold greater than acid (N-SMase activity 36 ± 3 , A-SMase activity 5 ± 3 pmol/mg protein/min, $n = 3$). Treatment with ET-1 (100 nmol/l) increased N-SMase activity, which was significant at 5 min and returned towards basal at 10 min (fig. 1a). A lower concentration of ET-1 (1 nmol/l) also increased N-SMase activity at 5 min (fig. 1b). Acid sphingomyelinase activity was not increased by ET-1 (100 nmol/l) up to 10-min stimulation (fig. 1a).

ET-1 Activates N-SMase through the ET_B Receptor

ET_A and ET_B receptor inhibitors were used to investigate which receptor coupled ET-1 to N-SMase. Treatment with BQ-123 (1 $\mu\text{mol/l}$), an ET_A receptor inhibitor [38],

Fig. 1. ET-1 activates N- but not A-SMase in RMSAs. **a** Time course of sphingomyelinase activation following ET-1 (100 nmol/l) measured in tissue lysates under neutral or acid conditions. Basal SMase activity was: N-SMase 36 ± 3 , A-SMase 5 ± 3 pmol/mg protein/min, $n = 3$. **b** Effect of 1 and 100 nmol/l ET-1 for 5 min on N-SMase activity. N-SMase activity was measured in tissue lysates by the conversion of NBD-C6-SM to NBD-C6-ceramide. Data are mean $\pm$ SEM from 9 (**a**) and 4 (**b**) separate experiments, expressed as % control where control activity was normalized to 100%, * $p < 0.05$ versus basal (0), ANOVA with Bonferroni's post hoc test.

Fig. 2. ET-1 activates N-SMase through the ET_B receptor and inhibition of p38MAPK prevents ET-1 activation of N-SMase. **a** Effects of ET_A (BQ-123 1 μ mol/l) and ET_B (BQ-788 2 μ mol/l) receptor antagonists on ET-1 (100 nmol/l, 5 min) induced N-SMase activity. **b** RMSA were treated with SB202474 (1 μ mol/l) or SB203580 (1 μ mol/l) for 30 min before stimulation with ET-1 (100 nmol/l 5 min), and N-SMase activity measured in tissue lysates by the conversion of NBD-C6-SM to NBD-C6-ceramide. Data are mean $\pm$ SEM from 4 (**a**) and 5 (**b**) separate experiments, expressed as % control where control activity was normalized to 100%. * $p < 0.05$ vs. ET-1 (**a**), * $p < 0.05$ SB203580 vs. SB202474 (**b**), ANOVA with Bonferroni's post hoc test.

had no effect on ET-1 (100 nmol/l) activation of N-SMase. However, the ET_B receptor inhibitor BQ-788 (2 μ mol/l) [39] blocked the response (fig. 2a).

Effects of p38MAPK Inhibition on N-SMase Activity

Neutral sphingomyelinases are commonly activated in response to cellular stress [31]. In RMSA ET-1 activates the stress-activated protein kinase p38MAPK leading to phosphorylation of Hsp27 [36] and in epithelial cells overexpressed nSMase2 is downstream of p38MAPK [26, 27]. Accordingly, we investigated whether p38MAPK was responsible for endogenous N-SMase activation in RMSA. p38MAPK inhibition with SB203580 (1 μ mol/l) [36] completely blocked N-SMase activation by ET-1 (fig. 2b). The activation of N-SMase by ET-1 was not affected by SB202474, a negative control for SB203580 (fig. 2b). Ad-

ditionally, p38MAPK-dependent Hsp27 phosphorylation by ET-1 [36] was inhibited by SB203580 but not SB202474 (p-Hsp27; SB202474 100%, SB202474 + ET-1 $215 \pm 25\%$, SB203580 + ET-1 $69 \pm 14\%$, $n = 5$).

nSMase2 Is Expressed in RMSA

nSMase2 is regulated by p38MAPK. This suggests that the ET-1-induced N-SMase activity may be due to nSMase2. Immunoblot analysis of RMSA homogenates with nSMase2 antibody showed the presence of a single immunoreactive band at approximately 66 kDa, which is slightly lower than the predicted molecular weight of 70 kDa exhibited by the human recombinant nSMase2 positive control (fig. 3a). To identify the cell type in which nSMase2 was expressed paraffin-embedded RMSA sections were stained with nSMase2 antibody. Immuno-

Fig. 3. Immunodetection of nSMase2 in RMSAs. **a** Postnuclear small-artery homogenates and lysate from cells overexpressing human recombinant nSMase2 (positive control) were probed with a polyclonal nSMase2 antibody. The immunoblot shows from left to right: molecular weight markers, small-artery homogenates from 4 separate animals and cell-positive lysate. **b** Cross sections of paraffin-embedded RMSA were probed with rabbit IgG (left-hand panel) or nSMase2 (H-195) rabbit polyclonal antibody (right-hand panel) and the immunoreactive signal devel-

oped with Ni-DAB giving black colouration. Cross sections of paraffin-embedded RMSA were probed with α -smooth muscle actin (α -sma) and nSMase2 (H-195) rabbit polyclonal antibody (**c**) or von Willebrand factor (vWF) and nSMase2 (I-16) goat polyclonal antibody (**d**). Upper panels: individual antibody immunoreactivity. Lower panels: merged images with Hoechst staining of nuclei. Arrows indicate endothelial cells and yellow colouration shows overlap of immunoreactivity. Scale bar = 20 μ m. The figure is representative of 4 similar experiments.

staining was present throughout the artery wall with the most intense staining observed in endothelial cells (fig. 3b). To confirm cell type staining small artery sections were double stained with nSMase2 and α -smooth muscle actin or von Willebrand factor, which are smooth muscle and endothelial cell markers, respectively (fig. 3c, d). Again, immunoreactivity was seen throughout the artery wall, which appeared most intense around the

lumen. A similar staining pattern was seen with both nSMase2 antibodies. These signals overlapped with α -smooth muscle actin and von Willebrand factor immunoreactivity indicating they originated from both smooth muscle and endothelial cells. No staining was seen in the negative controls (data not shown). GW4869 is an N-SMase inhibitor reported to inhibit nSMase2 activity in vitro [37] and in vascular tissue [32]. Incubation of RMSA

Fig. 4. N-SMase activity is inhibited by GW4869. RMSA were incubated with vehicle (0.01% DMSO) or GW4869 (10 μ mol/l) for 30 min before stimulation with ET-1 (100 nmol/l 5 min). N-SMase activity was measured in tissue lysates by the conversion of NBD-C6-SM to NBD-C6-ceramide (**a**) and phosphorylation of Hsp27 at serine82 measured by immunoblot (**b**). A representative immunoblot and densitometric data are shown. Data are mean $\pm$ SEM from 5 (**a**) and 9 (**b**) separate experiments, expressed as % control where control activity was normalized to 100%. * p < 0.05 vs. control, + p < 0.05 vs. GW4869, ANOVA with Bonferroni's post hoc test.

Fig. 5. ET-1 stimulates VCAM-1 expression that is prevented by p38MAPK and N-SMase inhibition. RMSA were incubated overnight in the presence of vehicle (0.1% dH₂O) or ET-1 100 nmol/l (**a**), ET-1 1, 10 or 100 nmol/l (**b**), SB202474 (1 μ mol/l) or SB203580 (1 μ mol/l) for 30 min before ET-1 100 nmol/l (**c**), or vehicle (0.01% DMSO) or GW4869 (10 μ mol/l) for 30 min before ET-1 100 nmol/l (**d**). Tissue lysates were prepared and immunoblotted for VCAM-1 and GAPDH as the loading control. **a** Immunoblot showing VCAM-1 expression from 2 control- and 2 ET-1-treated samples using arterial lysate from individual animals. **b, c, d** Representative immunoblots and densitometric data of VCAM-1 expression corrected for loading. Data are mean $\pm$ SEM from 4 separate experiments, expressed as % control where SB202474 or vehicle expression was normalized to 100%. * p < 0.05 vs. SB202474 (**c**) or vehicle (**d**), ANOVA with Bonferroni's post hoc test.

with 10 $\mu\text{mol/l}$ GW4869, a concentration that inhibited N-SMase activity in pulmonary arteries [32], had no effect on basal N-SMase activity but inhibited ET-1-stimulated activity (fig. 4a). GW4869 did not inhibit ET-1-induced HSP27 phosphorylation (fig. 4b) indicating its effect on N-SMase activation was not due to off-target inhibition of the p38MAPK pathway. Collectively, these data suggest nSMase2 is activated by ET-1 in RMSA.

ET-1 Stimulates RMSA VCAM-1 Expression through p38MAPK/N-SMase

In RMSA treatment with ET-1 for 16 h increased VCAM-1 expression (fig. 5a). The effect was concentration dependent from 1–100 nmol/l (fig. 5b). Inhibition of p38MAPK with SB203580 suppressed ET-1-induced VCAM-1 expression when compared to the negative control SB202474 (fig. 5c). In addition, inhibition of N-SMase with GW4869 also prevented the increase in VCAM-1 expression induced by ET-1 (fig. 5d). The increase in VCAM-1 expression in response to ET-1 (100 nmol/l) was less in the inhibitor studies (fig. 5c, d) than in the concentration experiments (fig. 5b). This possibly reflects the presence of DMSO as a vehicle for the inhibitors, which has been shown to be anti-inflammatory, and responsible for decreasing VCAM-1 expression [42] and neutrophil adhesion to endothelial cells [43].

Discussion

ET-1 is involved in cardiovascular disease through the induction of endothelial dysfunction and vascular inflammation, identifying it as a candidate target for therapeutic purposes in diseases such as hypertension, atherosclerosis, heart failure and vascular complications of diabetes. To date ET-1 receptor antagonists have been trialed for treatment of heart failure but despite promising results from animal studies the outlook from clinical trials has been disappointing [16]. This may in part reflect a lack of selectivity of the antagonists used for individual endothelin receptors [44] but it also highlights the need for further studies investigating the mechanisms of action of ET-1. In this study we show in intact vascular tissue that ET-1 activates N-SMase through a p38MAPK-dependent pathway and that this is required for the subsequent induction of vascular inflammation by this agonist. Although N-SMase has been implicated in the pro-inflammatory effects of cytokines this is the first evidence that sphingolipids are important mediators of ET-1-induced vascular dysfunction.

Even though both A-SMase and N-SMase activity were detected in RMSA, ET-1 selectively activated the latter. N-SMase activation by ET-1 was concentration dependent and transient, with peak activity at 5 min. Transient and a similar magnitude activation of endogenous N-SMase has been reported in response to cytokines in endothelial [34], epithelial [26] and liver cells [45]. Furthermore, this transient activation of N-SMase in turn regulated eNOS activity [34], VCAM-1 expression [26, 34] and Jun kinase phosphorylation [45]. Accordingly, it is likely that the transient activation of N-SMase by ET-1 has important functional consequences in vascular tissue. We used RMSA in this study because they undergo remodelling in hypertension and ET-1 is implicated in this process in animal models [1, 4, 12] and humans [46]. N-SMase stimulation was seen with 1 and 100 nmol/l ET-1. Whilst these concentrations are greater than reported plasma levels (0.15–1.5 pmol/l), there is evidence that tissue ET-1 content and plasma levels are not directly comparable. For instance, in DOCA-salt hypertensive rats the plasma levels of ET-1 are reported as 0.8 pmol/l but tissue levels vary between aorta, carotid and mesenteric arteries: 0.5, 1.6 and 4–5 fmol/mg tissue, respectively [47]. Furthermore, blood vessel ET-1 concentration is elevated approximately 2-fold in salt-sensitive hypertension with no accompanying rise in plasma levels [48, 49]. In normotensive rats ex vivo treatment of carotid artery with 1 nmol/l ET-1 elevated tissue ET levels to approximately 1.6 fmol/mg tissue weight, similar to levels seen in arteries from DOCA-salt hypertensive rats [50]. Accordingly, we believe that ET-1 activation of N-SMase in small arteries could occur in vivo and that it is a potential pathophysiological response.

To date the main physiological stimuli of N-SMase identified are cellular stress, for instance UV irradiation and cytokines such as TNF α [31], both of which are potent activators of p38MAPKs [21]. In RMSA, p38MAPK activation by ET-1 is rapid [36] and precedes the maximum N-SMase activity observed in the current study. Furthermore, p38MAPK inhibition blocked ET-1 activation of N-SMase suggesting a central role for this pathway in response to ET-1. Four N-SMase isoforms have been identified [51] and overexpressed nSMase2 was recently shown to be regulated by p38MAPK in response to cigarette smoke in human airway epithelial cells [27, 52, 53]. In addition, TNF α stimulation of overexpressed nSMase2 in lung epithelial cells was also dependent on p38MAPK [26]. In RMSA endogenous nSMase2 was expressed predominantly in endothelial cells. Given that ET-1-dependent VCAM-1 expression occurs in endothe-

lial cells in vivo [12], this suggests that nSMase2 may be the isoform involved in ET-1 responses. In pulmonary artery, nSMase2 was involved in hypoxic vasoconstriction and generation of ceramide in smooth muscle cells [32] while in cultured endothelial cells a role for nSMase2 in TNF α -induced eNOS activation and adhesion molecule expression has been reported [34]. Collectively these studies show that N-SMases and particularly nSMase2 play an important role in arterial tissue.

ET_B but not ET_A receptor inhibition blocked N-SMase activation in response to ET-1. This is in agreement with a study in neural tissue which showed the ET_B receptor was involved in ET-1 activation of SMase [35]. Given that ET_B receptors are predominantly expressed on endothelial cells this suggests that ET-1 activation of N-SMase would be occurring mainly in the endothelium. In healthy blood vessels, ET-1 induces indirect vasodilation through ET_B receptors located on the endothelium and vasoconstriction through binding to ET_A receptors on vascular smooth muscle, whereas pathological effects such as inflammation and tissue remodelling are predominantly mediated through ET_A receptor stimulation in diseased blood vessels [2]. However, there is evidence that ET_B receptors located on vascular smooth muscle cells may mediate some of the pathological effects of ET-1 [54]. In addition, two studies have shown that the ET_B receptor is important for adhesion molecule expression in endothelial and dermal cells [9, 10]. In cardiovascular disease the ET_B receptor may be upregulated [54, 55], which could lead to it playing a greater role in the pathological effects of ET-1.

To investigate whether N-SMase was involved in the pro-inflammatory effects of ET-1 we treated RMSA with 1–100 nmol/l ET-1 for 16 h, a protocol that increases VCAM-1 expression in isolated rat carotid arteries [13]. We observed a concentration-dependent increase in VCAM-1 levels that was inhibited by p38MAPK and N-SMase inhibitors. The N-SMase inhibitor did not block Hsp27 phosphorylation, a downstream effector of p38MAPK in RMSA [36], indicating that its effects were unlikely to be due to off-target interference with the p38MAPK pathway. N-SMases hydrolyse SM to produce ceramide, which can be further metabolised and phosphorylated to produce ceramide-1-phosphate (C1P) and/or sphingosine-1-phosphate (S1P) [56]. In this study we have not investigated which lipid might be important for ET-1-induced VCAM-1 expression. However, in cultured endothelial cells, TNF α stimulates nSMase2 and sphingosine kinase leading to the production of S1P, which was required for VCAM-1 expression [34]. S1P also activated

eNOS leading to NO production that acted to inhibit TNF α -stimulated adhesion molecule expression [34], suggesting that the role of S1P in vascular inflammation may depend on the level of eNOS activity and endothelial cell function. Indeed, in vascular smooth muscle cells S1P activates p38MAPK and increases VCAM-1 expression, an effect greater in cells from hypertensive than normotensive animals [23]. This raises the possibility that ET-1 activation of p38MAPK and N-SMase may lead to S1P production and adhesion molecule expression in vascular tissues. In healthy arteries this effect may be counteracted by S1P activation of eNOS and NO production. However, in hypertension when endothelial function is compromised and S1P/p38MAPK signalling augmented, VCAM-1 expression would be upregulated leading to inflammation and small artery remodelling. However, whether ET-1 activates sphingosine kinases and increases S1P production in small arteries remains to be determined. Furthermore, a role for ceramide and/or other metabolites such as C1P cannot be ruled out, particularly as C1P has been shown recently to stimulate artery remodelling, although the MAPK family members ERK1 and 2 rather than p38 were responsible for this response [57].

In summary, this study shows that ET-1 stimulates N-SMase activity in RMSA through p38MAPKs leading to VCAM-1 expression. The p38MAPK dependence of the response implicates nSMase2, which was expressed in endothelium and smooth muscle. The role of sphingolipids in ET-1 signalling warrants further study particularly in the setting of cardiovascular disease.

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