Endothelial nitric oxide synthase activity is linked to its presence at cell–cell contacts

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The enzyme endothelial nitric oxide synthase (eNOS) is essential for vascular integrity. Many studies have demonstrated a link between the localization and activity of eNOS. Here, we studied the influence of cell–cell contact on this link in the microvascular endothelial bEnd.3 cell line. By immunofluorescence microscopy, eNOS localization at the plasma membrane was found to be dependent on cell–cell contact. In particular, eNOS was highly enriched at the intercellular contact sites. Further analysis showed that the pattern of eNOS localization at the plasma membrane resembled that of PECAM-1 (platelet endothelial cell adhesion molecule 1), but not that of the adherens junction proteins VE (vascular endothelial)-cadherin and plakoglobin. eNOS that was localized at the contact sites was, in part, Triton X-100-insoluble, in contrast with eNOS at the Golgi complex, which may indicate an association of eNOS with the actin cytoskeleton. Interestingly, eNOS activity was up-regulated in confluent monolayers compared with subconfluent cells, while there was no difference in eNOS expression. This correlation between cell confluence and eNOS activity was also found when primary bovine aortic endothelial cells were studied. These data imply that cell–cell contact induces the localization of eNOS at intercellular junctions, which is required for agonist-induced eNOS activation.

Key words: endothelium, endothelial permeability, eNOS, junctions.

INTRODUCTION

The integrity of the endothelial lining of the vascular system is important for vessel function [1]. It protects the vessel wall against thrombus formation and atherogenesis. Moreover, changes in the integrity of the endothelium might facilitate transendothelial migration of inflammatory cells and intravasation of metastases [2–4]. The integrity depends on the adhesion of the endothelial cells to the underlying basement membrane, as well as on the adhesion of the cells to each other. The intercellular adhesion is accomplished predominantly by the calcium-dependent adherens junctions and, to a lesser extent, by tight junctions and gap junctions [3]. In addition to its protective properties, the adherens junctions also play an important role in cell motility, proliferation and differentiation [5]. The proteins that are mostly involved in these junctions are members of the cadherin family. Vascular endothelial cadherin (VE-cadherin) mediates the extracellular contact between the cells [6]. While its luminal part binds to the extracellular domain of a VE-cadherin molecule of a neighbouring endothelial cell, its cytosolic part is involved in the intracellular organization of the junction. This domain binds β-catenin, plakoglobin and p120, which are linked to the actin cytoskeleton via α-catenin, resulting in stabilization of the junction.

Platelet endothelial cell adhesion molecule 1 (PECAM-1; also known as CD31) is another molecule that plays a role in the intercellular adhesion of the endothelium [7]. As for the cadherins, PECAM-1 is also involved in proliferation and migration of endothelial cells [8] and in angiogenesis [9]. In contrast with cadherins, its role in intercellular adhesion is calcium-independent [10]. When endothelial cells are forming a confluent monolayer, PECAM-1 becomes localized at cell–cell contacts much later than VE-cadherin [10]. PECAM-1 does not become associated with the adherens junctions, but may bind the adherens junction component β-catenin [9], which indicates that cadherins and PECAM-1 might be linked to the actin cytoskeleton via the same intermediate protein. Whether PECAM-1 is associated with another specific subdomain of the contact sites is currently unknown.

An important characteristic of the endothelial junction is its dynamic organization. The junctions can be rapidly modulated to allow the inter-endothelial passage of macromolecules and circulating cells from the blood. Changes in the endothelial junctions can be induced by hypoxia, leucocyte adhesion, and by agents such as thrombin, bradykinin, histamine, vascular endothelial growth factor (VEGF) and inflammatory cytokines, and might occur within minutes [11,12]. Increases in endothelial permeability are often associated with increases in tyrosine phosphorylation of components of the intercellular junction, including VE-cadherin, β-catenin, plakoglobin, p120 and PECAM-1 [13, 14], and their subsequent disappearance from the cell surface [15,16]. In agreement with this observation, tyrosine phosphatase inhibitors increase endothelial permeability [17]. One of the key players that regulates acute changes in the organization of the intercellular junctions is NO. For instance, VEGF-induced increases in endothelial permeability require NO. This is evident from studies in which endothelial cells that were pre-treated with nitric oxide synthase (NOS) inhibitors had lost their ability to

Abbreviations used: BAEC, bovine aortic endothelial cells; DAF-2 DA, 4,5-diaminofluorescein-2 diacetate; (e)NOS, (endothelial) nitric oxide synthase; FCS, fetal calf serum; L-NAME, N^G-nitro-L-arginine methyl ester; PECAM-1, platelet endothelial cell adhesion molecule 1; VE-cadherin, vascular endothelial cadherin; VEGF, vascular endothelial growth factor.

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respond to VEGF [18]. Since NO plays a role in the regulation of endothelial junctions, we studied whether endothelial (e)NOS is localized at intercellular contact sites. In microvascular as well as in macrovascular endothelial cells, eNOS was enriched at contact sites. Our data indicate that eNOS, like PECAM-1, is probably not present in adherens junctions, but at other parts of the contact sites. Inter-endothelial cell–cell contact was required for proper eNOS activation. Therefore our findings suggest that eNOS needs to be localized at contact sites in order to respond to agonists.

EXPERIMENTAL

Material

The bEnd.3 cell line was generously given by Alan Schwartz (University of Washington, St Louis, MO, U.S.A.). Primary bovine aortic endothelial cells were obtained from Clonetics (Walkersville, MD, U.S.A.). A23187 and 4,5-diaminofluorescein-2 diacetate (DAF-2 DA) were purchased from Calbiochem (San Diego, CA, U.S.A.). Acetylcholine and N^{G}-nitro-L-arginine methyl ester (L-NAME) were from Sigma Chemical Co. (St Louis, MO, U.S.A.). Antibodies against eNOS, caveolin-1 and GM130 were obtained from Transduction Laboratories (San Diego, CA, U.S.A.). Polyclonal anti-caveolin antibody (#C13630) labelled caveolin-1 in caveolae, whereas monoclonal anti-(caveolin-1) antibody (#C37120) labelled caveolin-1 in the Golgi. Anti-(GOS-28) antibody was generously given by Peter van der Sluijs (UMC Utrecht, Utrecht, The Netherlands) [19]. Horseradish-peroxidase-conjugated anti-mouse antibody, as well as antibodies against VE-cadherin, plakoglobin and PECAM-1, were from Santa Cruz (Santa Cruz, CA, U.S.A.). Fluorescent secondary antibodies were purchased from Jackson Immunochemicals (West Grove, PA, U.S.A.).

Cell culture

Immortalized murine microvascular endothelial bEnd.3 cells [20] were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum (FCS) and penicillin/streptomycin, and split 1:4 to 1:8 upon reaching confluence. bEnd.3 cells expressed high levels of eNOS, a phenomenon that was probably caused by the polyoma virus middle T oncogene, which was used to immortalize the primary cells [21]. Western blot analysis demonstrated that these cells did not express a detectable amount of inducible NOS (results not shown).

Primary bovine aortic endothelial cells (BAEC) were cultured according to the manufacturer's instructions. BAEC were cultured in EGM BulletKit medium (Clonetics), split 1:6 upon reaching confluence, and used at passage 4. For immunofluorescence microscopy, cells were grown on non-coated glass coverslips. For nitrite measurements and for Western blotting, cells were grown in six-well culture dishes. For DAF-2 DA experiments, cells were grown in black clear-bottom 96-well plates (Costar; Acton, MA, U.S.A.). bEnd.3 cells were grown for 18 h in FCS-free medium supplemented with 0.1% (w/v) BSA before initiating the experiments. BAEC were not put on FCS-free medium. For experiments in which the correlation between cell confluence and NO synthesis was studied, cells were treated with trypsin at the day they reached confluence, and split as indicated in the Figures.

Immunofluorescence microscopy

Endothelial cells were immunostained by indirect fluorescent labelling. To analyse Triton X-100 solubility, cells were washed three times with ice-cold PBS, incubated with 0.2% (v/v) Triton X-100 in PBS for 10 min on ice, and then washed twice with PBS before fixation. Cells were fixed with 3% (w/v) paraformaldehyde, permeabilized with 0.1% saponin, blocked with normal serum, incubated with primary antibodies for 60 min, washed three times with PBS, incubated with FITC- and Texas Red-labelled secondary antibodies for 30 min, and then washed three times with PBS and embedded in Mowiol. Confocal laser scanning microscopy was performed using a Leica TCS 4D system. FITC- and Texas Red-labelling were examined by scanning sequentially and overlaid images, using Adobe Photoshop software. All experiments were repeated at least three times. Representative images are shown in the Figures.

Analysis of eNOS activity by nitrite measurement

The accumulation of nitrite in the medium has been used to assess eNOS activity [22,23]. bEnd.3 cells were washed with Hepes buffer [20 mM Hepes/133 mM NaCl/6.5 mM KCl/1 mM $CaCl_2$/1 mM $MgCl_2$/5.5 mM glucose/50 μM L-arginine/0.1% (w/v) BSA (pH 7.4)], and incubated in Hepes buffer with or without eNOS agonists in the absence or presence of 1 mM L-NAME for 1 h at 37 °C. The nitrite that had been released in the Hepes buffer was measured fluorimetrically (excitation wavelength 355 nm; emission wavelength 460 nm), according to the manufacturer's instructions (Cayman, Ann Arbor, MI, U.S.A.), except that all nitrite samples were mixed with nitrite assay buffer in a 1:1 ratio to increase sensitivity. eNOS activity was expressed as the L-NAME-dependent release of nitrite·mg of cellular protein^{-1}. To determine their protein content, cells were incubated with 1% (v/v) Triton X-100 in PBS for 30 min on ice, scraped and centrifuged for 15 min at 16000 g. Protein concentration in the supernatant was measured using bicinchoninic acid reagent (Pierce, Rockford, IL, U.S.A.) according to the manufacturer's instructions. Experiments were repeated three times. Within each experiment, all cell incubations were performed in duplicate. Nitrite concentration in each sample was measured in triplicate.

Analysis of eNOS activity by DAF-2 fluorescence

Intracellular NO was measured in real time using the NO-specific fluorescence probe DAF-2 DA. DAF-2 DA is able to diffuse freely across the membrane, and is hydrolysed by intracellular esterases, resulting in the formation of DAF-2. Intracellular DAF-2 reacts with the NO oxidation product N_2O_3, which generates the stable highly fluorescent derivative DAF-2 triazole [24,25]. Cells were washed with Hepes buffer, incubated with 5 μM DAF-2 DA in Hepes buffer for 30 min at room temperature, washed again with Hepes buffer and then incubated with Hepes buffer for 30 min at 37 °C in the absence or presence of 1 mM L-NAME, after which calcium ionophore A23187, acetylcholine or Hepes buffer was added to the wells. Fluorescence (emission wavelength, 485 nm; excitation wavelength, 538 nm) was measured temporally at 37 °C from 10 to 70 min after the addition of A23187, acetylcholine or Hepes buffer using the bottom-reading mode in a fluorescence microtitre plate reader (type Fluoroskan Ascent; Labsystems, Helsinki, Finland). eNOS activity was expressed as the L-NAME-dependent increase in fluorescence in 60 min·μg of cellular protein^{-1}. To determine the cellular protein content, parallel cell cultures were grown in 60 mm dishes, lysed in 1% (v/v) Triton X-100 and analysed for protein content as described above. DAF-2 DA experiments were repeated three times. Within each experiment, four wells were used for every NO measurement.

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Western blotting

Aliquots of bEnd.3 and BAEC lysates were subjected to SDS/PAGE and transferred to PVDF membranes. Blots were incubated with anti-eNOS and anti-(caveolin-1) antibodies, and subsequently with horseradish-peroxidase-conjugated anti-mouse antibody. Membrane-bound antibodies were visualized using chemiluminescence reagent (Roche, Mannheim, Germany).

RESULTS

To study the localization of eNOS at endothelial junctions, the murine microvascular endothelial cell line bEnd.3 was used [20]. To characterize this cell line in regard to eNOS activity, bEnd.3 cells were incubated with various eNOS agonists. eNOS activity was determined by measuring the L-NAME-dependent release of nitrite into the cell medium (Table 1). Calcium ionophore A23187 was used as a positive control. This agonist clearly activated eNOS in bEnd.3 cells. Acetylcholine, 5-hydroxytryptamine (serotonin), and, to a lesser extent, VEGF, also induced eNOS activation. Bradykinin and histamine had no effect.

The presence of eNOS at cell–cell contacts was studied by indirect immunofluorescence microscopy. Confluent and subconfluent bEnd.3 cells were immunolabelled with anti-eNOS and anti-(caveolin-1) antibodies and subjected to confocal laser scanning analysis (Figure 1A). In 'subconfluent' cells (i.e. cells having a limited number of cell–cell contacts), eNOS was present at a perinuclear region, determined previously as being the Golgi complex [26]. eNOS was hardly detectable at the plasma membrane. In contrast, caveolin-1 was mostly present at the plasma membrane, presumably at caveolae. Caveolin-1 staining was similar in confluent cells: it still remained at the plasma membrane. eNOS staining was clearly different in these cells compared with subconfluent cells. Its localization in the Golgi complex was unaffected, but now eNOS was also clearly visible at the plasma membrane, where it co-localized to a large extent with caveolin-1. Such an effect of cell confluence on eNOS localization was also found in primary BAEC (results not shown). To determine whether the cell–cell contact induced a general redistribution of eNOS towards the plasma membrane, or whether eNOS was specifically enriched at these cell–cell contacts, confluent bEnd.3 cells were immunolabelled with eNOS and PECAM-1 antibodies, and a top-to-bottom confocal scanning analysis was performed (Figure 1B). At the plasma membrane, PECAM-1 and eNOS overlapped to a large extent and were specifically enriched at the contact sites. In contrast with eNOS, PECAM-1 was not found at the Golgi complex.

Since eNOS was enriched at cell–cell contacts, it was likely that eNOS was present at adherens junctions. Therefore we determined by immunofluorescence microscopy whether eNOS co-localized with VE-cadherin in early confluent as well as in late confluent cell layers (Figures 2A and 2B). Surprisingly, eNOS and VE-cadherin co-localized at the plasma membrane in late confluent cells, but not in early confluent cells. Because plakoglobin associates with adherens junctions at a later stage of junction maturation than VE-cadherin [27], the overlap in the localization of eNOS and plakoglobin was studied (Figures 2C and 2D). As for VE-cadherin, plakoglobin co-localized with eNOS at the plasma membrane in late confluent cell layers, but not in early confluent cells, indicating that eNOS is not present in adherens junctions. Therefore the localization of eNOS at cell–cell contacts was compared with that of PECAM-1, which is known to be present at contact sites, but not at adherens junctions (Figures 2E and 2F). As for eNOS, PECAM-1 localized at the contact sites of late confluent cells only. PECAM-1 co-localized with eNOS at these sites to a large extent.

Contact sites are known to be enriched in proteins that anchor the actin cytoskeleton to these sites, making them insoluble in detergents such as Triton X-100. VE-cadherin and, to a lesser extent, PECAM-1 are indeed partially insoluble in Triton X-100 [10]. We then determined whether the eNOS that is present at the contact sites is insoluble in Triton X-100. Immunofluorescence microscopy was performed on cells that were extracted with Triton X-100 before fixation (Figure 3). eNOS, caveolin-1, as well as PECAM-1 were present in Triton X-100-insoluble membrane patches at the plasma membrane (Figures 3A–3D). Caveolin-1 is Triton X-100-insoluble because of the detergent insolubility of caveolae. In Triton X-100-treated cells, eNOS co-localized to a small extent with caveolin-1, and to a larger extent with PECAM-1. The use of a monoclonal antibody that specifically recognizes caveolin-1 at the Golgi enabled us to show that the caveolin-1 that is present at caveolae is detergent-insoluble, whereas that which is present at the Golgi complex is Triton X-100-soluble (Figures 3E and 3F). The loss of Golgi-associated eNOS and caveolin-1 upon Triton X-100 treatment might be explained by a complete wash-out of the Golgi complex by the detergent. Therefore detergent-treated cells were double-labelled for the Golgi 'SNARE' (soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor) GOS-28 and either eNOS or GM130 (Figures 3G–3J). While labelling of eNOS at the Golgi complex was lost upon Triton X-100 treatment, the labelling of GOS-28 as well as GM130 remained, indicating that eNOS and caveolin-1 at the Golgi were specifically solubilized by the detergent.

So far, our data have indicated that eNOS is present and enriched at cell–cell contacts, where it co-localizes with PECAM-1 in detergent-insoluble membrane domains that are different from adherens junctions. In contrast, in endothelial cells that do not make contact with neighbouring cells, eNOS is hardly found at the plasma membrane. To determine whether the presence of eNOS at these contact sites is involved in agonist-induced NO generation, confluent as well as 'sparse' bEnd.3 cells (i.e. cells having no cell–cell contacts) were incubated in the absence or presence of A23187 or acetylcholine. eNOS activity was assessed by measuring the L-NAME-dependent release of nitrite in the medium (Figure 4A). Nitrite accumulation was normalized for cellular protein content. Basal and stimulated eNOS activity was present in confluent cells, but hardly in sparse cells. Western blots showed that this difference in eNOS activity

Table 1 eNOS activation in microvascular endothelial bEnd.3 cells

Cells were incubated for 1 h at 37 °C with or without agonist in the absence or presence of 1 mM L-NAME. The amount of nitrite in the medium was determined and normalized for the amount of cellular protein. Data from three experiments are presented as means ± S.D. 'Δ' represents L-NAME-dependent (i.e. NOS-mediated) nitrite formation.

	Nitrite (nmol · mg of protein^{-1})		
Agonist	− L-NAME	+ L-NAME	Δ
None	0.31 ± 0.05	0.08 ± 0.05	0.23 ± 0.07
A23187 (5 μM)	1.44 ± 0.06	0.35 ± 0.06*	1.09 ± 0.08†
Acetylcholine (1 μM)	0.83 ± 0.12	0.06 ± 0.09	0.77 ± 0.15†
5-Hydroxytryptamine (1 μM)	0.94 ± 0.07	0.05 ± 0.05	0.88 ± 0.08†
VEGF (20 ng/ml)	0.60 ± 0.03	0.10 ± 0.03	0.50 ± 0.05†
Bradykinin (1 μM)	0.28 ± 0.06	0.04 ± 0.05	0.24 ± 0.07
Histamine (10 μM)	0.36 ± 0.04	0.08 ± 0.05	0.28 ± 0.06

* A23187 autofluorescence.

† Statistically significant effect compared with basal nitrite formation, as analysed by Student's *t* test ($P < 0.05$).

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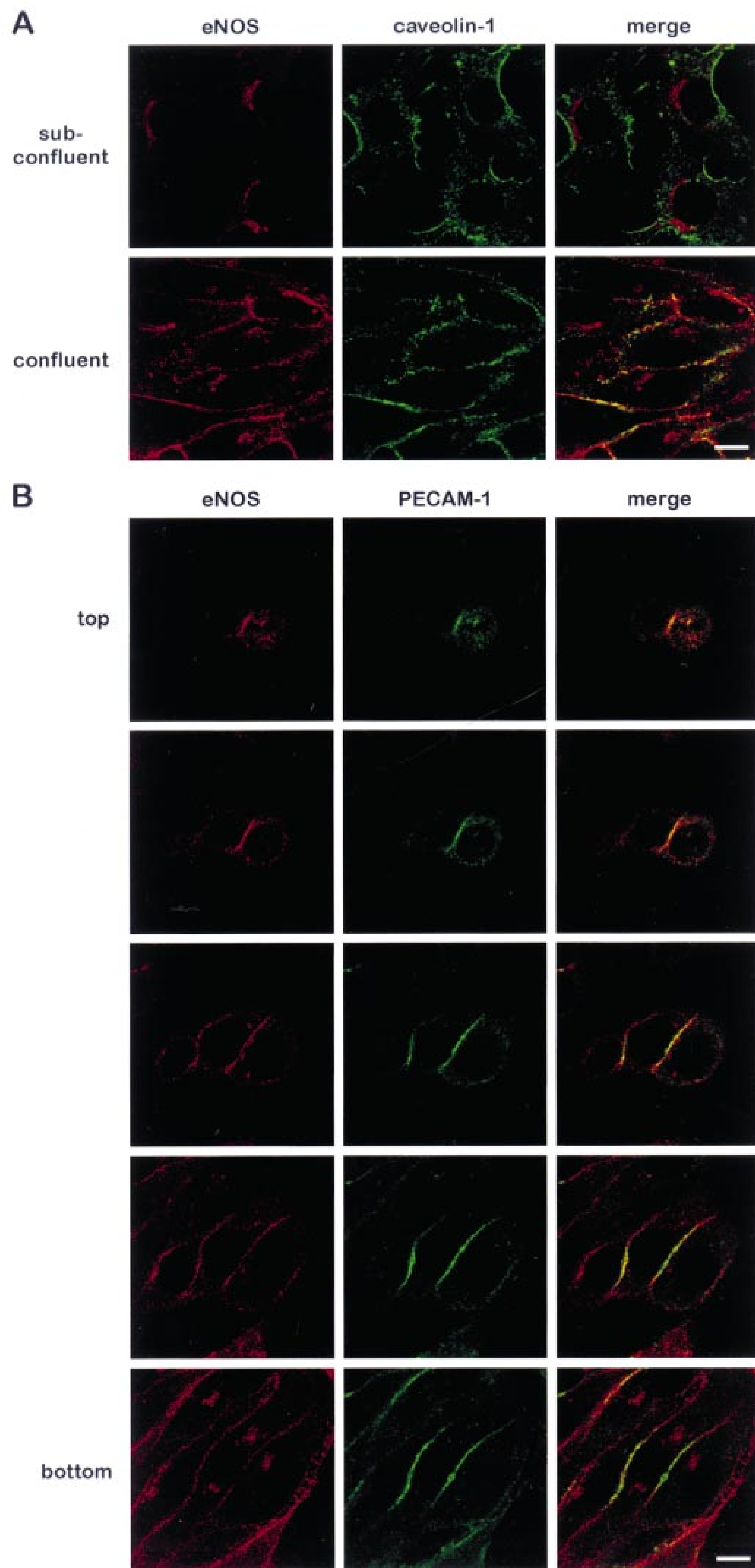

Figure 1 For legend see opposite page

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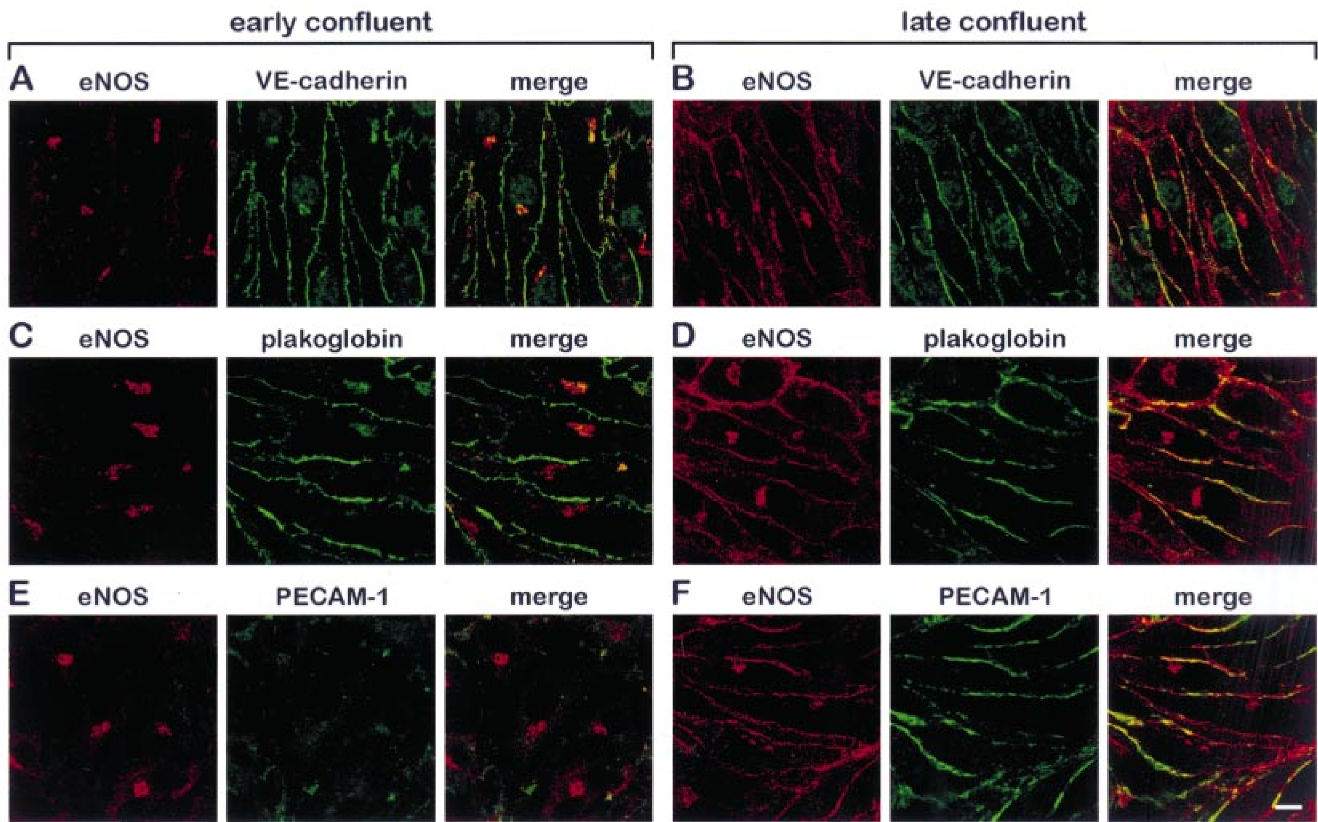

Figure 2 Co-localization of eNOS with junction proteins at the plasma membrane

Early (**A**, **C** and **E**) and late (**B**, **D** and **F**) confluent bEnd.3 monolayers were fixed, permeabilized and immunolabelled with monoclonal anti-eNOS (**A**–**F**) and polyclonal anti-(VE-cadherin) (**A** and **B**), anti-plakoglobin (**C** and **D**) and anti-(PECAM-1) (**E** and **F**) antibodies, and subsequently with Texas Red-conjugated donkey anti-mouse and FITC-conjugated donkey anti-goat antibodies. Fluorescent secondary antibodies were visualized by confocal microscopy. The bar represents 10 μm.

was not due to a difference in eNOS or caveolin-1 expression. Since these results are in contrast with data obtained by other research groups [28–30], we also evaluated eNOS activity in cell cultures of varying densities by means of DAF-2 fluorescence (Figure 4B). bEnd.3 cells were treated with trypsin and split 1:8, 1:6, 1:5, 1:4, 1:3 and 1:2. After 2 days, cells were loaded with DAF-2 DA and incubated with or without calcium ionophore A23187 or acetylcholine. NO generation was monitored by the increase in fluorescence. eNOS activity was expressed as L-NAME-dependent DAF-2 fluorescence $\cdot \mu$g of cellular protein^{-1}. Both A23187- and acetylcholine-induced eNOS activity were markedly reduced in cells that had not yet reached confluence. It is noteworthy that the decrease in eNOS activity was most dramatic in early confluent bEnd.3 cells (split 1:3) compared with late confluent cells (split 1:2). The effect of cell confluence on basal eNOS activity was less clear in these experiments. The reduced eNOS activity in subconfluent cells was not caused by differences in either eNOS or caveolin-1 expression (Figure 4C). To ascertain that the effect of cell confluence on eNOS activity was not specific for bEnd.3 cells, similar experiments were performed with BAEC. Since BAEC did not respond to acetylcholine, only A23187 was used to stimulate eNOS activity. As for the bEnd.3 cells, eNOS activation was markedly reduced in subconfluent BAEC (Figure 4D), while neither eNOS nor caveolin-1 expression was affected by the differences in cell density (Figure 4E), thereby implying a general endothelial regulatory mechanism in which eNOS is directed towards the inter-endothelial contact sites, where it might be activated by agonists.

DISCUSSION

NO plays an important role in maintaining inter-endothelial junctions. By regulating these junctions, NO determines the relative permeability of the endothelial lining of the vessel wall [18,31]. In the present study, we have shown that most of the eNOS that is localized at the plasma membrane is present at

Figure 1 Localization of eNOS at cell–cell contacts

(**A**) Subconfluent and confluent bEnd.3 cells were fixed, permeabilized and immunolabelled with monoclonal anti-eNOS and polyclonal anti-(caveolin-1) antibodies, and with Texas Red-conjugated goat anti-mouse and FITC-conjugated goat anti-rabbit antibodies. Fluorescent secondary antibodies were visualized by confocal microscopy. The bar represents 10 μM. (**B**) Confluent bEnd.3 cells were fixed, permeabilized and immunolabelled with monoclonal anti-eNOS and polyclonal anti-(PECAM-1) antibodies, and with TRITC-conjugated donkey anti-mouse and FITC-conjugated donkey anti-goat antibodies. Labelled cells were subjected to confocal microscopy. Images were collected from the top to the bottom of the cells. The bar represents 10 μm.

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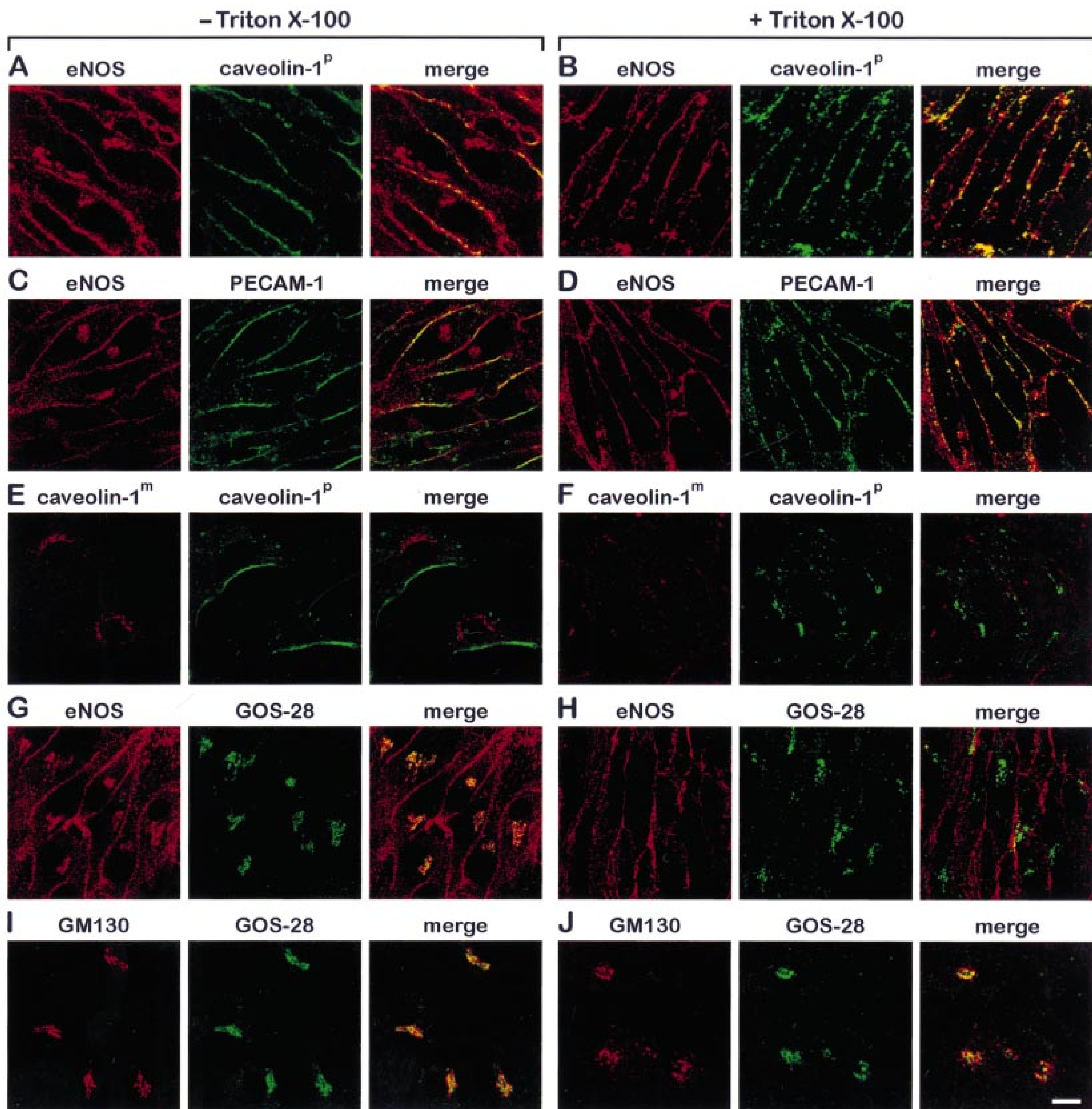

Figure 3 Effect of Triton X-100 extraction on eNOS localization

bEnd.3 cells were either fixed directly or incubated with 0.2% Triton X-100 in PBS for 10 min at 4 °C before fixation, as indicated. Cells were labelled with the indicated antibodies and with fluorescent secondary antibodies, and analysed by confocal microscopy. Caveolin-1^{p} is polyclonal anti-(caveolin-1) antibody, which recognizes caveolin-1 in caveolae; caveolin-1^{m} is monoclonal anti-(caveolin-1) antibody, which recognizes caveolin-1 in the Golgi complex. The bar represents 10 μm.

cell–cell contacts. At these contact sites, eNOS is probably not localized at adherens junctions, but at membrane domains which also contain PECAM-1, and which are at least in part insoluble in solutions containing low concentrations of the detergent Triton X-100. Furthermore, our data indicate that, in subconfluent proliferating cells that do not exhibit cell–cell contacts, eNOS is hardly activated by agonists, while in confluent quiescent cell layers eNOS is activated to a large extent by calcium-mobilizing agents. In conclusion, our data show a clear correlation between eNOS activity and its presence at cell–cell contacts, indicating that eNOS needs to be present at cell–cell contacts to become activated.

To date, eNOS localization studies have focused mainly on the presence of eNOS at the Golgi [26] and at cell-surface caveolae [32,33]. In addition, it has been reported that eNOS might be present at intercellular contact sites [34]. Nevertheless, it was

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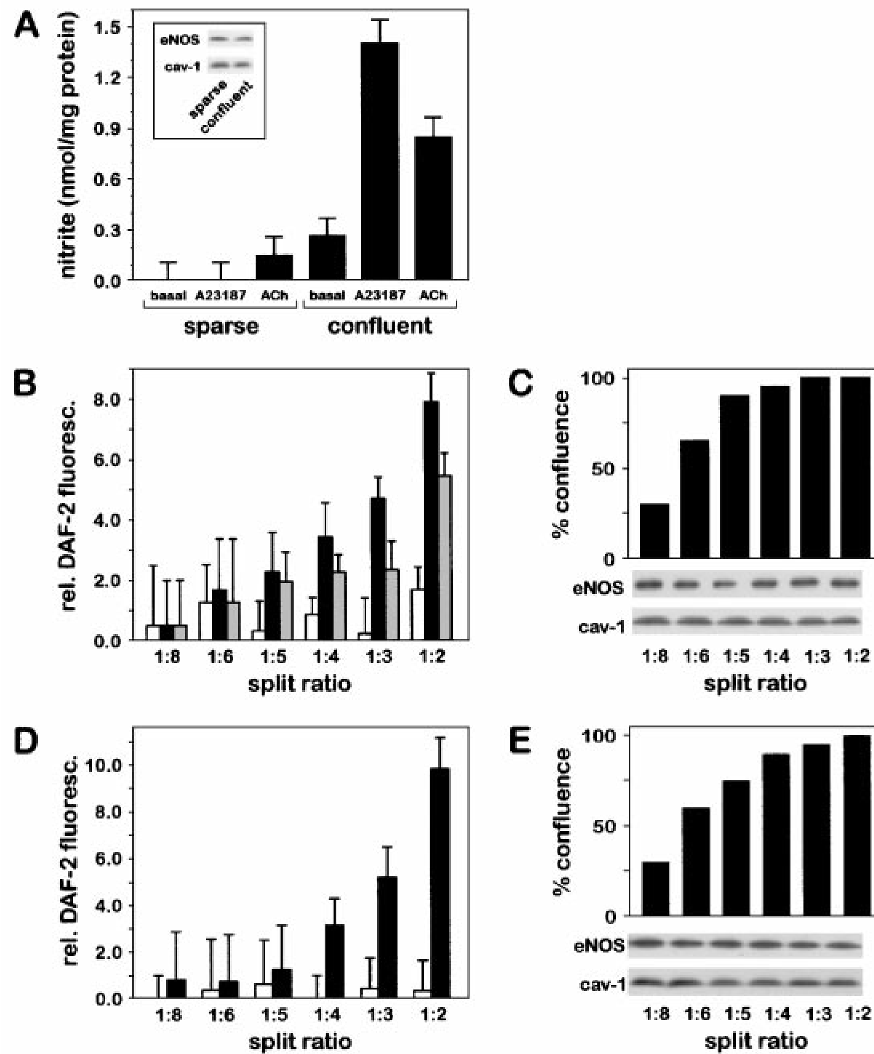

Figure 4 Influence of cell confluence on eNOS activity

(**A**) Sparse and confluent bEnd.3 cells were incubated for 1 h at 37 °C in the absence (basal) or presence of 5 μM A23187 (A23187) or 1 μM acetylcholine (ACh). The amount of nitrite in the medium was determined, and NOS activity was assessed as the amount of L-NAME-dependent release of nitrite in the medium · mg of cellular protein^{-1}. Lysates were prepared from both cell cultures, and from these lysates 3.5 μg of protein was subjected to SDS/PAGE, transferred to PVDF and analysed using the antibodies indicated in the inset. (**B**–**E**) bEnd.3 cells (**B**) and BAEC (**D**) were treated with trypsin, split in ratios from 1 : 8 to 1 : 2 (resulting in initial confluencies ranging from 12.5–50%), cultured for 48 h, loaded with DAF-2 DA and incubated without agonist (white bars) or with 2.5 μM A23187 (black bars) or 1 μM acetylcholine (grey bars). eNOS activity was assessed as the L-NAME-dependent increase in fluorescence · μg cellular protein^{-1}. Lysates were prepared from bEnd.3 (**C**) and BAEC (**E**) cultures, and from these lysates 4.0 μg (bEnd.3) and 2.4 μg (BAEC) of protein was subjected to SDS/PAGE, transferred to PVDF and analysed using the relevant antibodies. Confluence of bEnd.3 (**C**) and BAEC (**E**) cultures was estimated by light microscopy.

proposed that the apparent presence of eNOS at these sites could have been a visual artefact, and that the presence of eNOS at the plasma membrane was accentuated by the close proximity of the membranes at these spots. Our data exclude such an explanation, since we found a high concentration of eNOS at these sites and virtually no eNOS at the remaining part of the plasma membrane (Figure 1). The presence of eNOS at these contact sites in detergent-insoluble membrane domains, also called rafts or detergent-insoluble glycolipid-enriched complexes ('DIGs'), might implicate a link between eNOS and the actin cytoskeleton [35,36]. Involvement of the actin cytoskeleton in regulation of eNOS has already been suggested [37]. The detergent insolubility of eNOS at the contact sites closely resembles that of the junctional proteins VE-cadherin and PECAM-1 [10], indicating that eNOS is indeed part of a specific membrane subdomain at the cell–cell contacts. In addition, it is possible that eNOS at the contact sites is localized in caveolae, since caveolae are also Triton X-100-insoluble [38]. This might be reflected by the localization of caveolin-1 at the Triton X-100-insoluble lateral membranes of adherent cells (Figure 3). Previously, eNOS was found to be present in detergent-insoluble membranes, especially after bradykinin treatment [39]. However, we have not identified any change in solubility of eNOS in Triton X-100 upon incubation of the cells with agonists.

Our data show that, in both microvascular and macrovascular endothelial cells, eNOS and caveolin-1 expression are not affected by changes in cell confluence. In contrast with our results, Arnal and co-workers [28] have shown that, in BAEC, basal and calcium-ionophore-induced NO production is highest in subconfluent cells and falls rapidly when cells become confluent. This

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was accompanied by decreases in the amount of eNOS mRNA and protein. Zöllner and co-workers [40] also identified similar results for BAEC. In our experiments, BAEC, as well as microvascular endothelial cells, produced the highest amount of NO at a late confluent state. This apparent controversy might be explained by the different methods with which these experiments were performed. After treatment with trypsin, Arnal's and Zöllner's groups split the cells in an equal ratio for all experimental conditions, and assayed the cells in consecutive days. We have split the cells in different ratios, cultured them for 3 days and performed all cell assays on the same day, so that changes in eNOS activity could not be attributed to differences in time of cell culture after trypsin treatment of the cells, but only to differences in cell confluence. There has been a report on endothelial cells that also shows an increase in eNOS activity in confluent cells compared with subconfluent cells [41]; this increase was restricted to pulmonary endothelial cells. Furthermore, it was accompanied by a huge increase in eNOS protein levels, which is in contrast with our findings, since we have detected an increase in eNOS activity without any change in eNOS protein levels.

In conclusion, our experimental findings may be different from those of other groups because of slight differences in cell-culture methods, experimental conditions or the procedures by which the cells were isolated. In addition, the expression and regulation of eNOS have been suggested to depend on the vascular bed from which the cells are derived [42–44]. This could also account for the contrasting data. Nevertheless, the link between eNOS localization at cell–cell contacts and eNOS activity in both aortic macrovascular and brain microvascular endothelial cells suggests that our findings represent a general mechanism for eNOS regulation in endothelial cells. Previously, it has been shown that eNOS activity is regulated by phosphorylation [45,46]. Western blot analysis of eNOS using a phospho-eNOS-specific antibody demonstrated that, in bEnd.3 cells and in BAEC, eNOS phosphorylation is not changed when cells reach confluence (results not shown), which implies that eNOS phosphorylation is not the key mediator in the linkage between eNOS activity and cell confluence.

Apparently, eNOS needs to be localized at cell–cell contacts in order to become activated. Why should eNOS be activated at cell–cell contacts? One of the functions of NO within the vessel wall is to regulate vascular permeability. The eNOS agonists VEGF, histamine, ionomycin and ATP increase vascular permeability in an NO-dependent manner [47–49]. On the other hand, NO is also required for maintaining the integrity of the endothelium (i.e. decreasing permeability) [31,50]. This paradox may be explained either by the different conditions in which NO-dependent endothelial permeability was studied or by differences in NO levels. Moderate NO concentrations may decrease permeability, whereas low (in the presence of NOS inhibitor) or high (in the presence of eNOS agonist) levels of NO may increase permeability [51,52]. Interestingly, the role of NO in endothelial permeability has been linked with NO-mediated changes in the cytoskeleton [31,50]. This implies a functional role for the eNOS that is present in the Triton X-100-insoluble, cytoskeleton-associated intercellular junctions. In this regard, it is noteworthy that, in brain capillary endothelial cells, NO induces phosphorylation of vasodilator-stimulated phosphoprotein ('VASP'), a protein present at cell–cell contacts [53]. This might provide a mechanism by which eNOS locally regulates intercellular junctions.

In summary, NO plays an important role in the dynamic regulation of the intercellular junctions of the endothelium. We have shown that eNOS is enriched at these junctions, which is a prerequisite for its activation by agonists. At the junctions, eNOS co-localizes with PECAM-1, but not with VE-cadherin and plakoglobin. The nature of the molecular mechanisms that lead to the enrichment of eNOS at intercellular junctions, and which allow these junctions to be regulated by NO, remains to be determined.

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