Subcellular localization and characterization of nitric oxide synthase(s) in endothelial cells: physiological implications

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Endothelial cells (EC) contain a constitutive Ca^{2+} /calmodulin-dependent nitric oxide (NO) synthase (cNOS) which plays an important role in the local control of vascular tone. We compared the subcellular distribution of this enzyme in cultured and freshly isolated pig EC by determination of specific cNOS activity and immunoblot analysis. Similar studies were also performed with cultured and freshly isolated bovine and cultured human EC. Enzyme activity was predominantly (> 70%) associated with the particulate fraction of all EC types tested and was highest in freshly isolated porcine EC. Both specific cNOS activity and immunoreactivity were substantially higher (> 3-fold) in the microsomal as compared with the soluble fraction of all EC types tested. In freshly isolated pig EC, these two fractions also differed in terms of their Ca^{2+} -dependency, pH optimum and inhibitor specificity. EC may thus contain either two different

cNOS isoenzymes or a single enzyme, the conformation of which differs between the soluble and membrane-bound state. Moreover, detailed subcellular fractionation of freshly isolated pig EC revealed that the distribution of cNOS activity closely resembled that of the plasma membrane marker 5'-nucleotidase, suggesting that most, if not all, of the cNOS activity in these cells is associated with the plasma membrane. This localization might render the enzyme more susceptible to activation by physical stimuli, such as a shear stress-induced change in the fluidity of the plasma membrane. Moreover, the continuous exposure to shear stress *in vivo* may also upregulate cNOS expression in EC, since specific enzyme activity, immunoreactivity and basal NO release were significantly higher in freshly isolated EC as compared with cultured EC.

INTRODUCTION

The formation of NO from L-arginine in mammalian cells is catalysed by a family of isoenzymes, the so-called NO synthases [1–4]. These enzymes are NADPH-dependent oxygenases which require tetrahydrobiopterin, FAD and FMN as cofactors. The isoforms of these enzymes which are constitutively expressed in endothelial and neuronal cells bind calmodulin in a reversible, Ca^{2+} -dependent manner, and therefore can be activated by agonists which elevate the intracellular concentration of free Ca^{2+} . Recent findings indicate that NO synthases are cytochrome P-450-like haemoproteins which are unique in that they contain both the reductase and the oxygenase catalytic domain on the same polypeptide chain [5,6]. The genes encoding these NO synthases have been cloned from different types of endothelial [7,8] and neuronal [9] cells.

In cultured bovine endothelial cells (EC), the Ca^{2+} /calmodulin-dependent constitutive NO synthase (cNOS) is predominantly found in the particulate fraction [10,11], suggesting that this enzyme is a membrane-bound or -associated protein. This conclusion can also be derived from the deduced amino acid sequence of this enzyme which, in contrast to that of the mainly soluble neuronal enzyme [2], contains a consensus sequence for N-terminal myristoylation [7]. Due to their localization at the blood-vessel wall interface, vascular EC are predisposed to sense physical stimuli such as shear stress or hypoxia. The shear stress exerted by the blood flow at the luminal surface of the endothelium is considered to be primarily responsible for the high continuous production of NO, the release of which seems to play

a crucial role in the control of organ blood flow (for references see [12]).

We have recently postulated that an increase in shear stress may affect the fluidity of the EC plasma membrane, thereby modulating the activity of the membrane-bound cNOS [13]. We have, therefore, investigated whether this enzyme is localized to the plasma membrane fraction of EC. In the course of these experiments, we noticed that, apart from stimulating endothelial NO release acutely, continuous exposure of EC to shear stress may also upregulate cNOS expression in these cells [13].

EXPERIMENTAL

Materials

The monoclonal anti-cNOS antibody was kindly provided by Dr. J. S. Pollock (Abbott Laboratories, Abbott Park, IL, U.S.A.), superoxide dismutase (SOD; Peroxinorm) by Dr. M. Haurand (Grünenthal, Aachen, Germany), and SKF-525A by Dr. J. Ostrowski (Cassella, Frankfurt/M., Germany). L-[^3H]Arginine, [α - ^{32}P]GTP and the polyclonal anti-mouse horseradish peroxidase-conjugated antibody were from Amersham Buchler (Braunschweig, Germany). 6R-Tetrahydrobiopterin was obtained from Dr. B. Schircks Laboratories (Jona, Switzerland). All other substances and chemicals were from Sigma (Deisenhofen, Germany), Serva (Heidelberg, Germany) or Boehringer (Mannheim, Germany).

Subcellular fractionation

EC from freshly obtained bovine or pig aortae or from human

Abbreviations used: EC, endothelial cells; cNOS, constitutive Ca^{2+} /calmodulin-dependent nitric oxide synthase; SOD, superoxide dismutase; DTT, dithiothreitol; PMSF, phenylmethanesulphonyl fluoride; sGC, soluble guanylate cyclase; L-NNA, N^G -nitro-L-arginine.

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umbilical veins were detached by treatment with dispase (2.4 units/ml for 45 min at 37 °C). They were either taken directly or seeded in culture dishes (100 mm internal diam.; Greiner, Nürtingen, Germany) or on microcarrier beads (Biosilon; A/S Nunc, Roskilde, Denmark), and grown to confluence in Dulbecco's modified Eagle's medium/Ham's F12 medium (1:1, v/v) or in medium 199 containing 20% fetal calf serum (Vitromex, Vilshofen, Germany), as described previously [14].

The cells were homogenized by five cycles of freeze-thawing (for the preparation of all intracellular membranes) or by sonication (three cycles at 100 W for 10 s; for the preparation of the soluble and microsomal fractions only) in ice-cold 50 mM Tris/HCl buffer (pH 7.4) containing 1.15% (w/v) KCl, 1 mM EDTA, 5 mM glucose, 0.1 mM DL-dithiothreitol (DTT), 200 units/ml SOD, 2 mg/l leupeptin, 2 mg/l pepstatin A, 10 mg/l trypsin inhibitor and 44 mg/l phenylmethanesulphonyl fluoride (PMSF; homogenization buffer 1). Subcellular fractions were prepared by differential ultracentrifugation at 2000 *g* for 10 min, 10000 *g* for 20 min and 100000 *g* for 60 min. An aliquot of the 2000 *g* supernatant, the resuspended 10000 *g* pellet (in homogenization buffer 1) and the 100000 *g* supernatant (cytosol) were stored at -70 °C. The 100000 *g* pellet (microsomes) was resuspended either in 50 mM Tris/HCl buffer (pH 7.4) containing 10% (v/v) glycerol, 0.1 mM EDTA, 0.1 mM DTT, 2 mg/l leupeptin, 2 mg/l pepstatin A, 10 mg/l trypsin inhibitor and 44 mg/l PMSF (homogenization buffer 2) or in 10 mM Tris/HCl buffer (pH 7.4) containing 0.25 M sucrose and 1 mM EDTA (homogenization buffer 3).

A 1 ml aliquot of the microsomal fraction (2.0–2.5 mg of protein) resuspended in homogenization buffer 3 was layered over a 9 ml cushion of 27% (w/v) sucrose in 10 mM Tris/HCl buffer containing 1 mM EDTA and centrifuged at 65000 *g* for 3 h, as described for human platelets in [15]. The 65000 *g* pellet (endoplasmic reticulum) was resuspended in homogenization buffer 2 and stored at -70 °C. The band (1 ml) at the sucrose interface (plasma membrane) was diluted with 9 ml of homogenization buffer 2 (without glycerol), collected by centrifugation at 100000 *g* for 60 min, resuspended in homogenization buffer 2 and stored at -70 °C.

Marker enzyme analysis

The designation of the subcellular fractions as mitochondrial and microsomal pellet, cytosol, endoplasmic reticulum and plasma membrane fraction was based on the determination of the corresponding marker enzyme activities, i.e. glutathione transferase (EC 2.5.1.18; cytosol [16]), fumarase (EC 4.2.1.2; mitochondrial matrix [17]), antimycin-insensitive NADH-dependent cytochrome *c* reductase (EC 1.6.99.3; endoplasmic reticulum [18]) and 5'-nucleotidase (EC 3.1.3.5; plasma membrane [17]), with 25–50 µg aliquots of each fraction by using standard photometric assays.

Determination of cNOS activity

Soluble guanylate cyclase (sGC) assay

Haem-containing sGC from bovine lung was purified to apparent homogeneity as described [9]. The subcellular fractions from EC (10–100 µg/ml) were incubated, in the absence or presence of *N*^ε-nitro-L-arginine (L-NNA; 1 mM), for 10–30 min at 37 °C with the purified enzyme (1–3 µg/ml), 0.3 mM L-arginine, 0.1 mM NADPH, 0.1 µM 6*R*-tetrahydrobiopterin, 0.1 µM calmodulin and 60 µM CaCl₂ in a buffer containing 15 mM Hepes

(pH 7.4), 0.1 mM [α -³²P]GTP (0.2 µCi), 0.1 mM cyclic GMP, 2 mM glutathione, 0.5 mM 3-isobutyl-1-methylxanthine, 4 mM MgCl₂, 0.1 mM EGTA, 27 units/ml SOD, 0.1 mg/ml γ -globulins, 15 mM phosphocreatine and 0.5 unit/ml creatine kinase (final volume of 100 µl). The reaction was stopped by zinc acetate/sodium carbonate precipitation. [α -³²P]GTP was isolated by chromatography on acid alumina and quantified by liquid scintillation counting; sGC activity was calculated as nmol of cyclic GMP formed/min per mg of enzyme, and cNOS activity was expressed as the L-NNA-sensitive activation of sGC.

Citrulline assay

Subcellular fractions from EC (50–200 µg/ml) were incubated, in the absence or presence of L-NNA (1 mM), for 10–30 min at 37 °C with 1 µM L-[³H]arginine (1 µCi), 1 mM NADPH, 15 µM 6*R*-tetrahydrobiopterin, 1 µM FAD and 1 µM calmodulin in 50 mM Hepes buffer (pH 7.4) containing 1 mM DTT, 1 mM EDTA and 1.25 mM CaCl₂ (final volume of 150 µl). The incubations were terminated by the addition of 1 ml of ice-cold 100 mM Hepes buffer (pH 5.5) containing 10 mM EGTA and 500 mg of Dowex AG 50W-X8 (counter-ion Na⁺) cation-exchange resin (Serva), and incubated for 5 min at 0–4 °C, followed by a 5 min centrifugation at 10000 *g*. L-[³H]Citrulline in the supernatant was quantified by liquid scintillation counting, and specific cNOS activity was calculated as the L-NNA-sensitive formation of L-[³H]citrulline/min per mg of protein. L-Citrulline was quantitatively recovered by this batch assay, as determined by using L-[¹⁴C]citrulline (DuPont, Dreieich, Germany).

Immunodetection of cNOS

The proteins in the subcellular fractions from EC were separated by SDS/PAGE (8% gel), blotted on to nitrocellulose membranes (Bio-Rad, München, Germany), and incubated with a primary monoclonal anti-cNOS antibody (dilution 1:10000; raised against the enzyme from cultured bovine EC) and a secondary polyclonal anti-mouse horseradish peroxidase-conjugated antibody, followed by staining with 3,3'-diaminobenzidine and H₂O₂ as described for protein kinase C [20]. Prestained molecular mass markers (Bio-Rad; low and high range) were used as standards for the SDS/PAGE/immunoblot analysis.

Determination of basal NO release from cultured and native pig EC

EC from pig aortae were grown to confluence in 24-well plates as described above. Endothelium-intact segments of pig aortae were cleaned of adventitial adipose and connective tissue, and mounted in a circular frame (18 mm internal diam.) with the adventitia facing the bottom of the frame. Both cultured and native EC were incubated with Tyrode's solution (200 and 400 µl respectively), either in the absence or in the presence of L-NNA (30 µM), for 15 min at 37 °C. Thereafter, the buffer was changed and the incubation was continued for 1 min at 37 °C. After this time an aliquot of the supernatant (50 µl) was removed and analysed for NO content by using a modification of the aforementioned sGC assay [21].

Miscellaneous

Protein concentrations were measured according to the method of Bradford [22] by using the Bio-Rad protein assay and BSA as a standard. Unless indicated otherwise, all data in the figures and text are expressed as means $\pm$ S.E.M. Statistical evaluation was

Table 1 Distribution of cNOS activity in cultured and freshly isolated EC from different species

Unless indicated otherwise, enzyme activity in the subcellular fractions was determined by using the citrulline assay ($\dagger$: determined by sGC assay). n denotes the number of experiments (in duplicate) performed with different batches of EC. Significant differences: * $P < 0.05$, ** $P < 0.01$ versus cytosolic fraction. 'Native' denotes freshly isolated; 'particulate' is mitochondrial and microsomal pellets combined.

Species	EC type	cNOS activity (% of total)			n	Specific cNOS activity (pmol/min per mg)		n
		Particulate	Microsomal	Cytosolic		Microsomal	Cytosolic	
Bovine	Cultured	87 $\pm$ 5	48 $\pm$ 3	13 $\pm$ 5	3	10.0 $\pm$ 3.3*	1.8 $\pm$ 0.2	3
	Native	97 $\pm$ 3	47 $\pm$ 9	7 $\pm$ 3	4	27.2 $\pm$ 4.2**	4.2 $\pm$ 1.6	3
Human	Cultured	73	29	28	1	8.4 $\pm$ 1.9*	2.3 $\pm$ 0.9	5
Pig	Cultured	64 $\pm$ 5 $\dagger$	38 $\pm$ 5 $\dagger$	36 $\pm$ 5 $\dagger$	8	12.6	2.1	1
	Native	71 $\pm$ 7	22 $\pm$ 2	29 $\pm$ 7	3	137.5 $\pm$ 36.8*	35.0 $\pm$ 4.4	5

Table 2 Distribution of cNOS and marker enzyme activities among subcellular fractions from freshly isolated pig EC

cNOS activity in the mitochondrial pellet (Mito), cytosol (Cyt), microsomes (Mic), endoplasmic reticulum (ER) and plasma membranes (PM) was determined by monitoring the L-NNA-sensitive formation of L-[3 H]citrulline from L-[3 H]arginine. Cells isolated from 120 aortae were pooled for a single subcellular fractionation. Results are means $\pm$ S.E.M.; $n \geq 3$ with different batches of EC; * $P < 0.05$, ** $P < 0.01$ versus microsomes; $\dagger P < 0.05$ versus ER. Glutathione transferase, fumarase, antimycin-insensitive NADH-dependent cytochrome *c* reductase and 5'-nucleotidase were employed as marker enzymes for the cytosol, the mitochondrial matrix, the endoplasmic reticulum and the plasma membranes respectively (means $\pm$ S.E.M. of three replicates; HM, homogenate; ND, not determined). The numbers in parentheses indicate the enrichment (fold) of the marker enzyme and cNOS activities in the mitochondrial, cytosolic and microsomal fraction relative to the postnuclear supernatant (i.e. homogenate), and in the ER and PM fractions relative to the microsomes.

Subcellular fraction	Marker enzyme activity (nmol/min per mg)				Specific cNOS activity (pmol/min per mg)	Total protein content (mg)
	Cytosol	Mitochondrial	Endoplasmic reticulum	Plasma membrane		
HM	26.0 $\pm$ 3.1	61.5 $\pm$ 0.8	90.9 $\pm$ 1.3	108.6 $\pm$ 1.9	69.2 $\pm$ 13.0	25.2 $\pm$ 6.4
Mito	13.8 $\pm$ 2.1 (0.22)	129.6 $\pm$ 6.7 (1.76)	198.4 $\pm$ 9.0 (2.00)	329.8 $\pm$ 8.3 (3.01)	213.4 $\pm$ 42.9 (2.55)	4.2 $\pm$ 1.4
Cyt	86.6 $\pm$ 9.4 (1.40)	68.2 $\pm$ 9.1 (0.93)	6.4 $\pm$ 0 (0.06)	25.7 $\pm$ 1.0 (0.24)	35.0 $\pm$ 4.4* (0.42)	14.1 $\pm$ 2.4
Mic	10.4 $\pm$ 3.1 (0.16)	14.0 $\pm$ 0 (0.18)	428.9 $\pm$ 12.8 (4.31)	204.3 $\pm$ 11.4 (1.86)	137.5 $\pm$ 36.8 (1.64)	2.7 $\pm$ 0.7
ER	8.6 $\pm$ 2.1	5.5 $\pm$ 0.8	628.6 $\pm$ 39.0 (1.08)	130.0 $\pm$ 8.1 (0.82)	254.0 $\pm$ 47.0 (0.88)	0.9 $\pm$ 0.2
PM	ND	ND	289.3 $\pm$ 2.6 (0.50)	337.0 $\pm$ 3.6 (2.13)	511.5 $\pm$ 32.8** $\dagger$ (1.77)	0.14 $\pm$ 0.04

performed by Student's *t* test or one-way analysis of variance (followed by a Bonferroni *t* test), where appropriate, with a *P* value of < 0.05 considered statistically significant.

RESULTS

Distribution of cNOS activity in cultured and freshly isolated EC

Differential ultracentrifugation of homogenates from both cultured and freshly isolated bovine and pig EC as well as cultured human EC revealed that the majority of the L-NNA-inhibitable cNOS activity in these cells was associated with the particulate fraction, i.e. the combined 10000 *g* and 100000 *g* sediments (Table 1). Of this particulate cNOS activity, 30–60% was present in the microsomal pellet, which consisted predominantly of membranes derived from the endoplasmic reticulum and plasma membranes (see below). Total cNOS activity was approximately 15 and 3 times greater in freshly isolated as compared with cultured pig and bovine EC respectively. Specific cNOS activity in cultured EC was very similar regardless of the species from which the cells were isolated (Table 1).

The cNOS activity in the microsomal fraction appeared to be tightly associated with the membrane(s), since resuspending the 100000 *g* sediment from freshly isolated pig EC in homogenization buffer 1, which contains approx. 150 mM KCl, followed by 60 min centrifugation at 100000 *g*, did not result in any

appreciable increase in cNOS activity in the supernatant. In contrast, treatment of the microsomal fraction with CHAPS (20 mM) in homogenization buffer 2 for 30 min at 0–4 °C solubilized approx. 80% of the membrane-bound NOS activity (results not shown). Homogenization of the freshly isolated bovine or pig EC in 15 mM Hepes, pH 7.4 [23], increased the proportion of soluble cNOS activity to 24 $\pm$ 3 and 52 $\pm$ 5% respectively ($n = 3$; cf. Table 1), whereas overall enzyme activity (particulate and soluble fractions combined) decreased more than 2-fold. When freshly isolated pig EC were homogenized in homogenization buffer 3, however, the distribution of total and specific cNOS activity among the subcellular fractions was not different from the values shown in Table 1 (results not shown).

Due to the high cNOS activity in freshly isolated pig EC, the microsomal fraction of these cells was chosen for a further subfractionation into endoplasmic reticulum and plasma membranes. To verify that the designation of the various subcellular fractions was correct, the activity of four different marker enzymes was determined in these fractions (Table 2). Both the 10000 *g* and the 100000 *g* pellets were significantly enriched in antimycin-insensitive NADH-dependent cytochrome *c* reductase activity (a specific marker for the endoplasmic reticulum [18]) and 5'-nucleotidase activity (a specific marker for the plasma membrane [17]), whereas there was only very little, if any, contamination of the 100000 *g* pellet with fumarase or glutathione transferase activities, specific markers for mitochondrial

membranes and the cytosol respectively [16,17]. Apart from the mitochondrial marker, there was no major contamination of the cytosolic fraction with the endoplasmic reticulum or plasma membrane markers, whereas this fraction was clearly enriched in glutathione transferase activity. Moreover, the fraction corresponding to the endoplasmic reticulum contained the highest cytochrome *c* reductase activity, while 5'-nucleotidase was most active in the plasma membrane fraction.

Although 76% of the total cNOS activity present in the microsomes was associated with the endoplasmic reticulum (comprising 87% of the total microsomal protein), specific cNOS activity was not only significantly enriched in the plasma membrane fraction, but its distribution among all subcellular fractions also closely resembled that of 5'-nucleotidase, the plasma membrane marker (Table 2).

Characterization of the soluble and microsomal cNOS activity in freshly isolated pig EC

In the absence of exogenous calmodulin, cNOS activity in the microsomal fraction of freshly isolated pig EC decreased only slightly ($28 \pm 3\%$), while that in the cytosol was virtually unaffected ($n = 3$). Addition of FAD, FMN ($100 \mu\text{M}$ each) or a combination of the two flavine nucleotides significantly and virtually identically enhanced the activity of the membrane-bound and soluble enzymes by $34 \pm 1\%$ during a 10 min incubation and by $73 \pm 3\%$ during a 30 min incubation respectively ($n = 3$; $P < 0.05$). However, there was a clear difference in the Ca^{2+} -dependency of the membrane-bound and soluble cNOS, which were half-maximally stimulated at 1 and $0.3 \mu\text{M}$ free Ca^{2+} respectively (Figure 1). Moreover, the pH optimum (Figure 2) of the soluble enzyme (pH 7.1) was different from that of the microsomal cNOS activity (pH 7.6). Although both enzyme activities were similarly inhibited by L-NNA, N^G -monomethyl-L-arginine was a more potent inhibitor of the soluble cNOS, whereas the cytochrome *P*-450 inhibitor SKF-525A was more effective in blocking the microsomal enzyme (Table 3).

Effects of shear stress on cNOS expression in EC

The striking difference in cNOS activity between freshly isolated and cultured EC from the same species (Figure 3) was also reflected by the significantly different cNOS content in the cytosolic and microsomal fractions of these cells, as determined by immunoblot analysis (Figure 3, inset). The monoclonal anti-cNOS antibody recognized a single protein band with an apparent molecular mass of $142 \pm 2 \text{ kDa}$ in both fractions from either bovine or pig EC ($n \geq 3$; Figure 3, inset). The immunoblot analysis also revealed that the difference in specific cNOS activity between the cytosolic and microsomal fractions from the same EC type is reflected by the relative enzyme content of these subcellular fractions (Figure 3 inset; identical results were obtained with cultured and freshly isolated bovine EC; $n \geq 3$). One functional correlate of this apparent difference in cNOS expression was a substantially smaller basal release of NO from cultured pig EC as compared to native pig EC (Figure 3).

To substantiate the hypothesis that the continuous exposure of EC to shear stress *in vivo* might be responsible for the observed differences in cNOS activity and content between freshly isolated and cultured EC, the effect of such stress on the expression of cNOS in cultured bovine aortic EC was examined. In the 10000 *g* supernatant of these cells (combined cytosolic and microsomal fraction), cNOS activity increased from 2.9 to 5.6 pmol/min per mg after a 16 h exposure to a shear stress of 0.3 mN/cm^2

Figure 1 Ca^{2+} -dependence of the cytosolic (●) and microsomal (○) cNOS activities derived from freshly isolated pig EC

In contrast to the standard citrulline assay, aliquots of the corresponding subcellular fractions were incubated in 50 mM Hepes buffer (pH 7.4) containing 0.1 mM EGTA instead of 1 mM EDTA, to which increasing concentrations of CaCl_2 were added ($10\text{--}480 \mu\text{M}$). The concentration of free Ca^{2+} in the buffer was estimated by using the Fura-2 method as described [23]. Results are means $\pm$ S.E.M., $n = 4$; the two curves are significantly different at $P < 0.01$.

Figure 2 Determination of the pH optima of the cytosolic (●) and microsomal (○) cNOS activities derived from freshly isolated pig EC

The incubations for the citrulline assay (in duplicate) were performed in 50 mM bis-Tris-propane/HCl instead of 50 mM Hepes. This buffer is suitable for a pH range of 6.3–9.5. Virtually identical results were obtained with a buffer combination of 50 mM bis-Tris/HCl (pH 6.0–7.0), 50 mM Hepes (pH 7.0–8.0) and 50 mM Bicine (pH 8.0–9.0).

Table 3 Inhibitor specificity of the cytosolic and membrane-bound cNOS from freshly isolated pig EC

The IC_{50} values represent the means $\pm$ S.E.M. from three experiments with different batches of EC. Note that only $1 \mu\text{M}$ L-[^3H]arginine was used for the citrulline assay. L-NNA, N^G -monomethyl-L-arginine.

Subcellular fraction	IC_{50} (μM)		
	L-NNA	L-NMA	SKF-525A
Cytosol	0.08 ± 0.01	0.03 ± 0.01	> 1000
Microsomes	0.08 ± 0.01	0.3 ± 0.03	100 ± 5

Figure 3 Differences in basal NO release, cNOS activity and cNOS content between cultured and freshly isolated pig EC

The figure depicts the basal NO release (left; determined by the sGC assay; $n \geq 3$ with different batches of EC) and the specific cNOS activity in subcellular fractions (right; citrulline assay) from cultured (cult., single batch of EC, double determination) and freshly isolated pig EC (nat.; $n = 5$ with different batches of EC). The inset shows the immunoblot analysis of the corresponding cytosolic (cyt.) and microsomal (mic.) fractions from these cells (70 μ g of protein per lane). Lane MW contains the prestained molecular mass markers (from bottom to top): 80 kDa (BSA), 116 kDa (β -galactosidase) and 205 kDa (myosin). The values for basal NO release from cultured and native pig EC were normalized based on the EC surface and incubation volume (* $P < 0.05$).

(30 dyn/cm²), and this increase in enzyme activity was reflected by a more than 2-fold increase in cNOS-specific immunoreactivity in this fraction as compared with the 10 000 g supernatant derived from control cells ($n = 2$; results not shown).

DISCUSSION

The present findings demonstrate (i) that the majority of cNOS activity in bovine, pig and human EC is associated with the particulate fraction, i.e. the combined mitochondrial and microsomal sediments, and (ii) that the distribution of cNOS activity is not different between cultured and freshly isolated EC. Detailed fractionation of freshly isolated pig EC by using a simple density gradient technique revealed that most, if not all, of the cNOS activity present in these cells is associated with the plasma membrane fraction. This localization may render the enzyme more susceptible to an activation by physical stimuli, such as a shear stress-induced change in the fluidity of these membranes [24].

According to the marker enzyme analysis of the various subcellular fractions, there was considerable contamination of the mitochondrial sediment with endoplasmic reticulum and plasma membranes, but virtually no contamination of the microsomal sediment, the endoplasmic reticulum or plasma membrane fraction with mitochondrial membranes. By using a differential ultracentrifugation procedure very similar to ours, almost identical data have been obtained with freshly isolated bovine EC [25]. The contamination of the 10 000 g pellet is likely to be due to the unfavourable sedimentation coefficient of the endoplasmic reticulum and plasma membrane vesicles. As a consequence, the high cNOS activity in the mitochondrial fraction (approx. 40% of the total cNOS activity) should be

attributed to the cNOS content of these membranes and not to a cNOS activity present in the mitochondria of EC.

While the subcellular distribution of cNOS in freshly isolated bovine and cultured human EC corresponds to that previously described for cultured bovine EC [10,11], the Ca²⁺-dependency of the cNOS activity in freshly isolated pig EC contrasts with a previous report from this laboratory describing a partially Ca²⁺-independent soluble cNOS activity in these cells [23]. The most plausible explanation for this difference is that, in the previous study, a homogenization buffer with a comparatively low ionic strength was used, which largely precludes adequate vesicle formation. This may not only greatly affect the activity and physicochemical properties of the membrane-bound enzyme, but may also cause a substantial release of enzyme activity into the cytosol, thereby causing an underestimation of the particulate cNOS content.

Both the soluble and membrane-bound cNOS activity in freshly isolated pig EC as well as that in cultured bovine, human and pig EC (results not shown) was largely independent of the presence of exogenously added calmodulin, suggesting that there are either sufficient amounts of calmodulin in these crude preparations (cytosol) or that calmodulin is tightly bound to the microsomal enzyme [10]. In contrast, the activity of both the cytosolic and microsomal enzyme from freshly isolated pig EC was enhanced in the presence of FAD, FMN or both. This stimulatory effect increased with time, suggesting that the endogenous flavin nucleotides are inactivated or lost in the course of the enzymic catalysis. A similar time-dependent cofactor requirement has also been reported for tetrahydrobiopterin and the purified cNOS from rat cerebellum [26].

Apart from these common properties, there were some striking differences between the cytosolic and microsomal enzymes from freshly isolated pig EC relating to their respective Ca²⁺-dependency, pH optimum and inhibitor specificity. In conjunction with the substantially higher specific cNOS activity and content of the microsomal fraction, these data point to the possible existence of two different Ca²⁺-dependent NO synthases in freshly isolated pig EC. Alternatively, the observed differences may be explained by a change in the conformation of a single enzyme depending on whether it is soluble or membrane-bound. Although in the intact EC cNOS may be exclusively associated with the plasma membrane, part of it will be released into the cytosol upon cell disruption, i.e. during the homogenization procedure. Such a change in the three-dimensional structure of the enzyme, e.g. affecting the Ca²⁺/calmodulin-binding site, may account for the observed differences in pH and Ca²⁺-dependency between the soluble and membrane-bound cNOS types. Moreover, the conformation of the active site of the soluble enzyme may also be altered, resulting in a decrease in activity and a different inhibitor specificity.

Recently, cNOS in cultured bovine EC has been shown to be phosphorylated after exposure of these cells to bradykinin, resulting in a translocation of the enzyme from the particulate fraction to the cytosol [27]. Even though the membrane association of cNOS in bovine EC appears to be largely dependent on the N-terminal myristoylation of the enzyme [28,29], its intracellular localization may be amenable to a more dynamic regulation by receptor-dependent signal transduction mechanisms. Due to the differences in activity between the cytosolic and membrane-bound cNOS in EC, it is tempting to speculate that one functional significance of this 'reversed translocation' might be an overall decrease in the NO-synthesizing capacity of these cells, hence representing a feedback mechanism for the agonist-induced NO release.

The pH optimum of the membrane-bound cNOS from freshly

isolated pig EC was very similar to that of the purified inducible NO synthase from monocytes/macrophages (pH 7.8–8.0 [4]), but clearly different from the pH optimum of the neuronal cNOS (the soluble and membrane-bound cNOS from rat cerebellum have a pH optimum of 6.7 [30]). The finding that the activity of the endothelial enzyme is virtually lost within 1 pH unit above or below the optimum (pH 7.6) implies that changes in intracellular pH may play an important role in the control of cNOS activity in EC. This may be of particular relevance for ischaemic conditions, where the intracellular pH is often substantially decreased.

The substantial differences in cNOS activity and content and in the NO-releasing capacity between freshly isolated and cultured pig aortic EC suggest that the continuous exposure of the endothelium to certain stimuli *in vivo* affects cNOS expression. One likely candidate is the shear stress to which the cells are continuously exposed *in vivo*. Culturing these cells under virtually static conditions removes this stimulus and may consequently lead to a decline in cNOS expression. Indeed, exposure of cultured bovine EC to continuous shear stress for 16 h resulted in a significant increase in both cNOS activity and content. Moreover, preliminary evidence obtained in our laboratory suggests that unstimulated cultured bovine EC contain significantly less mRNA encoding cNOS than do freshly isolated cells. The notion that shear stress thus modulates cNOS expression in EC is supported by a recent paper [31] and our own findings with cultured bovine EC (results not shown), demonstrating that cNOS mRNA levels are markedly elevated in these cells upon exposure to continuous shear stress for 12–24 h.

In summary, our data support the hypothesis that the localization of the constitutive NO synthase in EC favours a modulation of its activity by physical stimuli such as a shear stress-induced change in the fluidity of the plasma membrane. Moreover, the expression of this enzyme in EC may also be influenced by the level of shear stress to which these cells are continuously exposed *in vivo*.

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