British Journal of Pharmacology (1997) 122, 1502–1508

Dobesilate enhances endothelial nitric oxide synthase-activity in macro- and microvascular endothelial cells

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1 Dobesilate is used for normalizing vascular dysfunction in a number of diseases. In search for an effect on endothelial NO production, macrovascular endothelial cells from rat aorta, microvascular endothelial cells from rat exocrine pancreatic tissue, and capillary endothelial cells from rat islets, were cultured in the presence or absence of Mg-Dobesilate. The activity of constitutive nitric oxide synthase (ecNOS) in resident cells as well as of inducible nitric oxide synthase (iNOS) in cytokine-activated cells was measured indirectly by recording the citrulline concentrations in culture supernatants.

2 In each of the different endothelial cells Mg-Dobesilate incubation (0.25–1 mM) for 24 h led to a significant and concentration-dependent increase in ecNOS-activities. With cytokine-activated endothelial cell cultures only moderate effects were seen with little or no concentration-dependency. Addition of the NOS-inhibitor N^G-monomethyl-L-arginine led to a significant suppression of citrulline formation in all cultures as an evidence for the enzyme specificity of these effects.

3 iNOS- and ecNOS-specific reverse transcription and semi-quantitative polymerase chain reaction (RT–PCR) with RNA from resident or cytokine-activated endothelial cells gave no evidence for an increase in NOS-specific mRNA after Mg-Dobesilate-treatment. Furthermore, Dobesilate-mediated enhancement of NO synthesis in resting endothelial cells was not due to iNOS induction in these cells, as no iNOS-specific signal was found by RT–PCR.

Keywords: Vascular dysfunction; endothelial cells; nitric oxide; Mg-Dobesilate

Introduction

Numerous cell types are capable of nitric oxide (NO) production including endothelial cells, macrophages, neutrophils and neurons (Moncada & Higgs, 1993). NO and equal amounts of citrulline are synthesised from the guanidino nitrogen of L-arginine by nitric oxide synthases (NOS) (Förstermann *et al*., 1994). In endothelial cells two different isoenzymes can be expressed, depending on the activation state of these cells. In resting, non-activated endothelial cells a constitutive enzyme (ecNOS) is expressed (Moncada & Higgs, 1993) and after challenge with proinflammatory cytokines and/or bacterial endotoxin a cytokine-inducible enzyme (iNOS) is expressed in addition (Suschek *et al*., 1993, 1994).

The constitutive, calcium-dependent isoenzyme produces low amounts of NO for short periods of time (Palacios *et al*., 1989). This endothelium-derived NO plays a crucial role in blood pressure regulation (Rees *et al*., 1989), in inhibition of platelet aggregation and platelet adhesion (Radomski *et al*., 1987a, b), and in modulating leukocyte adhesion, an essential step early in tissue inflammation (Kubes *et al*., 1991; Zimmerman *et al*., 1992).

The inducible, cytosolic, calcium-independent NO synthase is expressed only after cell activation and releases large amounts of NO for longer periods of time that functions as cytotoxic and immune regulatory effector molecule (for a review, see Kröncke *et al*., 1995).

Diabetes mellitus is associated with a two to six fold increased risk for micro- or macrovascular atherosclerotic disease (Keen & Jarret, 1979; Kannel, 1985). It appears that damage to the vascular endothelium may be an early pathological process in some of the diabetes patients (Bierman & Brunzell, 1978; Dolgov *et al*., 1982). Vascular function abnormalities, as manifested by haemodynamic changes (Pugliese *et al*., 1991) as well as increased vascular permeability (Parving, 1976; Pugliese *et al*., 1989), and changes in vascular structure (Arbogast *et al*., 1984), develop early in human diabetes and in animal models of this disease (Cameron & Cotter, 1992; Tomlinson *et al*., 1992). Vascular function abnormalities are also reflected by decreased synthesis of prostacyclin and nitric oxide (Durante *et al*., 1988; McVeigh *et al*., 1992) and increased levels of circulating endothelin (Takahashi *et al*., 1990). Diabetes-induced vascular dysfunctions are primarily found in retina, peripheral vessels, aorta and kidney (Rossini & Chick, 1980; Ramsay *et al*., 1989).

Calcium Dobesilate (calcium dihydroxy-2,5 benzenesulfonate, Doxium®) has been widely used as an angioprotective agent for the treatment of vascular disease, especially in diabetic retinopathy and chronic venous insufficiency. The drug has been shown to diminish capillary permeability (Bayer *et al*., 1980; Bijsterveld & Janssen, 1981) and capillary fragility and to reduce whole blood viscosity (Benarroch *et al*., 1985) in patients with diabetic retinopathy. Furthermore, Calcium-Dobesilate has also been shown to inhibit platelet aggregation (Heidrich *et al*., 1983), as well as thrombus formation in the microcirculation (Michal & Giessinger, 1985). As many of these known beneficial effects could be explained by increased NO supply, we have now searched for a modulating activity of Dobesilate on endothelial NO synthesis.

By determining endothelial citrulline production as well as NOS-specific mRNA levels we examined the effects of Dobesilate on both the ecNOS- and iNOS-activities and mRNA expression in resting or cytokine-activated endothelial cells obtained from Wistar rats.

Methods

Reagents

Mg-Dobesilate was a kind gift from Laboratoire OM, Geneve, Switzerland. Recombinant human interleukin-1β (IL-1β), recombinant murine tumor necrosis factor-α (TNFα) as well as recombinant rat gamma-interferon (γIFN) were obtained from

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HBT (Leiden, Netherlands) or from Genzyme (Cambridge MA, U.S.A.). The LPS content in these various cytokine batches never exceeded 0.3 ng μg^{-1} protein. Endothelial cell growth supplement (ECGS), type I collagen, collagenase (from *Cl. histolyticum*), urease type VII and rabbit anti-human von Willebrand Factor (vWF) antiserum were from Sigma (Deisenhofen, F.R.G.). Monoclonal antibodies Ox2 and Ox43 were from Serotec (Camon, Wiesbaden, F.R.G.). Peroxidase-conjugated porcine anti-rabbit IgG from DAKO (Hamburg, F.R.G.), and peroxidase-conjugated goat anti-mouse IgG was from Zymed Laboratories, Inc. (San Francisco, CA, U.S.A.). Trypsin, EDTA, foetal calf serum (FCS, endotoxin free), RPMI-1640 (endotoxin free), the oligo dT16-primer and Taq-polymerase were purchased from Boehringer Mannheim (F.R.G.) or Gibco Laboratories (Eggenstein, F.R.G.). 3,3′-diaminobenzidine (DAB) was from Serva GmbH (Heidelberg, F.R.G.), Ficoll 400 from Pharmacia (Uppsala, Sweden). Some batches of RPMI-1640 were mixed from individual substances (all p.a. grade, Sigma, Deisenhofen, F.R.G.) according to standard recipe with the exception of glucose and using $CaCl_2$ instead of $Ca(NO_3)_2$.

Animals

Male Wistar rats (about 30 days old and 150 g body weight) were obtained from the University breeding facility. All animals received a standard diet and tap water *ad libitum*.

Endothelial cells (EC)

Capillary islet endothelial cells (IEC) were isolated from hand-picked pancreatic islets by outgrowth on a collagen type I matrix (Suschek *et al*., 1994). Pancreatic islets were harvested by ductal injection of collagenase and subsequent centrifugation of the dispersed tissue on a Ficoll density gradient followed by hand-picking (Appels *et al*., 1988). Exocrine pancreatic microvascular endothelial cells (EPEC) were isolated from microvessels of exocrine pancreatic tissues hand-picked from the collagenase digest. Macrovascular endothelial cells (AEC) were isolated by outgrowth from rat aortic rings exactly as described (McGuire & Orkin, 1987).

Aortic segments, hand-picked whole islets or microvessels from the exocrine pancreas were placed on top of a collagen gel (1.8 mg collagen/ml) in 24-well tissue culture plates and incubated in RPMI 1640 with 20% FCS and 100 μg ECGS/ml in a humidified incubator at 37°C in a 95% air/5% CO_2 atmosphere for 5–7 days depending on the degree of cellular outgrowth. Aortic explants, islets or microvessels were then removed, cells detached with 0.25% collagenase in HBSS and replated onto plastic culture dishes in RPMI 1640 with 20% FCS. Cells were subcultured for up to 10 passages, and removal from culture dishes for each passage was performed by treatment with 0.05% trypsin/0.02% EDTA in isotonic NaCl for 3 min.

Cellular characterization of cultured endothelial cells

Cells were passaged from tissue culture dishes onto sterile glass coverslips and allowed to grow as subconfluent monolayers. Cells were washed with PBS and fixed with acetone at −20°C for 10 min. The coverslips were washed with PBS, incubated with a crossreacting rabbit anti-human-vWF antiserum (1 : 50 dilution) at room temperature for 45 min, washed extensively with PBS and incubated in a 1 : 50 dilution of peroxidase-conjugated porcine anti-rabbit IgG for 45 min at room temperature, then again washed extensively with PBS and subsequently peroxidase activity was revealed using diaminobenzidine. To exclude antifibronectin reactivity in the commercially available anti-vWF serum, aliquots were absorbed with rat plasma fibronectin immobilized on colloidal gold prior to use (Frens, 1973; Geoghegan & Ackerman, 1977). Control cultures were incubated with a nonrelevant rabbit hyperimmuneserum instead of first antiserum. As positive controls isolated human platelets and as negative controls isolated rat alveolar macrophages and the fibroblastoma cell line L929 were also tested with this anti-vWF antiserum. A rat vascular endothelium specific monoclonal antibody Ox43 (Robinson *et al*., 1986) and the rat thymocyte and, brain endothelium specific monoclonal antibody Ox2 (Barclay, 1981 were used in a 1 : 50 dilution. A peroxidase-conjugated goat anti-mouse IgG was diluted 1 : 50 prior to use. Otherwise conditions were as described above.

Macrophages

Macrophages were isolated from the peritoneal cavity of Wistar rats pretreated for 5 days with an intraperitoneal injection of a heat inactivated suspension of *Corynebacterium parvum* (0.5 ml/animal, Wellcome, Burgwedel, F.R.G.). Macrophages were enriched by adherence for 1 h (37°C, 5% CO_2) on FCS coated plastic culture dishes. The cells were removed by incubation in Ca^{2+} and Mg^{2+}-free HBSS (4°C, 10 min) and resuspended in RPMI 1640 with 10% FCS.

Experimental design

With EC cultures all measurements were performed with cells from passages 4–9. Endothelial cells or macrophages (each 1×10^5) were cultured in 24-well tissue culture plates in a humidified incubator at 37°C in a 95% air/5% CO_2 atmosphere in 600 μl RPMI 1640 with 20% FCS. Cytokine-challenge of EC was performed by addition of IL-1β, TNFα and γIFN as indicated. LPS concentrations of RPMI 1640, cytokines, and FCS were below 1 ng ml^{-1}. Macrophages were activated by the addition of 1 ng ml^{-1} LPS (*E. coli* 026:B6, Sigma). After 24 h of incubation nitrite was determined in culture supernatants using the diazotization reaction as modified by Wood *et al*. (1990) and $NaNO_2$ diluted in medium as well as in H_2O as standards. From resulting extinctions (540 nm) sham treated controls made for each culture with medium and respective additives but without cells were subtracted.

Resting or activated EC or macrophages were incubated for 24 h with Mg-Dobesilate at concentrations indicated. In this study we used Mg-Dobesilate instead of Ca-Dobesilate because the calcium-salt of dihydroxy-2,5 benzenesulfonate precipitates when dissolved in phosphate-buffered solutions like RPMI-medium.

When incubated with culture medium Mg-Dobesilate alone generates an intense pink colour especially at lower pH as has to be used in the Griess reaction, thereby interfering with nitrite determination. For these reasons endothelial citrulline production had to be used as the indicator for the endothelial NO production in the presence of this agent.

Citrulline determination

Citrulline was determined using a colorimetric assay exactly as described (Boyde & Rahmatulah, 1980). The assay is based on the reaction with diacetyl monoxime (5 g l^{-1}) in the presence of sulphuric acid (25%), phosphoric acid (20%) and $FeCl_3$ (250 mg l^{-1}): culture supernatants (500 μl) were incubated with urease (45 U ml^{-1}) for 30 min. The mixture was then deproteinised by adding TCA to a final concentration of 5%. After centrifugation 500 μl of the supernatant and 3 ml of the chromogenic solution were boiled for 5 min, cooled to room temperature and the absorbance measured at 530 nm in an ELISA-reader. From resulting extinctions controls with medium and respective additives sham incubated without cells were subtracted. Extinctions were calculated against citrulline standards diluted in medium as well as in H_2O.

Polymerase chain reaction (PCR)

Total cellular RNA (1 μg each), prepared from cytokine-stimulated or resting endothelial cells (Chomczynski & Sacchi, 1987) or from Mg-Dobesilate (1 mM) treated resting and activated cells was used for cDNA synthesis (Gubler & Hoffman,

1983) using the oligonucleotide ACTTCCTCCAGGATGTTGTA (antisense; bp 1338-1357 of mouse iNOS cDNA) (Xie, 1992) or the oligo dT16-oligonucleotid as primer, respectively. The rat ecNOS sequence is currently not available. We therefore searched for a general NOS primer by multialignment of the available NOS-sequences. This was used in some RT and as antisense primer in PCR. Otherwise, reverse transcription was carried out at 55°C for 45 min or at 37°C for 60 min, using the oligo dT16-primer. The cDNA was then used as template for PCR either primed by the sense oligonucleotide TGATGTGCTGCCCTCTGGTCT (sense; bases 1079–1098 of mouse iNOS cDNA) for specific iNOS amplification, or primed by the oligonucleotide GGCCGCTTCGACGTGCTGCCT (sense; consensus sequence of mouse iNOS cDNA, bases 1071–1090, and human ecNOS cDNA, bases 870–889) for iNOS/ecNOS amplification. For specific rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-cDNA amplification the oligonucleotides ACCACAGTCCATGCCATCAC (sense; rat GAPDH-cDNA bases 546–565) and TCCACCACCCTGTTGCTGTA (antisense; rat GAPDH-cDNA bases: 1049–1068) were used (Sirsjö *et al*., 1994). PCR was carried out following standard protocols (Saiki *et al*., 1988) with the cyclic profile: 30 s at 94°C, 30 s at 56°C, 1 min at 72°C. After a total of 35 cycles a final incubation step was performed at 72°C for 10 min. An aliquot of each reaction was subjected to electrophoresis on 2% agarose gels. Bands were visualised by ethidium bromide staining.

Results

Characterization of cultured endothelial cells

All endothelial cells were cultured under identical conditions in culture media containing 11 mM glucose. Immunocytochemistry with anti-vWF-antisera and the endothelial cell-specific monoclonal antibodies Ox2 and Ox3 demonstrate heterogeneity of antigen phenotypes among the different endothelial cell cultures. IEC exhibited the phenotype vWF_{high}, $Ox2_{high}$, $Ox43_{low}$ whereas AEC and EPEC showed the antigen phenotype vWF_{high}, $Ox2_{low}$, $Ox43_{high}$ exactly as published (Suschek *et al*., 1994). These labelling experiments also showed that cultures consisted of pure endothelial cells, since the endothelial specific markers were found in all cells.

We characterized the NOS activities in resting and cytokine-activated endothelial cell by measuring the nitrite and citrulline concentrations in supernatants of AEC-, EPEC-, and IEC-cultures after 24 h incubation (Table 1).

All endothelial cell cultures showed a constitutive NOS activity exactly as published previously (Suschek *et al*., 1993; 1994). Whereas resting AEC produced 1–2 nmol nitrite or citrulline, nitrite formation in supernatants of EPEC and IEC was three to fivefold higher and citrulline production was 5 to 10-fold higher, respectively.

Culturing AEC, IEC, and EPEC in the presence of IL-1β + TNFα + γIFN led to a significant increase in nitrite and citrulline formation in the culture supernatants by a factor of 12–15 with AEC and with IEC and EPEC by a factor of 2–3 compared to untreated cells.

Incubation of resting IEC or EPEC, cytokine-activated endothelial cell cultures or LPS-activated peritoneal macrophages with the NOS-inhibitor N^G-monomethyl-L-arginine (L-NMA: 1 mM) led to a highly significant and parallel suppression of both nitrite and citrulline production (Table 1).

Dobesilate-mediated increases in NO production

In resting AEC the presence of Mg-Dobesilate for 24 h led to a concentration-dependent increase in the NOS activity. Mg-Dobesilate ($\geqslant$ 0.25 mM) significantly enhanced citrulline production in these macrovascular endothelial cells and 1 mM Mg-Dobesilate led to a 4.6-fold increase in endothelial citrulline production (Figure 1a).

With resting EPEC cultures Mg-Dobesilate also led to a concentration dependent augmentation of NOS-activity, similar to that seen with AEC. At 1 mM Mg-Dobesilate-concentration the increase in citrulline production with these microvascular endothelial cells was 3.6 fold (Figure 1b).

Increases in citrulline formation found with Mg-Dobesilate in islet capillary endothelial cell cultures (IEC) again showed a concentration-dependency and were significant. The overall increases, however, were less than with large vessel endothelia, and were found to reach a maximum of 1.8 fold compared to untreated cells (Figure 1c).

In contrast to resident endothelial cells, with cytokine-activated endothelial cell cultures only moderate Dobesilate-mediated effects were seen with little or no concentration-dependency (Figure 1) and with activated macrophages incubation with Mg-Dobesilate had no significant effect on iNOS activity (Figure 1d).

Additional incubation of both resting or cytokine-activated endothelial cell cultures with the NOS-inhibitor N^G-monomethyl-L-arginine (L-NMA: 1 mM) led to a highly significant suppression of Mg-Dobesilate-induced effects on increases in citrulline formation as evidence for the NOS-specificity of these effects (Table 2).

RT-PCR analysis of total cellular RNA

Semi-quantitative RT-PCR was performed with total RNA extracted from untreated and Mg-Dobesilate-challenged AEC grown in culture medium with or without cytokines (Figure 2). The reverse transcription and polymerase chain reaction was primed by oligonucleotides for specific iNOS amplification or iNOS plus ecNOS-specific oligonucleotides to detect rat ecNOS-specific RNA. Only cytokine-treated cells yielded an amplification product for iNOS, whereas no iNOS-signal was obtained with RNA from cells grown in the absence of cytokines irrespective of the presence of MG-Dobesilate. Thus Mg-Dobesilate does not induce iNOS-mRNA-expression in resting cells. Using the consensus primers to also multiply ecNOS-specific sequences, a signal was obtained in all unchallenged AEC, representing ecNOS-specific amplification products, as iNOS-specific amplification was negative in these cultures.

Table 1 Nitrite and citrulline production of resting and activated rat endothelial cells and peritoneal macrophages

	Nitrite	*+L-NMA* (1 mM)	*Citrulline*	*+L-NMA* (1 mM)
AEC				
Resting	1.4±0.5	1.0±0.4	1.3±0.8	NT
Activated	12.7±1.5	*2.5±0.7	31.1±1.8	*3.5±0.9
EPEC				
Resting	5.3±1.0	*1.5±0.9	8.5±1.5	*2.9±1.2
Activated	15.2±3.5	*2.7±1.3	31.6±2.0	*6.3±1.9
IEC				
Resting	7.6±0.5	*3.8±2.0	14.5±1.8	*4.4±1.8
Activated	15.8±1.4	*4.2±0.9	37.2±1.3	*6.5±2.4
Macrophages				
Resting	0.4±0.4	NT	0.5±0.5	NT
Activated	7.3±0.3	*2.8±0.8	15.7±1.5	*3.5±1.0

1×10^5 aortic endothelial cells (AEC), exocrine pancreatic microvascular endothelial cells (EPEC), and islet capillary endothelial cells (IEC) were incubated for 24 h in RPMI 1640 medium only (resting) or with a combination of cytokines IL-1β (200 U ml^{-1}) + TNF-α (500 U ml^{-1}) + γIFN (100 U ml^{-1}) (activated). Additionally, peritoneal macrophages were activated with LPS. Nitrite and citrulline (nmol) were determined in culture supernatants as indicated in Methods. Statistical differences in nitrite and citrulline concentrations between resting or activated endothelial cell cultures or activated macrophages and respective cultures incubated with L-NMA were highly significant (*$P<0.0001$). Values are the mean±s.d. of 3–12 individual experiments. NT: not tested.

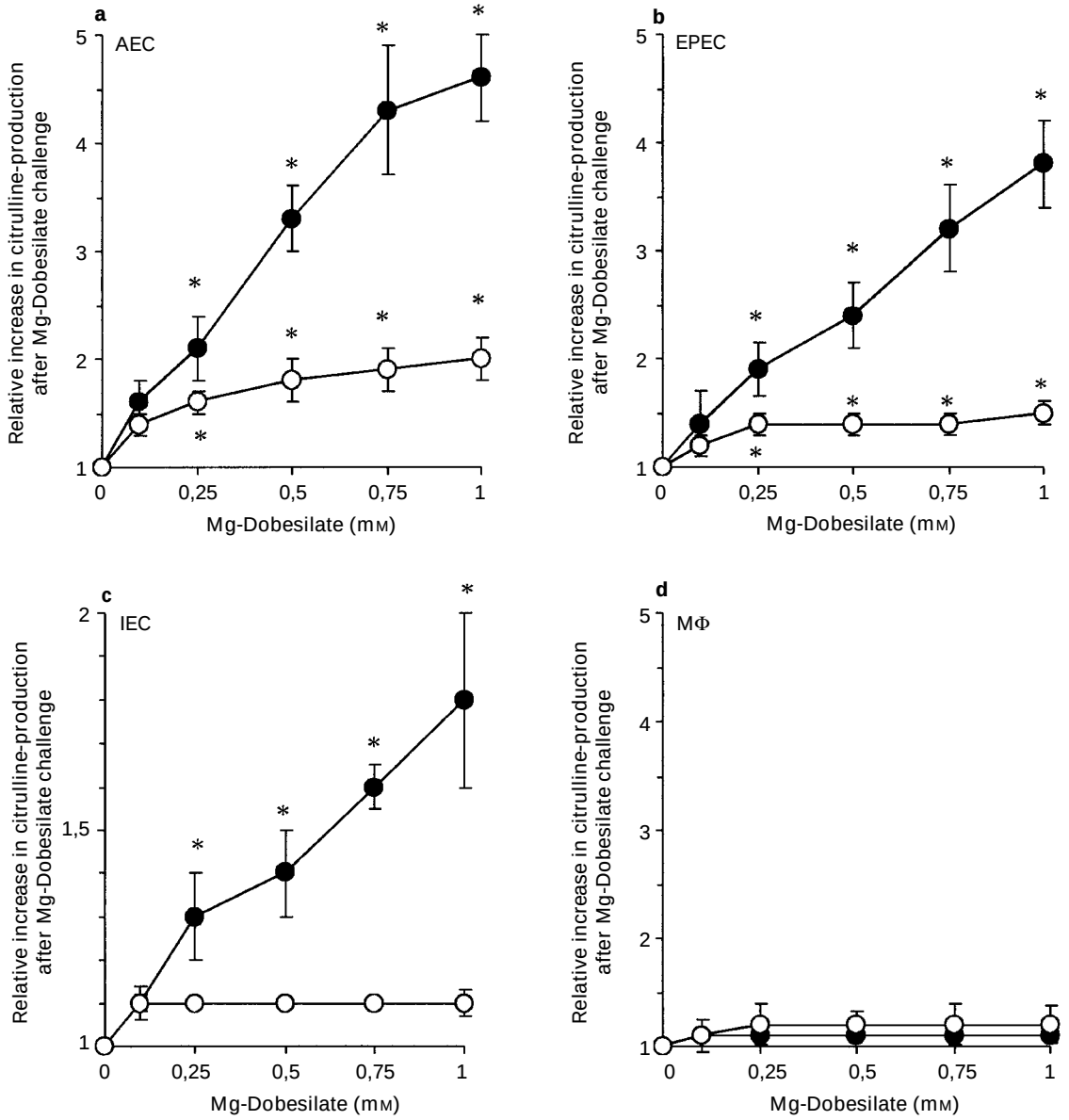

Figure 1 Concentration-dependent effect of Mg-Dobesilate on endothelial and macrophages citrulline production as an indicator of nitric oxide synthase activity. Endothelial cells and peritoneal macrophages were cultured for 24 h in the presence of Mg-Dobesilate and citrulline concentrations of culture supernatants were determined. Shown are the relative increases during a 24 h incubation period over the sham incubated controls (0 mM). (a) Strong correlation between increases in citrulline production and Mg-Dobesilate concentration is found with resting aorta endothelial cell (AEC) cultures (●) but effects were marginal with activated (200 U ml^{-1} IL-1β + 500 U ml^{-1} TNFα + 100 U ml^{-1} γIFN) AEC (○). Values are the mean ± s.d. of four individual experiments. (b) In resting exocrine pancreatic endothelial cell (EPEC) cultures (●) Mg-Dobesilate led to a concentration dependent increase in citrulline production. With cytokine-activated EPEC (○) the Mg-Dobesilate-dependent augmentation of citrulline production was less and not obviously concentration dependent. Values are the mean ± s.d. of four individual experiments. (c) With resting islet endothelial cell (IEC) cultures (●) the Mg-Dobesilate-generated increase was significant and exhibited a clear-cut concentration dependence but increases were smaller than in large vessel endothelia. After cytokine challenge (○) citrulline production was not significantly increased at any of the concentrations used. Values are the mean ± s.d. of six to seven individual experiments. Statistical differences in citrulline concentrations measured after Mg-Dobesilate challenge were highly significant (*$P < 0.0001$) compared to controls (0 mM). (d) With activated (○) macrophages (MΦ) Mg-Dobesilate showed no significant effects on iNOS activity; (●) resting MΦ. Values are the mean ± s.d. of three individual experiments.

After Mg-Dobesilate-treatment no apparent increases in signal intensities of the ecNOS-mRNA amplification products could be detected although probes were always simultaneously amplified. Analogous experiments with capillary islet endothelial cells gave similar results.

Discussion

Nitric oxide synthases (NOS) play an important role in many physiological processes. The constitutive endothelial NO-synthase (ecNOS) has a pivotal role in regulation of blood pressure and haemostasis (Moncada & Higgs, 1993), whereas NO produced by the inducible isoform (iNOS) in cytokine-activated cells serves to protect the host from viruses, bacteria, protozoa, helminths, and tumor cells (for a review, see Kröncke *et al*., 1995). An impaired constitutive NO formation is associated with hypertension, with increased platelet aggregation, with thrombus formation, as well as with augmentation of platelet and leukocyte adhesion to the endothelium (Moncada *et al*., 1991; Langrehr *et al*., 1993). The data presented here provide evidence for a specific action of Dobesilate on the

Table 2 Inhibition of Mg-Dobesilate-induced increase of endothelial NOS-activities

	Mg-Dobesilate		
	0 mM	*1 mM*	*1 mM + L-NMA*
AEC			
Resting	1.0 ± 0.3	*4.3 ± 0.6	*1.4 ± 0.7
Activated	27.8 ± 4.2	*44.5 ± 5.9	*3.2 ± 1.0
EPEC			
Resting	8.1 ± 1.5	*30.8 ± 2.2	*3.1 ± 0.8
Activated	32.5 ± 3.4	*46.5 ± 2.5	*2.6 ± 1.3
IEC			
Resting	15.8 ± 3.2	*26.9 ± 2.5	*2.2 ± 0.9
Activated	35.0 ± 2.4	40.6 ± 1.7	*2.4 ± 0.8
Macrophages			
Resting	0.5 ± 0.5	0.5 ± 0.5	NT
Activated	15.7 ± 1.5	18.5 ± 1.5	NT

1×10^5 macrovascular aorta endothelial cells (AEC), exocrine pancreatic microvascular endothelial cells (EPEC) and islet capillary endothelial cells (IEC) were incubated for 24 h in RPMI 1640 medium only (resting) or in the presence of cytokines IL-1β (200 U ml^{-1}) + TNFα (500 U ml^{-1}) + γIFN (100 U ml^{-1}) (activated), respectively. Cells were cultured in the absence or presence of Mg-Dobesilate (1 mM) and the NOS-inhibitor N^G-monomethyl-L-arginine (L-NMA: 1 mM), respectively. Values (mean ± s.d. from four individual experiments) represent citrulline concentrations (nmol 1×10^5 cells/24 h) determined in culture supernatants as described in Methods. Differences in citrulline concentrations measured in the absence of Dobesilate compared to the presence of 1 mM Mg-Dobesilate as well as the reduction in citrulline production after addition of L-NMA were highly significant (*$P < 0.0001$). Additionally, resting and LPS activated peritoneal macrophages were cultured in the absence or presence of Mg-Dobesilate. Values are the mean ± s.d. from three individual experiments. NT: not tested.

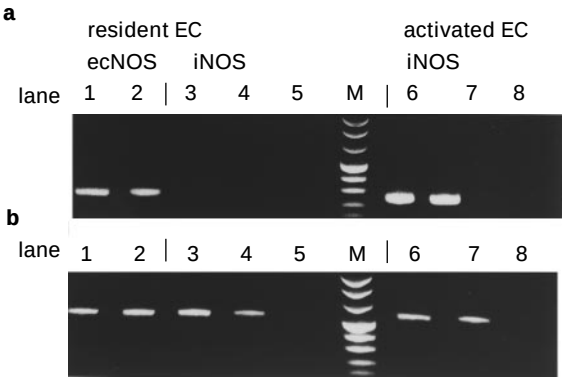

Figure 2 Detection of iNOS- and ecNOS-specific mRNA in aortic endothelial cells by polymerase chain reaction. Total RNA was extracted from aortic endothelial cells grown in the absence (resident) or in the presence (activated) of cytokines (200 U ml^{-1} IL-1β + 500 U ml^{-1} TNFα + 100 U ml^{-1} γIFN) or in addition in the absence or presence of Mg-Dobesilate (1 mM), respectively. PCR was performed with iNOS- or iNOS plus ecNOS-specific primers as described in Methods. (a) ecNOS/iNOS-specific amplification from resident cells in the absence (lane 1) or presence (lane 2) of Mg-Dobesilate; iNOS-specific amplification from resident cells in the absence (lane 3) or presence (lane 4) of Mg-Dobesilate; iNOS-specific amplification from cytokine-activated cells in the absence (lane 6) or presence (lane 7) of Mg-Dobesilate. An iNOS-specific amplification product (260 bp) was found in RNA from cytokine-challenged cells only (lanes 6 and 7). In resting cells an amplification product (268 bp) was obtained only by priming with the iNOS plus ecNOS specific oligonucleotides (lanes 1 and 2). This band represents the ecNOS specific product, since priming with iNOS-specific oligonucleotides gave negative results (lanes 3 and 4). Mg-Dobesilate-treatment (lanes 2, 4 and 7) showed no additional induction of the endothelial ecNOS- or iNOS- mRNA-expression, and did not induce a cumulative iNOS-mRNA-expression in resting cells (lane 4). Lanes 5 and 8: control for contaminating DNA-RNA at four times higher concentration was amplified with the respective oligonucleotides. Lanes in (b) show the specific GAPDH-cDNA amplification products (522 bp) of the corresponding cells. Lane 'M' represents DNA molecular weight marker VIII (Boehringer): fragments seen on the gel (bp): 1114, 900, 692, 489, 404, 320, 242, 190.

ecNOS-activity in endothelial cells. In resting endothelial cell cultures from different origin Mg-Dobesilate significantly increased in a concentration-dependent manner the citrulline production as an indirect measurement for nitric oxide synthase activity.

Under the experimental conditions used here, i.e. culturing in RPMI-medium containing arginine in a concentration of 1.15 mM citrulline measurements are a reliable indicator for endothelial NOS-activity as indicated by the parallel decrease in nitrite and citrulline in the presence of L-NMA and in agreement with earlier publications. It was shown that endothelial cells have no significant arginine deaminase activity (Hecker *et al*., 1990a), thus, exogenously added arginine, rapidly accumulated in endothelial cells, will not be converted to citrulline. In addition, endothelial cells are known to recycle citrulline to L-arginine, a pathway that could confuse the data. It has also been shown that this recycling occurs at L-arginine concentrations much lower than used here. Absence of L-citrulline metabolism was found in endothelial cells cultured in the presence of arginine concentration higher than 800 μM (Baydoun *et al*., 1990; Hecker *et al*., 1990a; b; Mitchell *et al*., 1990).

The Dobesilate-mediated increases in citrulline production by resident endothelial cell cultures are not due to an activating effect of the drug leading to expression of inducible NO-synthase as was confirmed by reverse transcriptase-polymerase chain reaction (RT-PCR) specific for iNOS mRNA, where absence of iNOS-specific signals was found. By using semi-quantitative RT-PCR we found the same signal intensity of ecNOS- or iNOS-mRNA expression irrelevant of the Dobesilate concentrations. Although this method does not allow for the accurate quantitation of mRNA content, increases in signal intensities are readily seen when probes are processed in parallel. We therefore conclude that the Mg-Dobesilate-mediated enhancement of citrulline production appears to be due to a modulation of the enzyme activity rather than de novo ecNOS expression.

Additionally, and in contrast to resident endothelial cells, incubation of cytokine-activated and iNOS-expressing endothelial cells led to small or absent Dobesilate-mediated increases in citrulline production. As activated endothelia express two NO-synthases, we cannot distinguish between citrulline produced by ecNOS versus iNOS. As increases in activated cells are small these are likely to represent augmented ecNOS activity. This is further supported by experiments using LPS activated rat peritoneal macrophages where incubation with Dobesilate in the same concentration range did not significantly affect the iNOS activities. As both ecNOS and iNOS use the same substrate and cofactors with the exception of Ca^{2+}, there are several possible mechanisms involved in Dobesilate-enhanced ecNOS activity, including increased life span for the individual protein, prolonged lifetime for the mRNA, increased mRNA translation efficiency, increased availability of free Ca-ions; a number of additional more indirect effects are also feasible.

Ca-Dobesilate (Doxium®) is widely used as an angioprotective agent for the treatment of vascular diseases and has been reported to act effectively in inhibiting platelet aggregation, thrombus formation, increased capillary permeability, capillary fragility, increased blood viscosity and other haemorrheological disturbances (Bayer *et al*., 1980; Barras & Graf, 1980; Heidrich *et al*., 1983; Michal & Gotti, 1988). After a single dose of 500 mg Dobesilate, serum concentrations in man are in the range 25–137 μM (Benakis *et al*., 1974). Thus, the Dobesilate concentrations used in the experiments are approximately 2 to 40 fold higher than these serum concen-

trations. However, during medication Dobesilate has to be taken for weeks until therapy effects become apparent, whereas under experimental *in vitro* conditions short-term effects are measurable only which require higher dosages for significant results.

This hitherto unknown effect of Mg-Dobesilate on the support of the natural constitutive NO-production by increasing the constitutive endothelial NO-synthase activity thus offers a possible explanation for the protecting effects of Dobesilate analogues on blood vessel function and integrity. The specific property of Dobesilate, i.e. moderate increases in eNOS activity with little or no influence on iNOS activity, provides evidence for its suitability in the treatment of vascular disorders, especially those caused by an insufficient or impaired constitutive endothelial NO production.

We thank Christa-Maria Wilkens, Marija Lovrencic and Ulla Lammersen for technical assistance, and Sabine Wenzel-Unger and Martha Turken for photographic assistance. This work was supported by a grant from the Deutsche Forschungsgemeinschaft (Ko806/6-1 to V.K.-B.).

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(Received April 4, 1997
Revised July 11, 1997
Accepted August 28, 1997)