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Primary Cultures of Rat Islet Capillary Endothelial Cells

Constitutive and Cytokine-Inducible Macrophagelike Nitric Oxide Synthases Are Expressed and Activities Regulated by Glucose Concentration

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We have succeeded in obtaining cultures of pure rat islet capillary endothelial cells. These multiply* in vitro *and exhibit the same antigenic phenotype as expressed* in situ*: von Willebrand factor^{high}, Ox43 (rat endothelial marker)^{weak}, and Ox2 (thymocyte and brain endothelium marker)^{high}. This phenotype differs from both exocrine endothelium stained* in situ *and rat aorta endothelial cells cultured* in vitro *under identical conditions. Islet and aorta endothelial cells were cultured in the presence of various glucose concentrations. Nitrite and citrulline concentrations in culture supernatants were measured as an indirect quantification of nitric oxide formation. In islet endothelia, both nitrite and citrulline levels were found to be strongly glucose-dependent, with high levels at high glucose concentrations and vice versa, in contrast to aorta endothelial cells, where no glucose effect was found. Shifting islet endothelial cultures from high to low glucose levels or the reverse led to a slow decrease or increase in nitrite and citrulline formation with several cell generations needed to reach steady levels. Adding a combination of the cytokines interleukin-1β, tumor necrosis factor-α, and interferon-γ to both endothelial cell cultures led to a dramatic increase of nitric oxide formation. Again with islet but not with aorta endothelial cells a modulating effect by glucose concentrations was found. Reverse-transcription-polymerase chain reaction with specific primers demonstrated the presence of constitutively expressed nitric oxide synthase-RNA in the islet capillary endothelial cells and confirmed the glucose effect. In addition, we found that cytokines indeed induce the expression of inducible synthase messenger RNA in both endothelial cells, which was not found in the absence of cytokines. Electron paramagnetic resonance spectroscopy of islet endothelial cells confirmed intracellular synthesis of nitric oxide in the presence of cytokines. In conclusion, we here for the first time provide evidence that constitutive nitric oxide synthase is also expressed in capillary endothelium and that cytokine challenge leads to the expression of the inducible isoform in these cells. (Am J Pathol 1994, 145:685–695)

Nitric oxide (NO) is a second messenger molecule with diverse functions such as vasodilation, neurotransmission, platelet aggregation, and it also functions as cytotoxic effector molecule (for review see refs. 1, 2). Endothelial cells are known to release endothelium-derived relaxing factor,[3] shown to be identical with NO,[4,5] synthesized by the recently cloned constitutively expressed nitric oxide synthase (ecNOS).[6–8] After challenge with various cytokines and/or bacterial endotoxin, endothelial cells are in ad-

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dition able to release larger quantities of NO[9,10] via an inducible nitric oxide synthase (iNOS) similar to or identical with the iNOS initially described for macrophages.[11]

Recently, NO has been shown to be involved also in the influence of interleukin-1β (IL-1β) on insulin release by pancreatic islet β-cells[12–15] as well as in mediating the islet-toxic effects of activated macrophages[16–18] or IL-1β.[19] This makes the excessive NO formation by iNOS within islets an important pathogenic effector mechanism during manifestation of type I diabetes.[20] In a normal islet, there are few macrophages but a large number of endothelial cells because of the high capillarization.[21,22] Thus we had speculated earlier[19] that the effect of IL-1β on islets may also involve induction of iNOS in the numerous capillary endothelial cells, leading to the local production of toxic concentrations of NO in the nearest neighborhood to insulin-producing β cells.

We now report results obtained with capillary endothelial cell cultures established by outgrowth from isolated rat islets. We found that these endothelial cells differ in phenotype from exocrine pancreatic endothelium and also from rat aorta endothelium cultured under the same conditions. Both islet and aorta endothelial cells can be induced for macrophagelike iNOS expression by challenging with cytokines. The constitutive NO production of islet endothelium shows in addition a unique regulatory influence of the glucose concentration in culture media.

Materials and Methods

Reagents

Recombinant human IL-1β and recombinant murine tumor necrosis factor-α (TNF-α) were from Genzyme (Cambridge MA), recombinant rat interferon (IFN-γ) was from HBT (Leiden, Netherlands). The lipopolysaccharide content in these various cytokine batches never exceeded 0.3 ng/μg protein. Endothelial cell growth supplement, type I collagen, collagenase (from *Clostridium histolyticum*), N^G-methyl-L-arginine (NMA), urease type VII and rabbit anti-human von Willebrand factor (vWF) antiserum were from Sigma (Deisenhofen, FRG). Monoclonal antibodies Ox2 and Ox43 were from Serotec (Camon, Wiesbaden, FRG). A monoclonal anti-vimentin antibody (clone V9) was from Boehringer (Mannheim, FRG), peroxidase-conjugated porcine anti-rabbit immunoglobulin G (IgG) from DAKO (Hamburg, FRG), and peroxidase-conjugated goat anti-mouse IgG was from Zymed Laboratories, Inc. (San Francisco, CA). Trypsin, Ethylenediaminetetraacetic acid, fetal calf serum (FCS, endotoxin-free), RPMI 1640 (endotoxin-free) was purchased from Boehringer Mannheim or Gibco Laboratories (Eggenstein, FRG). 3,3′-diaminobenzidine was from Serva GmbH (Heidelberg, FRG), Ficoll 400 from Pharmacia (Uppsala, Sweden). Some batches of RPMI 1640 were mixed from individual substances (all p.a. grade, Sigma), according to standard recipe, with the exception of glucose and using $CaCl_2$ instead of $Ca(NO_3)_2$.

Animals

Male Wistar rats (about 200 g) from the University breeding facility received a standard diet and tap water *ad libidum*.

Endothelial Cells

Islet endothelial cells (IECs) were isolated from hand-picked pancreatic islets by outgrowth on a collagen type I matrix. Pancreatic islets were harvested by ductal injection of collagenase and subsequent centrifugation of the dispersed tissue on a Ficoll density gradient.[23]

Vascular endothelial cells were isolated by outgrowth from rat aortic rings exactly as described.[11,24] Wistar rats were anesthetized, the thoracic aorta was removed, rinsed in Hanks' balanced salt solution, cleaned of periadventitial fat and connective tissue, and then cut into rings <2 mm in width.

Hand-picked whole islets and aortic segments were placed on top of the collagen gel (1.8 mg collagen/ml) in 24-well tissue culture plates and incubated in RPMI 1640 with 20% FCS and 100 μg endothelial cell growth supplement/ml in a humidified incubator at 37 C in a 95% air/5% CO_2 atmosphere for 5 to 8 days, depending on the degree of outgrowth of the endothelial cells. Islets and aortic explants were then removed, cells detached with 0.25% collagenase in Hanks' balanced salt solution 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% ethylenediaminetetraacetic acid in isotonic NaCl for 3 minutes.

Phenotype Characterization of Endothelium on Pancreatic Cryostat Sections

Rat pancreata were removed, embedded in Tissue-Tek (Reichert-Jung, Vienna, Austria), and immediately frozen in liquid nitrogen. Cryostat sections (7 μ) were fixed with 0.2% glutaraldehyde in Tris-buffered saline (TBS), pH 7.0, followed by three washing steps in TBS. After blocking of unspecific binding with 0.5% bovine serum albumin in TBS for 30 minutes and rinsing, sections were incubated with monoclonal antibodies Ox43 or Ox2 (diluted 1:10) in a moist chamber overnight. Slides were washed three times with TBS for 5 minutes. As secondary antibody peroxidase-conjugated goat anti-mouse IgG was used in a final dilution of 1:30 for 1 hour in TBS. All steps were performed at 4 C. After washing in TBS, sections were incubated with 0.05% diaminobenzidine + 0.015% H_2O_2 for 5 minutes at room temperature.

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 phosphate-buffered saline (PBS) and fixed with acetone at −20 C for 10 minutes. The coverslips were washed with PBS, incubated with the rabbit anti-vWF antiserum (1:50 dilution) at room temperature for 45 minutes, washed extensively with PBS, and incubated in a 1:50 dilution of peroxidase-conjugated porcine anti-rabbit IgG for 45 minutes at room temperature, then again washed extensively with PBS, and peroxidase activity was visualized as above. To exclude anti-fibronectin reactivity in the commercially available anti-vWF serum, aliquots were absorbed with rat plasma fibronectin immobilized on colloidal gold before use. Control cultures were incubated with a nonrelevant rabbit hyperimmunserum instead of first antiserum. As positive controls isolated human platelets, and the fibroblastoma cell line L929, and as negative controls isolated rat alveolar macrophages were also tested with this anti-vWF antiserum. A rat vascular endothelium-specific monoclonal antibody Ox43[25] and the rat thymocytes and brain endothelium-specific monoclonal antibody Ox2[26] were used in a 1:10 dilution. Peroxidase-conjugated goat anti-mouse IgG was diluted 1:30 before use. Otherwise, conditions were as described above.

Experimental Design

All measurements were performed with cells from passages 6 to 9. Endothelial cells (8×10^4) were incubated in 24-well tissue culture plates for 24 hours in a humidified incubator at 37 C in a 95% air/5% CO_2 atmosphere in 600 μl RPMI 1640 with 20% FCS in the presence of IL-1β, TNF-α and IFN-γ as indicated. Lipopolysaccharide concentrations of RPMI 1640 and FCS were below 1 ng/ml. After the incubation, nitrite was determined in culture supernatants using the diazotization reaction as modified by Wood et al[27] and $NaNO_2$ as standard. Citrulline was determined using a colorimetric assay exactly as described.[11,28] The assay uses the reaction with diacetyl monoxime (5 g/L) in the presence of sulfuric acid (25%), phosphoric acid (20%), and $FeCl_3$ (250 mg/L). Culture supernatants (400 μl) were incubated with 400 μl urease (45 U/ml) for 30 minutes. The mixture was deproteinized by adding trichloroacetic acid to a final concentration of at least 5%. After centrifugation, 750 μl of the supernatant and 3 ml of the chromogenic solution were boiled for 5 minutes, cooled to room temperature and absorbance measured at 530 nm in an enzyme-linked immunosorbent assay-reader. From resulting extinctions controls with medium and respective additives but without cells were substracted. Extinctions were calculated against a citrulline standard.

Polymerase Chain Reaction (PCR)

Total cellular RNA, prepared from cytokine-stimulated and unstimulated islet endothelial cells[29] (10 μg each) was used for complementary (c)DNA synthesis[30] using the oligonucleotide ACTTCCTCCAGGATGTTGTA (anti-sense; bp 1338 to 1357 of mouse iNOS cDNA) as primer.[31] Neither rat iNOS nor rat ecNOS sequences are currently available. We therefore searched for a general NOS primer by multialignment of the available NOS-sequences. This was used in reverse transcription (RT) as well as anti-sense primer in polymerase chain reaction (PCR). RT was carried out at 55 C for 45 minutes. The cDNA was then used as template for PCR either primed by the sense oligonucleotide TGATGTGCTGCCTCTGGTCT (sense; bp 1079 to 1098 of mouse iNOS cDNA) for specific iNOS amplification, or primed by the oligonucleotide GGCCGCTTCGACGTGCTGCCT (sense; consensus sequence of mouse iNOS cDNA, bp 1071 to 1090, and human ecNOS cDNA, bp 870 to 889) for amplification of both, iNOS plus ecNOS. PCR was carried out following standard protocols.[32] Cycle profile: 30 seconds at 94 C, 1 minute at 55 C, 1 minute at 72 C.

After a total of 32 cycles, a final incubation step was performed at 72 C for 10 minutes. An aliquot of each reaction was subjected to electrophoresis on 2% agarose gels. Bands were visualized by ethidium bromide staining.

EPR Spectrometry

IECs were cultured to near confluency in dishes (10 cm of diameter) in RPMI/10% FCS containing 4 mmol/L glucose. Cells were then incubated with or without cytokines (200 U/ml IL-1β, 150 U/ml TNF-α, 25 U/ml IFN-γ) for 24 hours. Cells (8×10^6) were harvested by ethylenediaminetetraacetic acid/trypsin treatment, washed twice with PBS, and transferred to quartz electron paramagnetic resonance (EPR) tubes. After centrifugation (250 *g* for 3 minutes), tubes were stored in liquid nitrogen. EPR spectra were taken using a computer controlled X-band spectrometer (ESP 300, Bruker, Karlsruhe, FRG). All samples were cooled to 90 K while recording the spectra. The machine parameters used were: 10 mW microwave power, 100 kHz modulation frequency, 10 G modulation amplitude, and a conversion time constant of 164 ms. The g-factor calibration was controlled using the DPPH-signal with g = 2.0036.

Results

In Situ *Antigenic Phenotype of Endothelium in Rat Pancreata*

On snap-frozen cryostat sections of rat pancreata, the phenotype of endothelial cells was determined by indirect immunohistochemistry using the monoclonal antibodies Ox43, Ox2, an anti-vimentin antibody as well as an anti-human vWF antiserum cross-reacting with the rat epitope. As shown in Figure 1, the endocrine portion of pancreata is capillarized with endothelia of a phenotype distinguishable from those in the exocrine portion of the tissue. Whereas both capillaries strongly stain with anti-vWF and anti-vimentin (not shown), only the exocrine portion expresses the Ox43 antigen. On the other hand, the Ox2 antibody stains endothelium within islets and gives a weak signal only in the exocrine part of the tissue.

Characterization of Cultured Islet Capillary Endothelial Cells

IECs were obtained by outgrowth from isolated rat islets (Figure 2) on a collagen matrix in the presence of endothelial growth supplement as described in de-

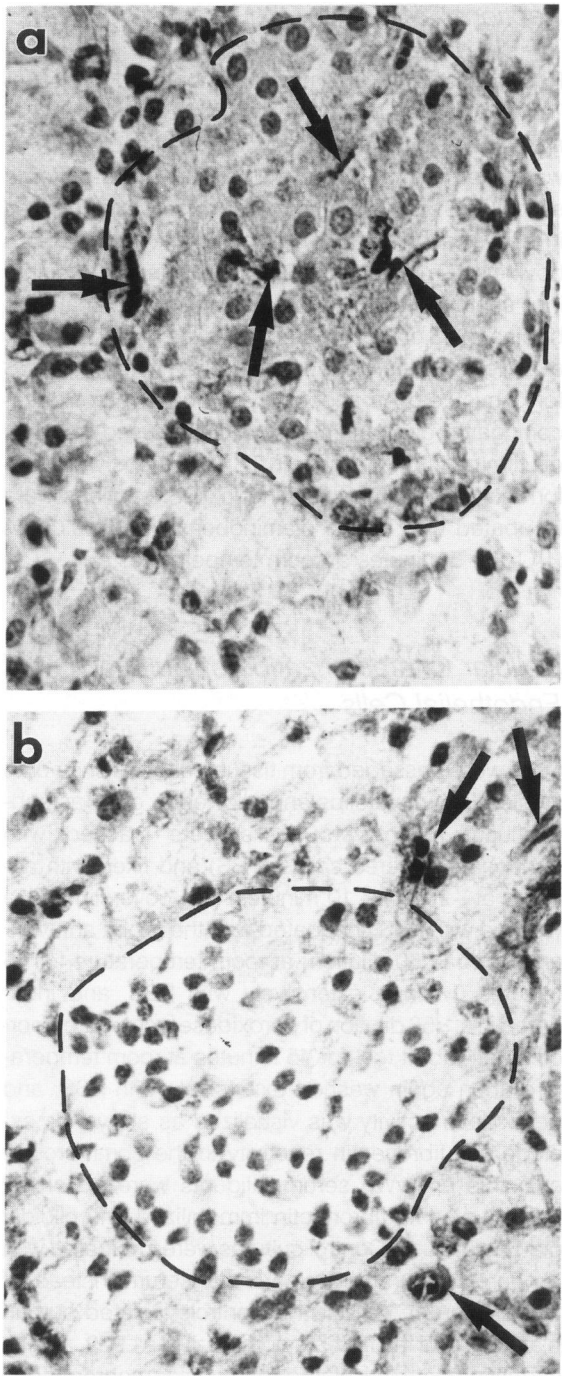

Figure 1. In vivo *antigenic phenotype of islet capillary endothelium. Cryostat sections of Wistar rat pancreata were stained for rat endothelial antigens by indirect immunocytochemistry with Ox2* (**a**) *or with Ox43* (**b**). *Whereas the islet area (indicated by dashed line) shows a clear-cut endothelial staining (arrows) with Ox2, staining is completely absent from islet area using Ox43. The few capillaries in the exocrine portion exhibit the opposite antigen pattern. Magnification: 650×.*

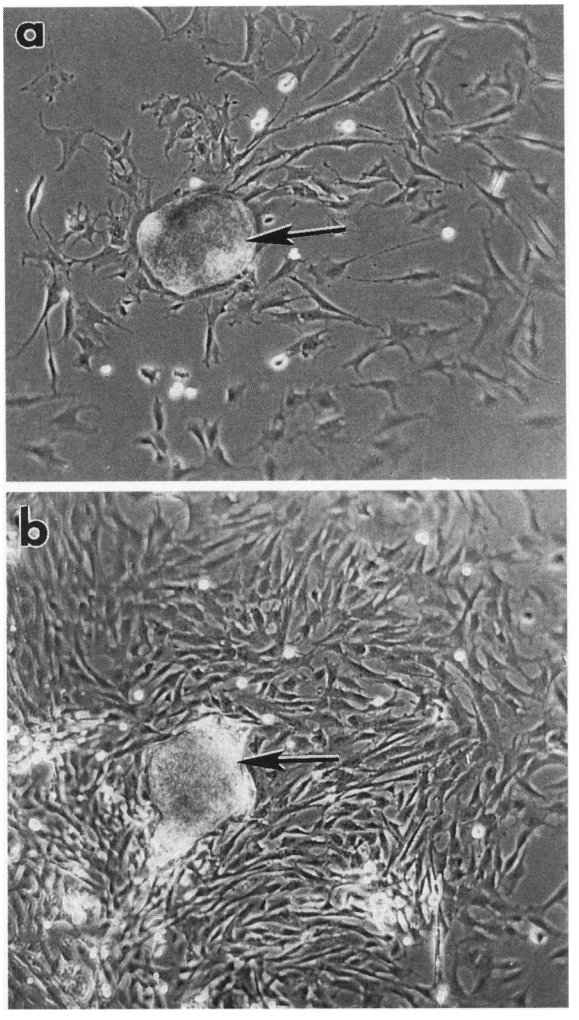

Figure 2. *Outgrowth of endothelia from isolated islets. Isolated rat islets (arrows) were placed on a collagen matrix and cultured as indicated in Materials and Methods. After 3 days, outgrowth of endothelial cells is be visible* (a), *after 10 days of culture, the collagen matrix around individual islets is evenly occupied by these cells* (b). *Magnification: 140×.*

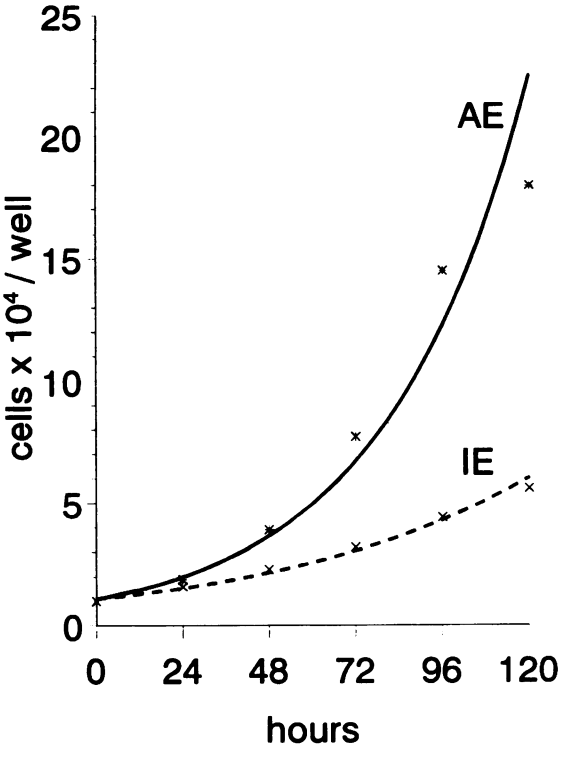

Figure 3. *Growth rates of endothelial cells. Aorta endothelial cells (AE) and islet endothelial cells (IE) were seeded and grown under identical conditions. Cells were quantitated at different times by neutral red staining. AECs grow with a doubling time of approximately 24 hours, IECs grow somewhat slower with a generation time of approximately 30 hours.*

tail in Materials and Methods. IECs were compared to rat aorta endothelial cells (AECs) cultured under identical conditions. Cell multiplication was slower with IECs (Figure 3), where duplication times of 30 to 36 hours were measured versus 22 to 24 hours with AECs.

Immunocytochemistry with the antibody panel described above confirmed the specific islet capillary phenotype for the cultured cells (Figure 4), whereas AECs expressed a similar phenotype as found for vessel lining cells in the exocrine portion (Table 1). These labeling experiments also showed that cultures consisted of pure endothelial cells, inasmuch as the respective staining patterns with the endothelial specific markers were found in absolutely all cells.

Nitrite and Citrulline Concentrations in Culture Supernatants

IEC and AEC cultures were maintained in culture media containing 1, 4, 11, or 20 mmol/L glucose respectively. Nitrite and citrulline concentrations in supernatants of these cultures (10^5 cells, 24-hour culture period) were measured. Nitrite and citrulline concentrations in supernatants of AECs were identical at all glucose concentrations. In supernatants of IECs grown in the presence of 1 mmol/L glucose nitrite and citrulline levels were not significantly different from AEC cultures. Culturing IECs at higher glucose concentrations resulted in increased nitrite and citrulline levels (Figure 5) with a strong correlation to the glucose concentrations. Between 1 mmol/L and 20 mmol/L glucose nitrite and citrulline concentrations increase by a factor of 5. Neither growth rates nor viability of IECs and AECs showed significant differences in these experiments. Shifting IEC cultures from high to low glucose (from 11 mmol/L to 1 mmol/L) will not immediately result in decreases of nitrite and citrulline values, but instead a slow decrease is found that levels out after three passages equalling about 8

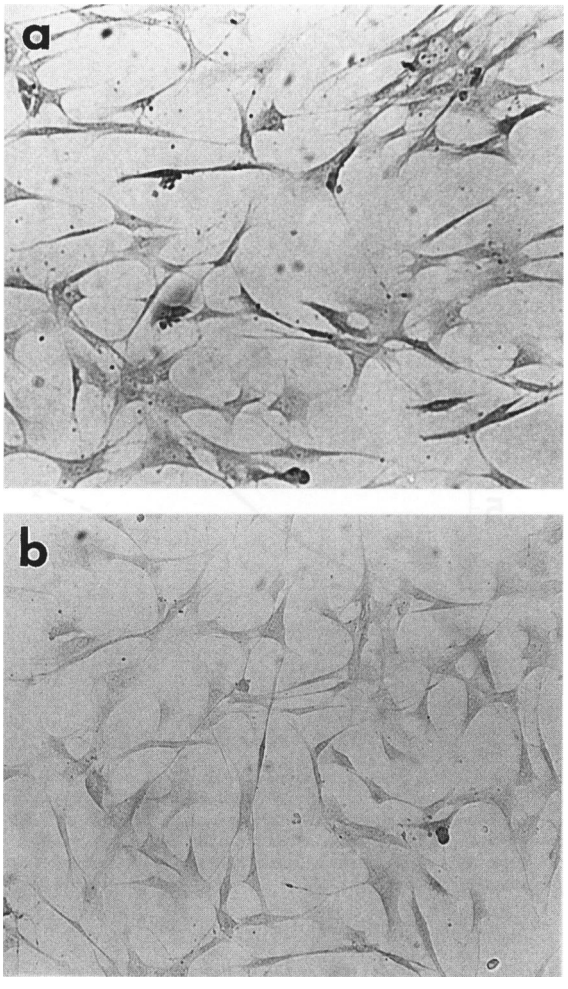

Figure 4. In vitro *antigenic phenotype of islet endothelial cells. IECs were grown for 24 hours on sterile glass coverslips, then fixed, and stained by indirect immunocytochemistry for expression of antigens recognized by Ox2* (a) *or Ox43* (b). *Cells stain strongly for Ox2 and weakly for Ox43. Magnification: 180×.*

Table 1. *Expression of Endothelial Antigens on AECs or IECs* in Vitro *as Determined by Indirect Immunocytochemistry*

Antigen	AECs*	IECs*
von Willebrand factor	High	High
Vimentin	High	High
Ox43	High	Low
Ox2	Low	High

* The expression pattern was found in 100% of cells in all cultures as an indication for the purity of the respective endothelial cell cultures.

to 10 cell division cycles (Figure 6). The same slow alteration was found for the reverse shift (from 1 mmol/L to 11 mmol/L glucose) as well. Shifts could be repeated with the same cells, always leading to the same results, which makes it improbable that subpopulation selection occurs.

We have reported previously, that AECs can be challenged with cytokines to express an inducible type of NOS. Here we tested whether IECs can also be provoked with cytokines to produce elevated amounts of nitrite and citrulline comparable to those found for AECs and whether glucose concentrations modulate this response as well. Culturing IECs and AECs in the presence of IL-1β (200 U/ml) plus TNF-α (500 U/ml) plus IFN-γ (100 U/ml) led to a significant increase in the two stable end products of NO synthesis in the culture supernatants (Figure 7). With AECs, no significant differences between cultures at various glucose concentrations were found. In contrast, with IECs, glucose concentrations again significantly modulated this response. As a direct proof for the intracellular formation of NO, EPR spectroscopy of resident and cytokine-activated IECs was performed. As shown in Figure 8, activated but not resident IECs showed the characteristic NO-specific axial feature at g = 2.04. The arginine antagonist NMA (1 mmol/L) decreased the glucose- and cytokine-dependent formation of nitrite and citrulline in islet endothelial cell culture supernatants (Figures 5 and 7).

Presence of Endothelial ecNOS and Macrophagelike iNOS messenger (m)RNA after Cytokine Challenge

RT-PCR was performed with total RNA extracted from IECs grown in culture medium containing 1, 4, or 11 mmol/L glucose with or without cytokines. The RT and PCR was primed by oligonucleotides for specific iNOS amplification or iNOS plus ecNOS-specific oligonucleotides to detect rat ecNOS-specific RNA. Primers were designed by using a multialignment of published NOS sequences, searching for a common sequence as mentioned in Materials and Methods. As shown in Figure 9, only cytokine-treated cells yielded an amplification product for iNOS (Figure 9, lanes 10 to 12). No signal was obtained with RNA from cells grown in the absence of cytokines (Figure 9, lanes 6 to 8) but at high glucose concentration, despite the relatively high concentrations of nitrite and citrulline in culture supernatants. Using the iNOS plus ecNOS-specific oligonucleotides as primers, a signal was obtained in all nonchallenged IEC cultures (Figure 9, lanes 2 to 4), which represents ecNOS-specific amplification products, inasmuch as the iNOS-specific amplification was negative. A significant and reproducible tendency for increasing amounts of PCR product with increasing glucose concentration was found.

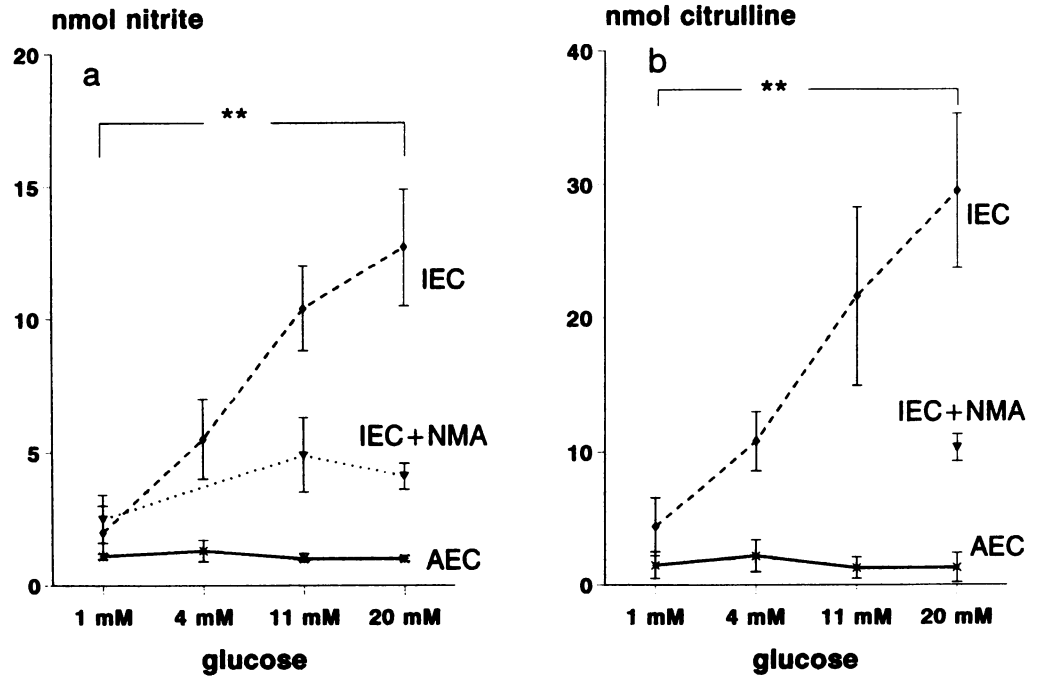

Figure 5. *Effect of glucose concentrations on nitrite and citrulline levels in culture supernatants. Aorta endothelial cells (AEC) and islet endothelial cells (IEC) were maintained at various glucose concentrations. After 24 hours of culture (1 × 10^5 cells each) nitrite* (a) *and citrulline concentrations* (b) *in culture supernatants were determined as indicated in Materials and Methods. Values are the mean ± S.D. of four to eight individual experiments. A strong correlation between nitrite/citrulline production and glucose concentration is found with IECs but not with AECs. Statistical differences in nitrite and citrulline concentrations measured in IEC cultures between 1 and 20 mmol/L glucose were highly significant (**P < 0.0001), the same significance was found between IECs and AECs at 4, 11 and 20 mmol/L. The glucose-dependent nitrite and citrulline levels in islet endothelial cell culture supernatants were significantly decreased by the addition of 1.0 mmol/L NMA (IEC+NMA).*

Discussion

Evidence is accumulating that endothelial cells play a significant role in inflammatory processes, and cytokines are known modulators of many of these endothelial cell functions.[33,34] One of the prominent effects that cytokines can excert in these cells is the induction of massive NO synthesis[9,10,35,36] by an enzyme, which we could recently show to be closely related or identical to the inducible enzyme isoform of macrophages.[11] This NO synthase isoenzyme is expressed in addition to the constitutive endothelial calcium/calmodulin-dependent isoform[1] in these same cells. NO produced by the latter form regulates the local dynamic control of vascular tone and blood supply,[37] whereas the former pathway seems to play a crucial role in inflammatory processes[17] and in pathophysiological control of blood supply.[9,10,35] Most of our current knowledge comes from *in vitro* experiments, and we have little insight into organ specificities of these pathways, neither concerning the expression of the constitutive nor of the inducible isoforms. Evidence is, however, accumulating that NO has a pivotal role in some aspects of the physiology of insulin release and islet β cell function as well as in the process of islet cell destruction preceding manifestation of insulin-dependent (type I) diabetes. It has been shown that IL-1β enhances or inhibits insulin release from islet β cells[38–40] and that this effect can be abrogated by addition of arginine analogs,[12–15,41,42] as an indication for the involvement of NO formation. Furthermore, we could show that activated macrophages kill syngeneic islets at a very low target:effector ratio[23,43] via NO[16,18] and that the cytotoxic effect of high doses of IL-1β on isolated islets can also be inhibited by arginine analogs.[19] It has generally been assumed that this islet cell cytotoxicity is due to macrophages of the activated phenotype infiltrating very early during onset of islet-destructive processes preceding diabetes manifestation in animal models of this disease.[44–46]

With the results presented here, we now provide for the first time evidence that the capillary-lining endothelial cells of islets may contribute considerably both to physiological responses as well as to the formation of potentially toxic concentrations of NO. We here demonstrate that mRNA for the ecNOS similar to the one expressed in human umbilical vein endothelium[8] is also found in these rat islet capillary endothelia, although there is no underlining smooth muscle cell

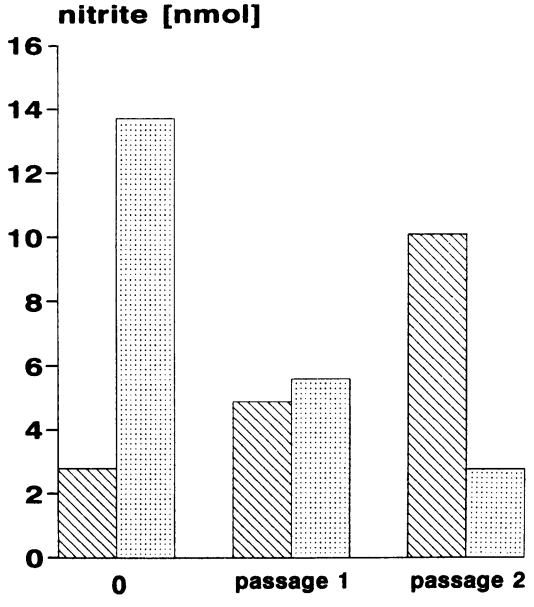

Figure 6. *Glucose concentration shift of IEC cultures. IEC cultures maintained at 1 mmol/L glucose (hatched bars) were shifted to 11 mmol/L glucose and other cultures maintained at 11 mmol/L glucose (dotted bars) were shifted to 1 mmol/L glucose. Nitrite concentrations were measured for the first 24 hours after passage in a parallel culture made of 1 × 10^5 cells grown in 24-well plates, otherwise cells were grown in dishes 10 cm of diameter. Each passage was performed after four to five cell divisions. Values are from one out of three experiments with similar results. Nitrite concentrations as an indirect measurement of NO synthase activities indicate a very slow response to the shift in glucose concentrations.*

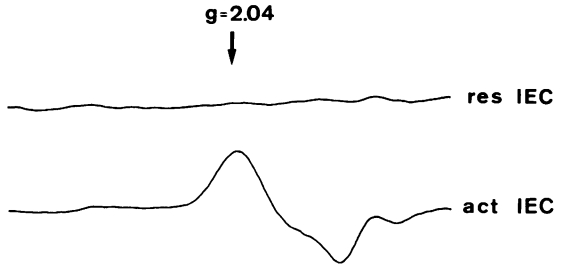

Figure 8. *EPR-spectroscopy of islet endothelial cells with or without cytokines. Islet endothelial cells were grown in the presence of 4 mmol/L glucose with or without cytokines (same combination as in Figure 7) for 24 hours. Cells (8 × 10^6 each) were detached, transferred and centrifuged in EPR tubes, and snap-frozen. The characteristic NO-specific axial feature at g = 2.04 is clearly visible with cytokine-challenged cells but not in unchallenged cultures.*

layer as a target cell for the NO synthesized by the endothelial ecNOS. Furthermore, adding a combination of cytokines previously shown to induce effectively iNOS in rat AECs[11] leads to a rapid and readily detectable iNOS-specific mRNA formation and massive increases in nitrite and citrulline levels in culture supernatants. The relative concentrations of nitrite and citrulline are comparable to or even higher than the values reported for activated macrophages and the intracellularly formed NO can be readily detected by EPR spectroscopy.

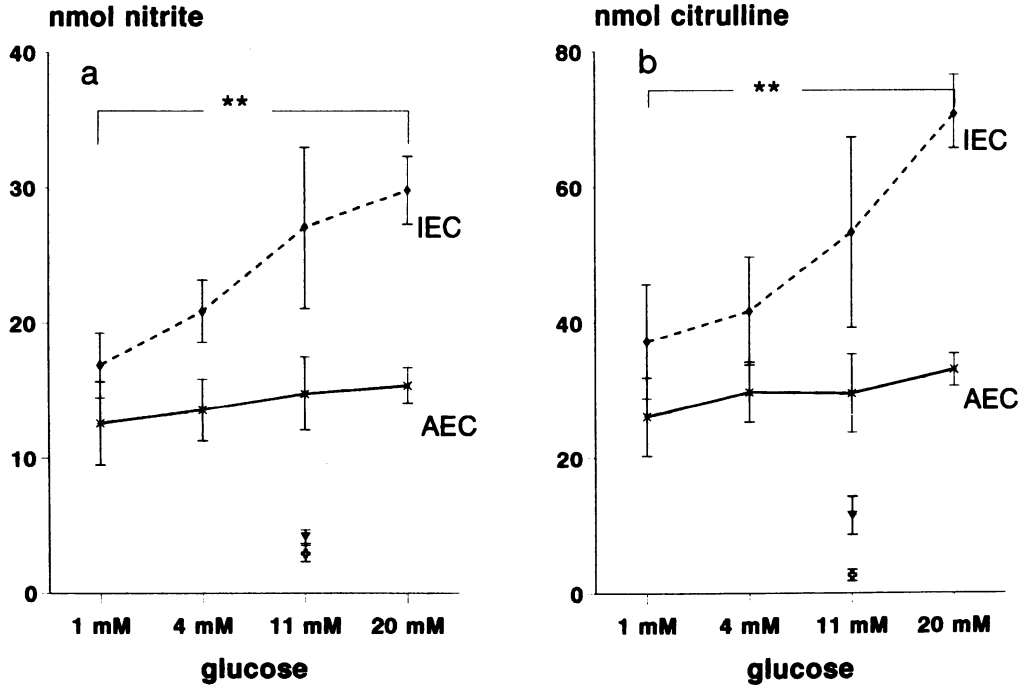

Figure 7. *Effect of glucose-concentration on nitrite and citrulline formation after cytokine challenge. Addition of a combination of cytokines (200 U/ml IL-1β, 500 U/ml TNF-α, 100 U/ml IFN-γ) leads to a dramatic increase in the levels of nitrite (a) and citrulline (b) found in culture supernatants of both, aorta endothelial (AEC) and islet endothelial cells (IEC). Values are the mean of three to eight experiments ± S.D. Glucose concentrations in the culture media influenced islet endothelia but not aorta endothelia. Values in IEC cultures at 1 mmol/L glucose are significantly different from 20 mmol/L (**P < 0.0001) but not in AEC cultures. Addition of 1.0 mmol/L NMA significantly decreased the cytokine-dependent nitrite and citrulline formation of aorta endothelial (■) and islet endothelial cells (▲).*

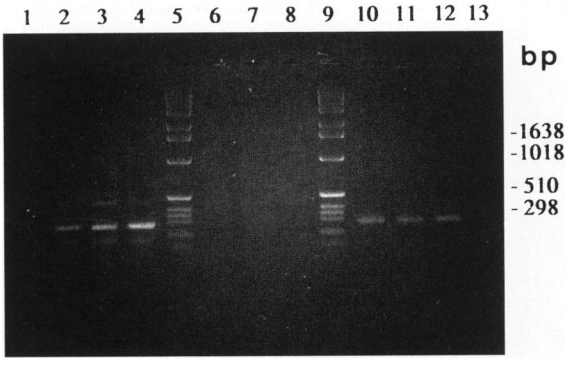

Figure 9. *Detection of iNOS- and iNOS plus ecNOS-specific mRNA in islet endothelial cells by PCR. Total RNA was extracted from IECs. Cells were grown in medium containing different glucose concentrations (1 mmol/L =* lanes 2, 6, 10; *4 mmol/L =* lanes 3, 7, 11; *11 mmol/L =* lanes 1, 4, 8, 12, 13*) in the absence (*lanes 2 *to* 4 *and* 6 *to* 8*) or in the presence (*lanes 10 *to* 12*) of cytokines as in Figure 7. PCR was performed with iNOS- or iNOS plus ecNOS-specific primers for 32 cycles as described in Materials and Methods. An iNOS-specific amplification product was found in RNA from cytokine-challenged cells (*lanes 10 *to* 12*) but not in noninduced cultures (*lanes 6 *to* 8*). In noninduced cells, an amplification product was obtained only by priming with the iNOS plus ecNOS-specific oligonucleotides (*lanes 2 *to* 4*). These amplification products represent the ecNOS-specific product, as priming with iNOS-specific oligonucleotides (*lanes 6 *to* 8*) gave negative results. As a control for contaminating DNA RNA (at four times higher concentration) was also amplified with the respective oligonucleotides (*lanes 1 *and* 13 *from untreated or cytokine treated cells, respectively). Lanes 5 *and* 9 *represent size markers.*

Our results are in favor of an organ-specific pattern of NO formation. In association with our finding that islet capillary endothelium expresses a phenotype that distinguishes these cells from the exocrine vessel forming cells as well as aorta endothelium, we also found a unique pattern of ecNOS activity exhibiting a close regulation by the glucose concentration present in the culture media. This effect indeed seems to be an organ-specific control of NO formation, as rat aorta endothelium cultured under identical conditions does not show any glucose sensitivity. The glucose-mediated regulation is not due to induction of iNOS isoform at high glucose concentration, inasmuch as there is absolutely no detectable iNOS-specific PCR product present and it is a comparatively slow, long-term effect as the culture shift experiments show, where we found that several cell generations are needed for full expression of the difference. Again also after cytokine challenge, nitrite values in culture supernatants of islet endothelia showed a glucose regulation not found in aorta cells.

It is conceivable that this latter effect reflects the contribution of ecNOS-mediated NO production; however, a downregulation of ecNOS message during cytokine challenge has been described[6] in large vessel endothelia. Due to species problems, we could not directly address this problem in our RT-PCR, but it is an intriguing idea that impaired glucose homeostasis aggravates tissue destruction via increased local NO production.

In conclusion, we here present evidence that constitutive and cytokine-inducible NO synthases are expressed in cultured islet capillary endothelial cells, that there is good evidence for an organ-specific regulation, and that it is conceivable that endothelia can contribute considerably to inflammatory processes via this pathway in organ-specific diseases.

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