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European Journal of Pharmacology 411 (2001) 231–243

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In vitro effects of dexamethasone on hypoxia-induced hyperpermeability and expression of vascular endothelial growth factor

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Received 26 September 2000; received in revised form 8 November 2000; accepted 28 November 2000

Abstract

Clinically, dexamethasone is known to reduce cerebral edema. To further investigate the mechanism of this neuroprotection, an in vitro model of brain-derived microvessel endothelial cells (BME cells) was used to investigate the effect of dexamethasone on hypoxia-induced hyperpermeability. Furthermore, the expression of vascular endothelial growth factor (VEGF), which is known to be the mediator of hypoxia-induced hyperpermeability, was evaluated. Dexamethasone (40 μg/ml = 100 μM) decreased hypoxia-induced permeability and VEGF expression significantly during time periods of more than 3 h. The time dependence of the dexamethasone effect correlated with a changed mechanism by which hypoxia induced VEGF expression. This was deduced because hypoxia-induced hyperpermeability and VEGF mRNA level were decreased in the presence of an antisense oligonucleotide coding for a region which binds a mRNA stabilizing protein, but only up to 3 h of hypoxia. Furthermore, during this time period the half-life of VEGF mRNA was increased. Results suggest that dexamethasone only decreases transcriptional-induced VEGF expression and that this may be related to the efficacy of dexamethasone to treat brain edema.

Keywords: Dexamethasone; VEGF (Vascular endothelial growth factor); Hypoxia; Endothelial cell

1. Introduction

The endothelial cells that line the cerebral microvessels differ morphologically and functionally from the cells of other vascular beds and form in vivo the blood–brain barrier. The inter-endothelial tight junctions, the absence of fenestrations, the paucity of pinocytotic vesicles, and the specialized membrane transport system collectively endow these cells with unique properties, which limit the amount of para- and trans-cellular flux across the blood–brain barrier and regulate the passage of most substances and drugs into the brain (Bradbury, 1984; Brightman and Reese, 1969). Pathological conditions such as ischemia, brain tumors or head injury are accompanied by endothelial cell dysfunction resulting in increased permeability across the blood–brain barrier (Betz and Coester, 1990; Sampaola et al., 1991). As a result, plasma components leak from the blood stream into the extracellular space of the brain, leading to the development of vasogenic cerebral edema. The use of glucocorticosteroids in the treatment of cerebral edema was described first by Galicich and French (1961) and further clinical investigation has demonstrated the effectiveness of glucocorticosteroids in reducing cerebral edema (Galicich and French, 1961; Rasmussen and Gulati, 1962). Although this observation has generally been confirmed by laboratory experiments, the mechanism by which steroids produce their beneficial effect is still unclear. Previously, the capacity of corticoids to reduce edema or to prevent new blood vessel formation was suggested to result, at least in part, from the ability of these agents to abolish the expression of vascular endothelial growth factor (VEGF) (Criscuolo, 1993; Nauck et al., 1998).

VEGF, also known as vascular permeability factor (VPF) or vasculotropin, is a specific endothelial cell mitogen (Ferrara and Henzel, 1989; Keck et al., 1989) that stimulates migration (Senger et al., 1996), enhances vascular permeability (Keck et al., 1989; Leung et al., 1989), increases edemagenesis (Criscuolo, 1993), and promotes angiogenesis (Leung et al., 1989). Molecular cloning of the cDNA suggests that in human cells at least four species of

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0014-2999/01/$ - see front matter
PII: S0014-2999(00)00915-8

VEGF can occur as 121-, 165-, 189-, and 206-amino acid forms, which are generated by alternative splicing of mRNA. Recent studies have shown that VEGF expression is inducible by hypoxia (Plate et al., 1992; Shweiki et al., 1992), a condition that eventually leads to necrosis and stimulates angiogenesis in neighboring tissue (Adair et al., 1990; Knighton et al., 1983). In tumors, VEGF expression is localized to ischemic areas and was shown to be responsible for extravasation of fluid. Previous permeability studies using an in vitro model of brain-derived microvascular endothelial cells (BME cells) cultured on polycarbonate membranes revealed that hypoxia increased the permeability of the BME cell monolayer, which is mediated by VEGF in an autocrine manner (Fischer et al., 1999). We demonstrate in this study that dexamethasone reduces the hypoxia-induced hyperpermeability of the BME cell monolayer in a time-dependent manner correlating to an inhibition of VEGF expression. It has been shown that VEGF expression induced by increasing VEGF mRNA stability is not inhibited by dexamethasone (Finkenzeller et al., 1995), whereas transcriptional gene upregulation is decreased by dexamethasone (Jonat et al., 1990; Schüle et al., 1990). Therefore, examining the effect of dexamethasone on VEGF expression after different periods of hypoxia will increase knowledge about the time course of the mechanism of hypoxia-induced VEGF mRNA expression in BME cells.

2. Materials and methods

2.1. Cell culture

Capillary endothelial cells were isolated from porcine brains as described previously (Mischek et al., 1989). The grey matter was digested with dispase II (0.8 mg/100 ml) for 3 h at 37°C. Capillaries were isolated after centrifugation with 15% Dextran. The Pellet was resuspended in medium M199 and treated with collagenase/dispase (1 mg/ml) for 5 h at 37°C. Endothelial cells were obtained after a Percoll gradient centrifugation. Cells were seeded on Biocoat Petri dishes (Becton Dickinson, Heidelberg, Germany) or on rat tail collagen I (Becton Dickinson, Heidelberg, Germany) coated polycarbonate transwell inserts with a pore size of 0.4 μm and a diameter of 12 mm (Millipore, Eschborn, Germany) and incubated at 37°C in a 5% CO_2-humidified incubator. The cells were grown in M199 medium supplemented with 20% [v/v] fetal calf serum, 200 units/ml penicillin, 200 units/ml streptomycin, and 2.5 μg/ml amphotericin B. All ingredients were purchased from Life Technologies (Eggenstein, Germany). After 7 days, the primary cultures formed confluent monolayers. Cells were characterized by analyzing endothelial marker enzymes such as alkaline phosphatase and γ-glutamyltransferase, using assay kits from Sigma (München, Germany). Furthermore, the uptake of acetylated low density lipoprotein (Dil-Ac-LDL) (Paesel and Lorei, Frankfurt, Germany) (Voyta et al., 1984) and the expression of the glucose transporter I were used as markers for cerebral endothelial cells. Pericytes and astrocytes were characterized by immunofluorescence staining with anti-α-smooth muscle actin (Sigma) and anti-glial fibrillary acidic protein (Boehringer, Mannheim, Germany) antibodies. Cultures used for our experiments contained less than 5% pericytes. Cells of astroglial origin could only be detected in some cultures but their content was always less than 1%.

2.2. In vitro hypoxia model

To induce hypoxia, confluent monolayers of BME cells were washed once with phosphate-buffered saline, pH 7.4 (PBS), and M199 medium without fetal calf serum in the absence or presence of added compounds was added. Culture plates were placed in special chambers and incubated at 37°C either under normoxic or hypoxic conditions (2% oxygen) by gas inflow with a gas mixture consisting of 95% air and 5% CO_2 or 93% N_2, 2% O_2 and 5% CO_2. For control, the concentration of oxygen in the culture medium after different time periods of hypoxia was determined using a Digital O_2-Meter (Schott Geräte, Germany). The respiratory activity of cells did not change the oxygen content in the culture medium significantly. The pH of the medium was unchanged during up to 24 h of hypoxia. For control, cultures were incubated under normoxic conditions for the same length of time.

2.3. Assay of endothelial monolayer permeability

After seeding BME cells onto polycarbonate membranes, maximal resistance values across the BME cell monolayer ranged from 70 to 120 Ω cm^2, corresponding to values determined by Erben et al. (1995), which were obtained after 6 to 8 days of culture (Fischer et al., 1996). Chambers with a resistance of more than 100 Ω cm^2 were used for measurements of the passage of [^{3}H]inulin (Amersham, Buchler, Germany) across the BME cell monolayer. The chambers, consisting of the apical part containing the filter membrane inserts with the cell monolayer and the basolateral part, were washed three times with PBS. 0.8-μCi [^{3}H]inulin dissolved in 300-μl medium M199 without serum was added to the apical part and 500 μl of medium was placed in the basolateral chamber. Different concentrations of dexamethasone (dexamethasone, Merck, Darmstadt, Germany), mifepristone–RU486 (graciously supplied by EXELGYN (Paris, France), recombinant $VEGF_{165}$, a polyclonal antibody against human VEGF (both from PreproTech, London, England), or α-lipoic acid (thioctacid, Merck, Darmstadt, Germany) were added to both chambers at the start of the experiment and chambers were incubated under normoxic or hypoxic conditions at 37°C. Experiments in the presence of the anti-

sense VEGF cDNA were performed in the same manner. Ten micromolar of the sense oligonucleotide, ligated with phosphothionate residues to increase stability (Roth, Karlsruhe, Germany), which either codes for the binding site of the RNA-binding protein HuR (AS: GGAGAGAGATTTAGTATGTAG) or the nonsense oligonucleotide (NAS: TTAAGAGATGTAAGGAGTGT), was added to the culture medium. The appearance of [^{3}H]inulin in the basolateral chamber was measured after different time periods by scintillation counting of small aliquots of the basolateral buffer. Results are expressed as the ratio of the inulin concentration determined after different incubation times in the lower chamber and the total concentration of [^{3}H]inulin added to the upper chamber at the start of the experiment (c_R/c_{total}). During the course of the experiment, chambers were kept at 37°C and care was taken to ensure that fluid levels in the apical and basolateral chambers were equal. The amount of inulin that crossed the cell-free membrane did not change during hypoxia or in the presence of used test substances.

2.4. Extraction of total cellular RNA and Northern blot analysis

When cells reached confluence, the plates were washed once with PBS and incubated in M199 medium without fetal calf serum under normoxic or hypoxic conditions in the presence or absence of different concentrations of dexamethasone and/or mifipristone–RU486 for the indicated time periods. Experiments in the presence of the antisense VEGF cDNA were performed in the same manner. Ten micromolar of the sense oligonucleotide, ligated with phosphothionate residues to increase stability (Roth, Karlsruhe, Germany), which either codes for the binding site of the RNA-binding protein HuR (AS: GGAGAGAGATTTAGTATGTAG) or the nonsense oligonucleotide (NAS: TTAAGAGATGTAAGGAGTGT), was added to the culture medium and incubated under normoxic or hypoxic conditions for the indicated time periods. For the isolation of RNA, the cells were washed once with PBS, harvested directly into the guanidinium-thiocyanate buffer and the RNA was isolated as described by Chomczynski and Sachhi (1987). For Northern blot analysis, 15 μg of the total RNA was denatured at 65°C in formamide and ethidium bromide containing loading buffer and subsequently electrophoresed on a 1% (w/v) agarose gel containing 2.2 M formaldehyde. The RNA was then transferred to a hybond-N membrane by vacuum blotting and subsequently UV cross-linked. Filters were prehybridized at 42°C for 3 h in 50% (v/v) formamide, 6 × SSC (sodium chloride/sodium citrate; 1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 1 M NaCl, 1 mg/ml denatured salmon sperm DNA and 5% (w/v) dextran sulfate. A fragment of human VEGF cDNA (graciously supplied by Dr. H. Weich) was labeled by using a multiprime labeling kit (Amersham) to a specific activity of 1×10^9 cpm/μg DNA. A denatured labeled DNA probe was added to the prehybridization buffer to a final concentration of 1×10^6 cpm/ml and incubated overnight. Filters were washed twice with 2 × SSC, 0.1% SDS (sodium dodecyl sulfate) at room temperature for 15 min, and once at 42°C for 20 min and exposed to Kodak XAR films at −70°C for 1–4 days. An 18S cDNA probe (kindly supplied by Dr. I. Oberbäumer) was used to rehybridize membranes for reference purposes. Quantitative analysis was performed using a PhosphorImager SF (Molecular Dynamics). To correct for differences in RNA loading, the signal intensity of VEGF expression of the 3.9 kb transcript for each sample was divided by that for the intensity of each sample hybridized with the 18S cDNA probe.

2.5. mRNA stability assay

The half-life of VEGF mRNA was determined by treating BME cells with actinomycin D (Sigma), as described by Lindholm et al. (1988). To determine changes in the half-life of BME cells during 3 and 6 h of normoxia and hypoxia in the absence and presence of dexamethasone (40 μg/ml = 100 μM) or the antisense oligonucleotide (10 μM), actinomycin D was added to the culture medium (10 μg/ml) to block transcription, and cells were returned to the same culture conditions (normoxia or hypoxia). BME cells were harvested every hour. Total RNA was prepared and Northern blot analysis was performed as described. All experiments were performed at least three times. For the determination of the half-life, VEGF mRNA levels after the addition of actinomycin D were plotted against time, and from the slope the half-life of VEGF mRNA was determined.

2.6. Determination of VEGF levels in cultured media

After BME cells were cultured for different time periods under normoxic or hypoxic conditions at 37°C in the absence and presence of added compounds, the medium was collected, concentrated and desalted using centriprep columns with a cut off of 10 000 (Amicon, Beverly, USA). Protein was determined using the BioRad protein assay kit (München, Germany). VEGF levels secreted into the culture medium were measured using a VEGF enzyme-linked immunosorbent assay (ELISA) kit (Tebu, Frankfurt, Germany) according to the manufacturer's protocol. The ELISA kit used detects all known isoforms of VEGF molecules. All the measurements were corrected for the protein content in each dish or well. The pig VEGF released from BME cell cultures cross-reacted with anti-human VEGF antibodies, as shown by SDS-polyacrylamide gel electrophoresis, which was performed as described by Laemmli (1970). 20-μg of total protein was loaded onto a SDS/15% polyacrylamide gel followed by Western blotting. The nitrocellulose filter was blocked with 2.5% (w/v) bovine serum albumin in PBS buffer (10

mM sodium phosphate pH 7.4, 150 mM NaCl, 0.075% Tween 20) and incubated with a polyclonal antibody against the N terminal peptide of human VEGF (PreproTech, London, England) at a dilution of 1:1000. Subsequently, a biotinylated goat anti rabbit antibody, an avidin-horseradish peroxidase antibody and a horseradish peroxidase reaction were used for the detection of the VEGF protein as described by the manufacturer (Bio-Rad, Harbour, USA).

2.7. Statistical analysis

Results are expressed as the means ± S.E.M. The unpaired Student's *t*-test or analysis of variance and subsequent multiple comparisons using Scheffe's method were used for statistical analysis. Results were considered statistically different at $P < 0.05$.

3. Results

3.1. Dexamethasone decreases hypoxia-induced hyperpermeability

Earlier studies revealed that hypoxia increased the permeability of the BME cell monolayer after 6 h significantly (Fischer et al., 1996). During 6 h of normoxia, dexamethasone did not change the permeability of the BME cell monolayer to [^{3}H]inulin, but during hypoxia, dexamethasone decreased the hypoxia-induced hyperpermeability in a dose-dependent manner, which became significant at the concentration of 40 μg/ml = 100 μM (Fig. 1).

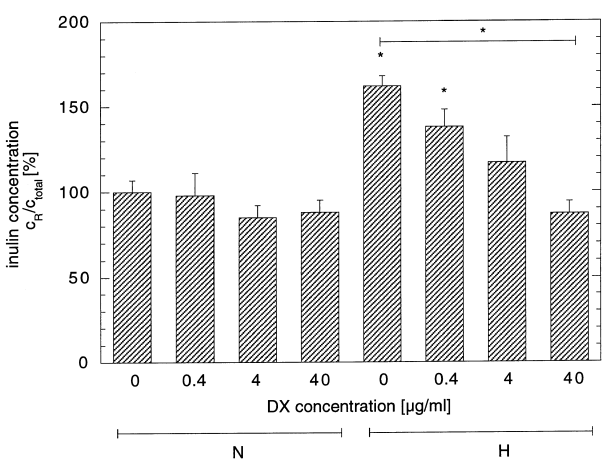

Fig. 1. Permeability of the BME cell monolayer to [^{3}H]inulin during 6 h of normoxia (N) and hypoxia (H) in the absence (0) and presence of different concentrations of dexamethasone. Dexamethasone (DX, 10–40 μg/ml = 25–100 μM) was added to BME cell cultures at the start of the experiment. The amount of [^{3}H]inulin that passed across the BME cell monolayer after 6 h of normoxia divided by the total concentration of [^{3}H]inulin added to the upper chamber at the start of the experiment (c_R / c_{total}) in the absence of added compounds was set to 100%. Values are expressed as the means ± S.E.M. ($n = 10$; $^*P < 0.05$ different from the value determined during 6 h of normoxia in the absence of dexamethasone).

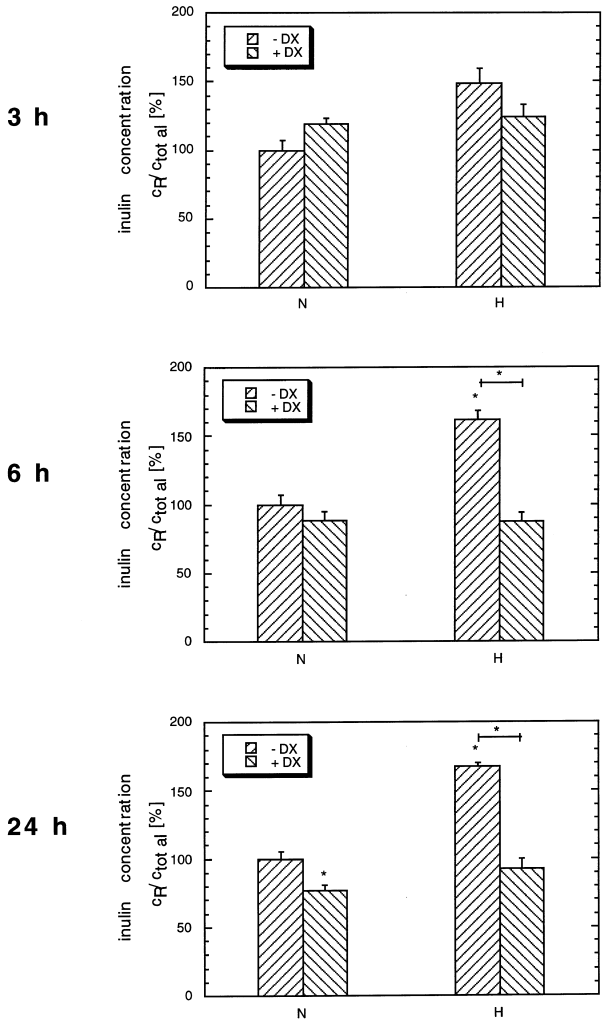

Fig. 2. Time dependence of the permeability of the BME cell monolayer to [^{3}H]inulin during normoxia (N) and hypoxia (H) in the absence and presence of dexamethasone. Dexamethasone (DX, 40 μg/ml = 100 μM) was added to BME cell cultures at the start of the experiment. The amount of [^{3}H]inulin that passed across the BME cell monolayer after 3, 6 or 24 h of normoxia divided by the total concentration of [^{3}H]inulin added to the upper chamber at the start of the experiment (c_R / c_{total}) in the absence of added compounds was set to 100%. Values are expressed as the means ± S.E.M. ($n = 10$; $^*P < 0.05$ different from the value determined during normoxia in the absence of dexamethasone).

Dexamethasone at the concentration of 40 μg/ml = 100 μM hardly changed hypoxia-induced hyperpermeability after 3 h. Dexamethasone also showed no effect on the permeability after 3 h of normoxia and hypoxia when dexamethasone (40 μg/ml = 100 μM) was preincubated with the cells before the start of the experiment. The permeability determined after 17 h of preincubation followed by 3 h of normoxia was 108 ± 5% and after 3 h of hypoxia 152 ± 4% in comparison to the value determined after 3 h of normoxia in the absence of dexamethasone without preincubation. In the case of hypoxia for 6 h and

longer, dexamethasone abolished completely the hypoxia-induced permeability increase of the BME cell monolayer (Fig. 2). Also during normoxia, dexamethasone reduced the permeability of the BME cell monolayer to a low level in a time-dependent manner, an effect, which became significant after 24 h. That the hypoxia-induced permeability increase after 24 h was nearly equal to that one determined after 6 h of hypoxia is because the flux across the BME cell monolayer during hypoxia reached its maximal value after 8 h. This value is as high as the flux measured across the cell-free membrane. Therefore, hypoxia-induced permeability was changed only slightly during hypoxia lasting longer than 6 h.

To evaluate whether the effect of dexamethasone on hypoxia-induced permeability changes after more than 6 h is receptor mediated, the specific glucocorticoid receptor antagonist mifepristone–RU486 was added to BME cell cultures at the start of the experiment. Mifepristone–RU486 (5 μM) did not alter the effect of dexamethasone on hypoxia-induced permeability changes, suggesting that the effect of dexamethasone is not receptor mediated. The control showed that mifepristone–RU486 did not alter permeability during normoxia and hypoxia in the absence of dexamethasone (Fig. 3).

3.2. Dexamethasone decreases hypoxia-induced VEGF expression

Because earlier studies demonstrated that hypoxia-induced permeability changes in BME cells are mediated by VEGF and NO in an autocrine manner (Fischer et al., 1999), we investigated whether the permeability-decreasing effect of dexamethasone is related to changes in hypoxia-induced VEGF expression. During hypoxia, the expression of VEGF mRNA and protein increased in a time-dependent manner (Fischer et al., 1997).

Quantitative analysis of Northern blots revealed that dexamethasone decreased the VEGF mRNA level during 6 h of hypoxia in a dose-dependent manner (Fig. 4A). The effect became significant at the dexamethasone concentration of 40 μg/ml = 100 μM. During normoxia, dexamethasone did not change the VEGF mRNA level signifi-

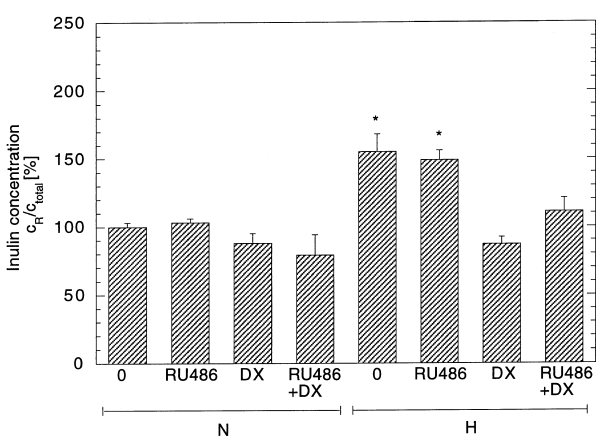

Fig. 3. Permeability of the BME cell monolayer to [^{3}H]inulin during 6 h of normoxia (N) and hypoxia (H) in the absence (0) and presence of mifepristone–RU486, dexamethasone, or both together. Mifepristone–RU486 (5 μM), dexamethasone (DX, 40 μg/ml = 100 μM) or both together (mifepristone–RU486 + DX) were added to BME cell cultures at the start of the experiment. The amount of [^{3}H]inulin that passed across the BME cell monolayer after 6 h of normoxia divided by the total concentration of [^{3}H]inulin added to the upper chamber at the start of the experiment (c_R / c_{total}) in the absence of added compounds was set to 100%. Values are expressed as the means ± S.E.M. ($n = 8$; $^*P < 0.05$ different from the value determined during 6 h of normoxia in the absence of dexamethasone).

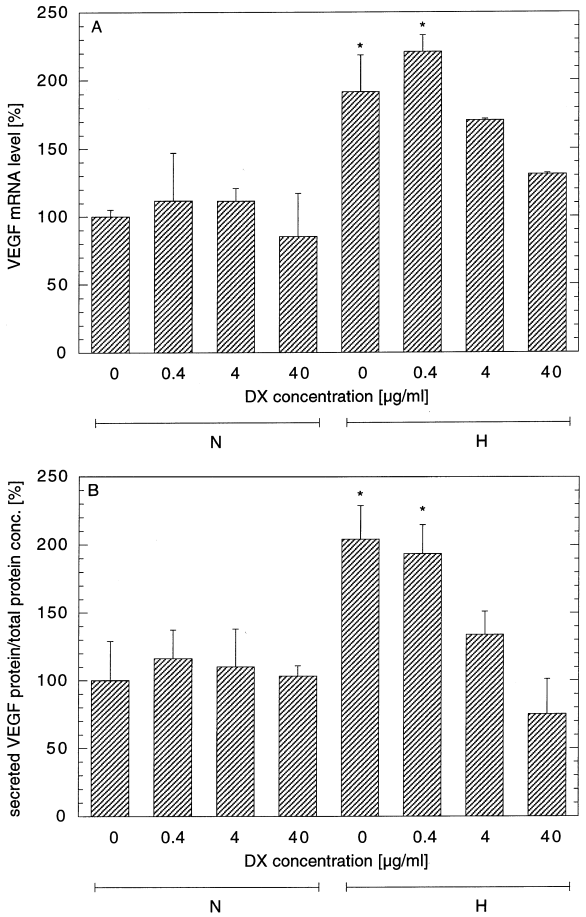

Fig. 4. Quantitative analysis of the VEGF mRNA level (A) and of the amount of VEGF protein (B) released into the culture medium after 6 h of normoxia (N) and hypoxia (H) in the absence (0) and presence of different concentrations of dexamethasone. Dexamethasone (DX, 10–40 μg/ml = 25–100 μM) was added to the culture medium at the start of the experiment and cultures were incubated under normoxic or hypoxic conditions for 6 h followed by RNA isolation and Northern blotting. The amount of VEGF released into the culture medium was determined using ELISA techniques as described under Section 2. The total amount of VEGF protein released into the culture medium after 6 h of normoxia was determined as 8.5 ± 2.4 ng/ml. Values determined for VEGF mRNA and protein level after culturing the cells for 6 h under normoxic conditions were set to 100%. Data are expressed as the means ± S.E.M. ($n = 6$; $^*P < 0.05$ different from the value determined after 6 h of normoxia in the absence of dexamethasone).

cantly. The baseline production of VEGF mRNA is shown in our last manuscript (Fischer et al., 1999).

Accordingly, the amount of VEGF protein released into the culture medium during 6 h of hypoxia was decreased by dexamethasone dose dependently and became significant at the dexamethasone concentration of 40 μg/ml = 100 μM (Fig. 4B). Dexamethasone did not change the protein level during normoxia.

The time dependence of the effect of dexamethasone on the hypoxia-induced VEGF mRNA level demonstrated that dexamethasone at the concentration of 40 μg/ml = 100 μM caused minimal changes in the hypoxia-induced VEGF mRNA levels after 3 h. In contrast, after 6 h and longer times of hypoxia, dexamethasone reduced the hypoxia-induced increase in the VEGF mRNA level significantly (Fig. 5).

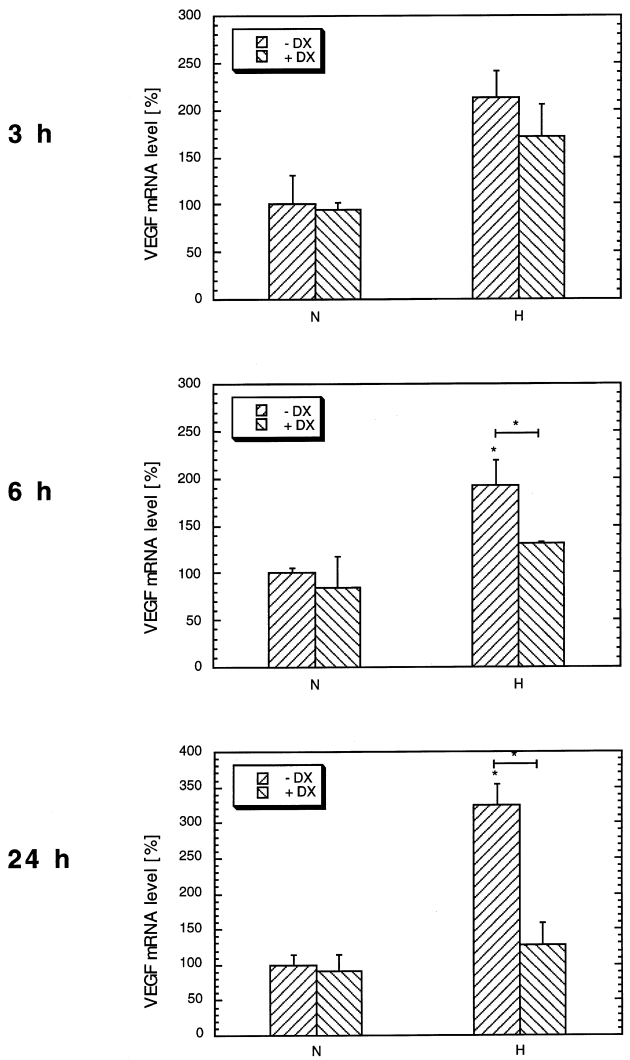

Fig. 5. Time dependence of the VEGF mRNA level after normoxia (N) and hypoxia (H) in the absence and presence of dexamethasone. Dexamethasone (DX, 40 μg/ml = 100 μM) was added to the culture medium at the start of the experiment and cultures were incubated under normoxic or hypoxic conditions for 3, 6 or 24 h followed by RNA isolation and Northern blotting as described under Section 2. The VEGF mRNA level was normalized to the corresponding level of 18S mRNA. Values determined after culturing the cells for 3, 6 or 24 h under normoxic conditions were set to 100%. Data are expressed as the means ± S.E.M. ($n = 6$; * $P < 0.05$ different from the value determined after normoxia in the absence of dexamethasone).

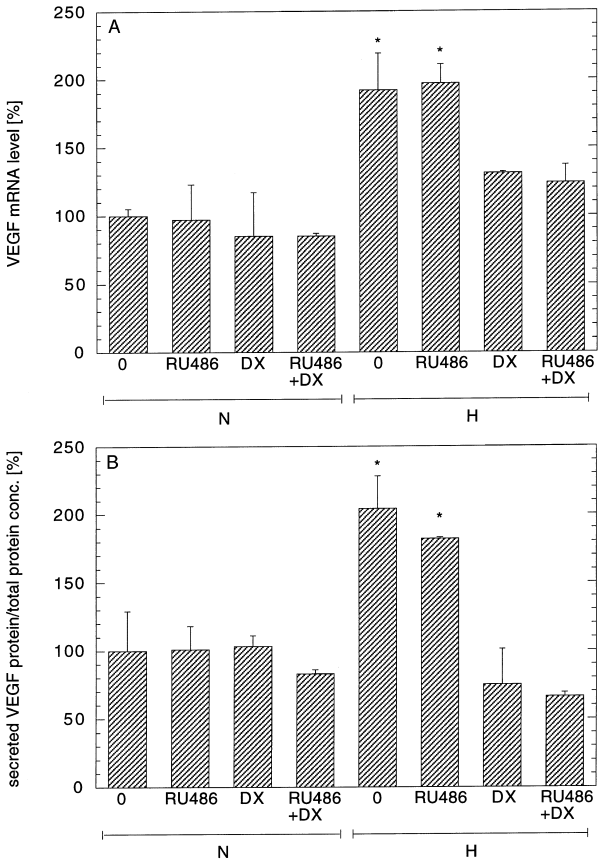

Fig. 6. Quantitative analysis of the VEGF mRNA level (A) and of the amount of VEGF protein (B) released into the culture medium after 6 h of normoxia (N) and hypoxia (H) in the absence (0) and presence of mifepristone–RU486, dexamethasone, or both together. Mifepristone–RU486 (5 μM), dexamethasone (DX, 40 μg/ml = 100 μM) or both together (mifepristone–RU486 + DX) were added to BME cell cultures at the start of the experiment and cultures were incubated under normoxic or hypoxic conditions for 6 h followed by RNA isolation and Northern blotting. The amount of VEGF released into the culture medium was determined using ELISA techniques as described under Section 2. Values determined after culturing the cells for 6 h under normoxic conditions were set to 100%. Data are expressed as the means ± S.E.M. ($n = 6$; * $P < 0.05$ different from the value determined after 6 h of normoxia in the absence of any compound).

To evaluate whether the effect of dexamethasone on the hypoxia-induced VEGF expression after more than 6 h is receptor mediated, mifepristone–RU486 was added to BME cell cultures at the start of the experiment. Mifepristone–RU486 (5 μM) did not change the effect of dexamethasone on the hypoxia-induced VEGF mRNA level. As a control, it was shown that mifepristone–RU486 did not change the VEGF mRNA level during normoxia and hypoxia in the absence of dexamethasone (Fig. 6A).

In accordance, the protein level of VEGF during hypoxia in the absence and presence of dexamethasone was not changed by mifepristone–RU486 (Fig. 6B).

After longer times of normoxia and hypoxia, mifepristone–RU486 did not alter the effect of dexamethasone on hypoxia-induced VEGF expression. After 24 h of hypoxia, the VEGF mRNA level was induced to 325 ± 30% compared to the value determined after 24 h of normoxia. Dexamethasone (40 μg/ml = 100 μM) reduced VEGF mRNA expression to 128 ± 30%, which did not change significantly in the presence of 5 μM mifepristone–RU486 (93 ± 30%).

3.3. Dexamethasone does not change VEGF- and hypoxia-induced endothelial cell monolayer permeability

To demonstrate that dexamethasone does not interfere with VEGF-induced permeability changes of the BME cell monolayer, VEGF was added to normoxic BME cell cultures. Earlier studies demonstrated that hypoxia-induced permeability in BME cells is mediated by the upregulation of the VEGF/VEGF receptor system, leading to the release of the permeability increasing agent, nitric oxide (NO). The release of NO alone, however, is not sufficient to induce hyperpermeability and requires conditions stabilizing the second messenger NO such as hypoxia or the presence of antioxidants (Fischer et al., 1999). Therefore, dexamethasone was added to VEGF-treated normoxic BME cell cultures in the presence of the antioxidant α-lipoic acid (2 μM). α-Lipoid acid alone did not alter the permeability as shown previously (Fischer et al., 1999). Under these conditions, VEGF (5 ng/ml) increased significantly the flux of inulin across the BME cell monolayer after 6 h. Dexamethasone at the concentration of 40 μg/ml = 100 μM did not alter these permeability changes (Fig. 7).

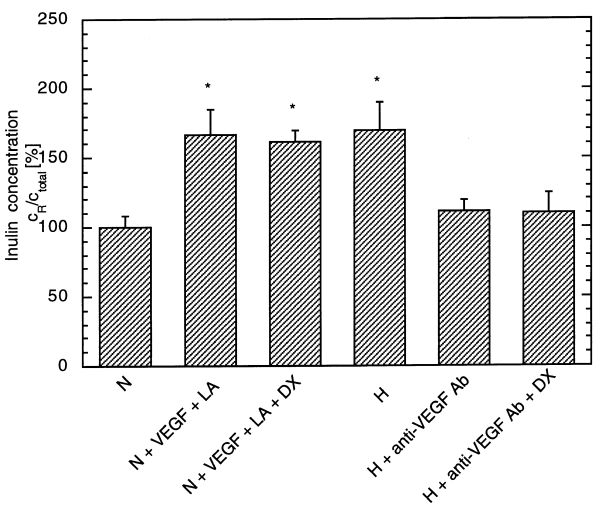

Fig. 7. Permeability of the BME cell monolayer to [^{3}H]inulin during 6 h of normoxia in the absence (N) and presence of VEGF and α-lipoic acid (N + VEGF + LA) and additionally dexamethasone (N + VEGF + LA + DX), and during 6 h of hypoxia in the absence (H) and presence of the VEGF neutralizing antibody (H + anti VEGF Ab) and additionally dexamethasone (H + anti VEGF Ab + DX). VEGF (5 ng/ml), α-lipoic acid (2 μM), dexamethasone (40 μg/ml = 100 μM) or the polyclonal antibody to human VEGF (10 μg/ml) was added to BME cell cultures at the start of the experiment. The amount of [^{3}H]inulin that passed across the BME cell monolayer after 6 h of normoxia divided by the total concentration of [^{3}H]inulin added to the upper chamber at the start of the experiment (c_R/c_{total}) in the absence of added compounds was set to 100%. Values are expressed as the means ± S.E.M. ($n = 9$; * $P < 0.05$ different from C).

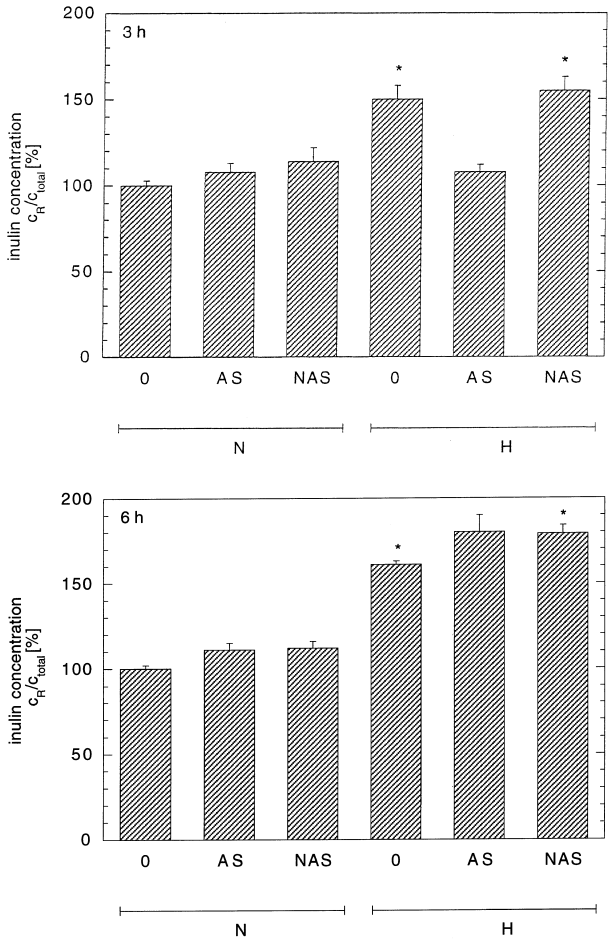

Fig. 8. Permeability of the BME cell monolayer to [^{3}H]inulin after 3 and 6 h of normoxia (N) and hypoxia (H) in the absence (0) and presence of the antisense oligonucleotide, coding for the binding site of the RNA-binding protein HuR (AS), and in the presence of the "nonsense" oligonucleotide (NAS). AS or NAS (each at the concentration of 10 μM) was added to the culture medium at the start of the experiment. The amount of [^{3}H]inulin that passed across the BME cell monolayer after 3 or 6 h of normoxia divided by the total concentration of [^{3}H]inulin added to the upper chamber at the start of the experiment (c_R/c_{total}) in the absence of added compounds was set to 100%. Values are expressed as the means ± S.E.M. ($n = 8$; * $P < 0.05$ different from the value determined during 3 or 6 h of normoxia in the absence of added compounds).

To confirm that dexamethasone does not alter the hypoxia-induced permeability changes in the absence of VEGF, hypoxia-induced permeability changes were evaluated in the presence of a neutralizing antibody to VEGF and additionally dexamethasone. The antibody, which did not alter the permeability during normoxia, prevented hypoxia-induced permeability changes, as shown before (Fischer et al., 1999). This effect was unchanged in the presence of dexamethasone at the concentration of 40 μg/ml = 100 μM (Fig. 7).

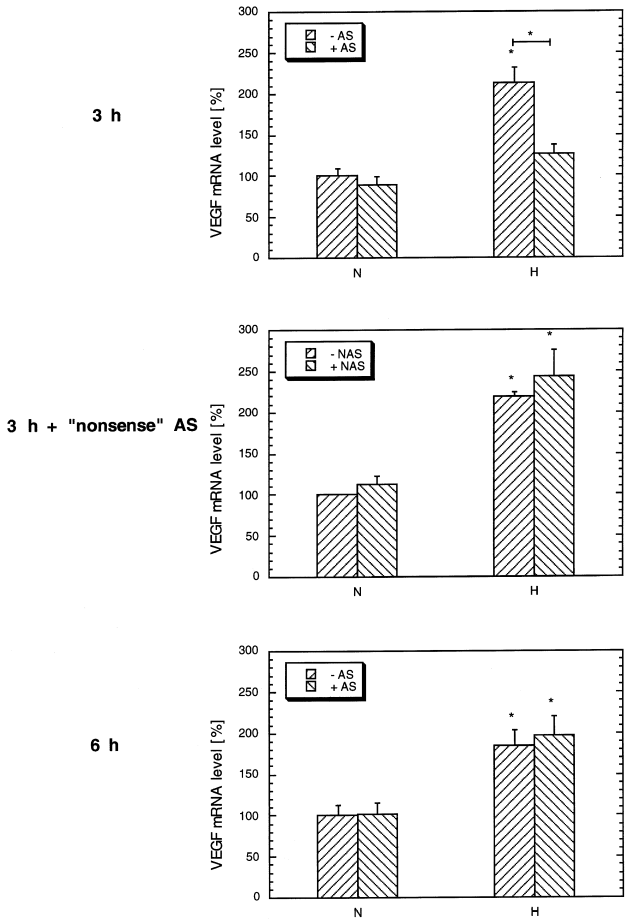

Fig. 9. Quantitative analysis of the VEGF mRNA level after 3 and 6 h of normoxia (N) and hypoxia (H) in the absence (−AS) and presence of the antisense oligonucleotide, coding for the binding site of the RNA-binding protein HuR (+AS), and after 3 h, as control, in the presence of the "nonsense" oligonucleotide (NAS). AS or NAS (each at the concentration of 10 μM) was added to the culture medium at the start of the experiment and cultures were incubated under normoxic or hypoxic conditions for 3 or 6 h, followed by RNA isolation and Northern blotting as described under Section 2. The VEGF mRNA level was normalized to the corresponding level of 18S mRNA. Values determined after culturing the cells for 3 or 6 h under normoxic conditions were set to 100%. Values are expressed as the means ± S.E.M. ($n = 9$; $^{*}P < 0.05$ different from the value determined during normoxia in the absence of dexamethasone).

3.4. Dexamethasone does not inhibit hypoxia-induced VEGF expression, which is regulated by a post-transcriptional mechanism

The time dependence of the dexamethasone effect on the hypoxia-induced VEGF mRNA abundance suggests that the mechanism to induce VEGF mRNA level is changed during longer times of hypoxia. Dexamethasone has been shown to decrease the transcriptionally activated VEGF mRNA level primarily (Klekamp et al., 1997). An antisense oligonucleotide coding for the binding site of the RNA-binding protein HuR was added to BME cell cultures. HuR is known to mediate the hypoxic stabilization of VEGF mRNA in the 3′-untranslated region of VEGF mRNA (Levy et al., 1998). After 3 and 6 h of incubation under normoxic and hypoxic conditions, permeability and the VEGF mRNA level were determined. Hypoxia-induced permeability changes after 3 h were completely abolished in the presence of the antisense oligonucleotide, whereas no effect was evoked after 6 h of hypoxia. For control, cells were treated with a nonsense oligonucleotide (NAS), which contained the same numbers of each nucleotide but in a different order to that of the antisense oligonucleotide. The same concentration of this oligonucleotide did not change the permeability level after 3 h of normoxia as well as after 3 h of hypoxia compared to the corresponding value determined under the same conditions in the absence of the oligonucleotide (Fig. 8).

Accordingly, the hypoxia-induced increase in the VEGF mRNA level after 3 h was completely abolished in the presence of the antisense oligonucleotide whereas after 6 h of hypoxia, no effect of the oligonucleotide could be distinguished (Fig. 9). The antisense oligonucleotide did not change the VEGF mRNA level between 3 and 6 h of normoxia. The same concentration of the nonsense oligonucleotide did not change the VEGF mRNA level after 3 h of hypoxia, as already described for the effect on hypoxia-induced permeability changes (Fig. 9). These results suggest that 6 h of hypoxia no longer enhanced the VEGF mRNA expression by a post-transcriptional mechanism.

To test this suggestion, the half-life of VEGF mRNA after 6 h of hypoxia was compared to the half-life determined after 6 h of normoxia. The rates of decline in VEGF mRNA in the presence of actinomycin D are shown in Fig. 10. In cells exposed to normoxic conditions for 6 h,

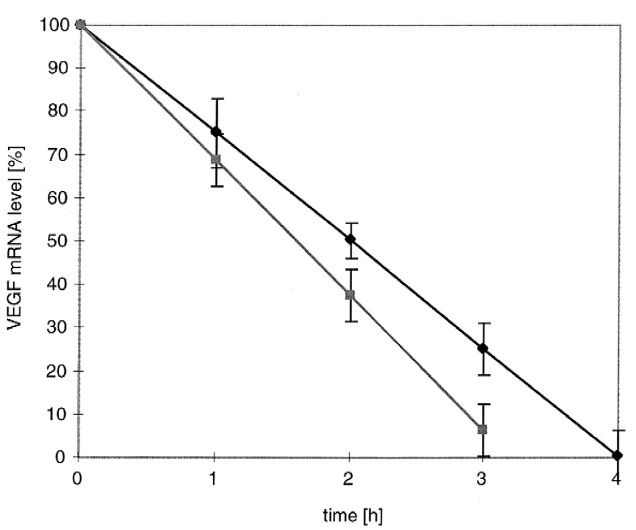

Fig. 10. Quantitative analysis of VEGF mRNA stability after 6 h normoxia (◆) and 6 h hypoxia (■). BME cells cultured under normoxia and hypoxia for 6 h were treated with actinomycin D and returned to the same culture conditions. At hourly intervals, RNA was prepared and analyzed for VEGF mRNA levels. The same blot was stripped and hybridized with an 18S cDNA probe. Quantitative analysis was performed with a phosphorImager and the VEGF mRNA level was normalized to the corresponding level of 18S mRNA. The corrected values were plotted as percentages of the 0 h value against time following the addition of actinomycin D. Data are expressed as the means ± S.E.M. ($n = 4$).

Table 1
Quantitative analysis of VEGF mRNA half-life after 3 and 6 h of normoxia and hypoxia in the absence (−) and presence of dexamethasone (+DX) and of the antisense oligonucleotide, coding for the binding site of the RNA-binding protein HuR (+AS). BME cells cultured under normoxia and hypoxia for 3 and 6 h in the absence and presence of DX (40 μg/ml = 100 μM) or the antisense oligonucleotide (10 μM) were treated with actinomycin D and returned to the same culture conditions. At hourly intervals, RNA was prepared and analyzed for VEGF mRNA levels. The same blot was stripped and hybridized with an 18S cDNA probe. Quantitative analysis was performed with a phosphorImager and the VEGF mRNA level was normalized to the corresponding level of 18S mRNA. The corrected values were plotted as a percentage of the 0 h value against time following the addition of actinomycin D. The half-life of VEGF mRNA was determined from the slope of the graph. Data are expressed as the means ± S.E.M. ($n = 4$)

	Half-life [h]		Half-life [h]	
	After 3 h of normoxia	After 3 h of hypoxia	After 6 h of normoxia	After 6 h of hypoxia
–	1.6 ± 0.1	4.3 ± 0.3[a]	2.0 ± 0.8	1.6 ± 0.2
+DX	1.7 ± 0.4	4.1 ± 0.1[a]	2.7 ± 0.6	2.1 ± 0.4
+AS	2.2 ± 0.4	2.6 ± 0.3	2.4 ± 0.7	1.9 ± 0.2

[a] $P < 0.05$ different from the corresponding value determined during normoxia.

the half-life was $t_{1/2} = 2.0 \pm 0.8$ h. Hypoxia did not significantly increase the half-life of VEGF mRNA, which was estimated as: $t_{1/2} = 1.6 \pm 0.2$ h.

The suggestion that dexamethasone does not reduce the VEGF mRNA level, which is induced by a post-transcriptional mechanism was confirmed by determination of the half-life of VEGF mRNA in the presence of dexamethasone. As shown in Table 1, the half-life of VEGF mRNA determined after 3 and 6 h of normoxia and hypoxia was not changed significantly in the presence of dexamethasone (40 μg/ml = 100 μM). In contrast, the antisense oligonucleotide, which was shown to inhibit the post-transcriptional induced VEGF mRNA level, reduced the half-life of VEGF mRNA determined after 3 h of hypoxia to the value determined after 3 h of normoxia. After 6 h of hypoxia, the half-life of VEGF mRNA was not changed by the oligonucleotide.

4. Discussion

Using an in vitro model of brain-derived microvascular endothelial cells, we demonstrated that the glucocorticoid dexamethasone reduces hypoxia-induced hyperpermeability in a dose-dependent manner, an effect, which became significant at the concentration of 40 μg/ml = 100 μM. Steroids have been shown to reduce ischemic edema formation in vivo by reducing the permeability of the blood–brain barrier (Yamada et al., 1979). Hypoxia is known to induce the expression of a number of permeability-increasing proteins such as VEGF. This protein is dramatically upregulated by hypoxia in a variety of cell types including endothelial cells (Aiello et al., 1995; Nomura et al., 1995), retinal pericytes (Aiello et al., 1995; Simorre et al., 1994), and retinal pigment epithelial cells (Adamis et al., 1993). VEGF is also enhanced during pathological processes characterized by hypoxia-related neovascularization (Pierce et al., 1995) and microvascular extravasation (Senger et al., 1983, 1986).

Because of the permeability increasing properties of VEGF, the protein was suggested to induce disturbances in the blood–brain barrier resulting in the development of brain edema associated with the extravasation of water, electrolytes, and plasma proteins from altered tumor microvessels (Criscuolo, 1993). In agreement with this hypothesis, the efficacy of the glucocorticoid dexamethasone in the treatment of peritumoral brain edema (Reichman et al., 1986; Yamada et al., 1979) was hypothesized to result from the suppression of VEGF expression as demonstrated in cultured human malignant glioma cells (Bruce et al., 1987; Goldman et al., 1993). Earlier studies demonstrated that hypoxia-induced permeability changes in BME cells are mediated by autocrine produced VEGF via the stabilization of NO (Fischer et al., 1999). In this study, we demonstrate that the effect of dexamethasone on hypoxia-induced permeability changes correlates with an effect of dexamethasone on hypoxia-induced VEGF mRNA and protein levels. Dexamethasone may decrease hypoxia-induced permeability changes either by reducing the response of BME cells to VEGF released during hypoxia or by decreasing VEGF expression. Results demonstrated that VEGF-induced permeability changes of the BME cell monolayer during normoxia were not altered by dexamethasone. These experiments were performed in the presence of autoxidants because VEGF only increases permeability under conditions that prevent the rapid autoxidation of the second messenger NO, such as hypoxia or in the presence of autoxidants (Fischer et al., 1999). Because this study only investigated the effects of dexamethasone on hypoxia-induced permeability changes, the results confirmed that dexamethasone does not interfere with the VEGF receptor and that the inhibition of hypoxia-induced permeability changes by dexamethasone might be caused by a decreased synthesis of VEGF.

In some studies, dexamethasone inhibition of VEGF induction has been shown to result from the binding of dexamethasone to the glucocorticoid receptor, which was confirmed by the finding that the effect of dexamethasone was reversed by the glucocorticoid receptor antagonist mifepristone–RU486 (Heiss et al., 1996). To evaluate whether the effects of dexamethasone are caused by binding to the glucocorticoid receptor, the specific receptor antagonist mifepristone–RU486 was added to hypoxic

BME cell cultures. This antagonist is known to competitively bind to the glucocorticoid receptor with even higher affinity than dexamethasone (Powell et al., 1999). Mifepristone–RU486 also is a progesterone antagonist (Baulieu, 1989). But the binding of mifepristone–RU486 to the glucocorticoid receptor is more potent than its binding to the progesterone receptor (Carbajo et al., 1996). Furthermore, it is questionable whether the progesterone receptor is expressed on microvascular endothelial cells (Wolff et al., 1992). Therefore, it is assumed that mifepristone–RU486 preferentially binds to the glucocorticoid receptor on BME cells. However, our results did not indicate the presence or activity of the glucocorticoid receptor on BME cells because mifepristone–RU486 prevented neither the effect of dexamethasone on hypoxia-induced permeability changes nor that one on VEGF expression. Furthermore, the concentration of dexamethasone needed to inhibit hypoxia-induced hyperpermeability and VEGF expression was much higher than the concentration needed to inhibit glucocorticoid receptor-dependent effects mediated by dexamethasone. In pulmonary artery endothelial cells, the EC_{50} for the dexamethasone effect was approximately equal to 0.5 nM, with the maximum effect at 10 nM (Lanier-Smith and Currie, 1991). Clinically used concentrations for the treatment of edema formation and neutrophil accumulation are in the range 0.2–3 mg/kg (Betz and Coester, 1990; Eisenberg et al., 1970; Göcer et al., 1996; Yarwood et al., 1993). After application of therapeutic anti-inflammatory dexamethasone concentrations of about 1 mg/ml, the concentration in serum plasma was calculated to range from 0.1 to 0.2 μg/ml (Brady et al., 1987; McDonald et al., 1988). The concentration is low because 75% of dexamethasone is bound to blood-borne proteins, preferentially albumin, and a certain proportion of the administered dose appears in urine (Cummings et al., 1990; Miyabo et al., 1981). Nevertheless, it has been reported that doses of dexamethasone that are used clinically for treating brain tumor-associated cerebral edema vary widely and often exceed doses that would saturate the glucocorticoid receptor. These high doses are believed to be required because inhibition of cerebral edema occurs through a pharmacological mechanism that does not require the glucocorticoid receptor (Heiss et al., 1996). Our results also suggest that the effects of dexamethasone are not receptor mediated. Although there are several reports demonstrating the presence of the glucocorticoid receptor on endothelial cells (Brostian et al., 1966; Inoue et al., 1999; Lanier-Smith and Currie, 1991; Stanimirovic et al., 1994), the presence of the glucocorticoid receptor on BME cells still has to be confirmed. Another reason for the ineffectiveness of the glucocorticoid receptor might be that the receptor is not activated in our model. Several reports demonstrate that the response to corticosteroids is increased significantly in the presence of heparin (Folkman et al., 1983; Folkman and Ingber, 1987; Maragoudakis et al., 1989). Therefore, further experiments are in progress to evaluate whether the dexamethasone effect will increase in the presence of heparin. Furthermore, studies with the intracerebral injection of VEGF in the absence and presence of dexamethasone are needed to evaluate whether the effect of dexamethasone on VEGF expression and the development of edema is of significance in vivo.

The finding that dexamethasone did not alter hypoxia-induced permeability changes in the absence of VEGF demonstrates that dexamethasone does not interfere with other permeability-increasing factors, which might be released during hypoxia.

The time dependence of the dexamethasone effect on the hypoxia-induced hyperpermeability and VEGF mRNA abundance suggests that the mechanism by which the VEGF mRNA level is increased changes during hypoxia. The effects of dexamethasone on the permeability and on the VEGF mRNA level during hypoxia became significant after periods of hypoxia longer than 3 h. Past studies have demonstrated an effective suppression of permeability with dexamethasone when administered several hours before the start of the experiment (Heiss et al., 1996). However, our results did not show any change of the dexamethasone effect on the permeability after several hours of preincubation. This finding confirmed the suggestion that the mechanism, by which VEGF mRNA expression is induced, is time dependent. Earlier studies revealed that 3 h of hypoxia modulated the VEGF mRNA level by enhancing the stability of VEGF mRNA (Fischer et al., 1997). Because dexamethasone has been found to inhibit VEGF mRNA upregulation primarily by blocking the transcription of VEGF mRNA (Klekamp et al., 1997), it is suggested that hypoxia lasting longer than 3 h upregulates the VEGF mRNA level transcriptionally. To confirm this suggestion, an antisense oligonucleotide coding for the binding site of the RNA-binding protein that is involved in the hypoxic stabilization of VEGF mRNA by hypoxia, was added to BME cell cultures during normoxia and hypoxia. It has been demonstrated that post-transcriptional induction of the VEGF gene is related to several *cis*-acting instability elements in the VEGF 3′-UTR region (Levy et al., 1996a). Several hypoxia-inducible proteins formed during hypoxia were identified that bind to the 3′ UTR region, resulting in increased stability of the mRNA (Claffey et al., 1998; Levy et al., 1996a). In vitro RNA degradation assays have allowed the identification of the specific regions of VEGF mRNA, which are responsible for the lability of VEGF mRNA under normoxic conditions and also regions that are critical for the stabilization of VEGF mRNA by hypoxia (Levy et al., 1996a). The same authors previously described a novel hypoxia-inducible protein complex that binds to the adenylate-uridylate-rich element in the VEGF 3′-UTR region (Levy et al., 1996b). One of the important factors that bind selectively to this region has been identified as HuR (Levy et al., 1998). Inhibition of HuR expression by antisense HuR constructs inhibits the stabilization of VEGF mRNA by hypoxia (Levy et al., 1998). In this

study, we blocked the stabilization of VEGF mRNA during hypoxia by adding an antisense construct, which codes for the mRNA binding region of HuR. The presence of this oligonucleotide completely prevented hypoxia-induced hyperpermeability and stabilization of VEGF mRNA after 3 h but not after 6 h of hypoxia. This suggests that the binding of the oligonucleotide prevents the binding of the mRNA stabilizing protein HuR. In contrast, after 6 h of hypoxia, the same antisense oligonucleotide did not block hypoxia-induced hyperpermeability and VEGF mRNA expression, suggesting that these mRNA stabilizing proteins are no longer involved in the hypoxic upregulation of VEGF mRNA. In accordance, there was no increase in the half-life of VEGF mRNA in cells exposed to hypoxia for 6 h, suggesting that the decrease in VEGF mRNA abundance after 6 h of hypoxia in the presence of dexamethasone is likely to be due to a blockade of transcription. This was confirmed by the finding that the half-life of VEGF mRNA, which was increased after 3 h of hypoxia, was not changed by dexamethasone but was reduced in the presence of the antisense oligonucleotide. After 6 h neither compound, dexamethasone as well as the antisense oligonucleotide, changed the half-life of VEGF mRNA. In agreement with these results, there are several studies showing that dexamethasone has no effect on hypoxia-induced VEGF expression, which is regulated by an increase in mRNA stability, as demonstrated using NIH3T3 or retinal epithelial cells (Shima et al., 1995). However, dexamethasone is known to block transcription, which is mediated by the transcription factor activator protein-1 (AP-1) (Finkenzeller et al., 1995). It is assumed that dexamethasone interacts with the glucocorticoid receptor, followed by binding of the dexamethasone-glucocorticoid receptor complex to the AP-1 transactivator complex. This binding in turn prevents the AP-1 transactivator complex from stimulating the transcription of genes containing an AP-1 site (Jonat et al., 1990; Schüle et al., 1990). Agents such as phorbol ester induce VEGF expression by a transcriptional mechanism involving the binding of transcription factors to the AP-1 binding site (Finkenzeller et al., 1995). Activation of VEGF expression by phorbol ester indicates the involvement of protein kinase C (Claffey et al., 1992). Earlier studies revealed that hypoxia-induced VEGF expression is not inhibited by bisindolylmaleimide, a specific inhibitor of the protein kinase, suggesting that hypoxic induction of VEGF in our model does not involve AP-1-mediated transcription (Fischer et al., 1995). Recent studies have implicated hypoxia-inducible factor-1 (HIF-1) in the hypoxic induction of VEGF (Forsythe et al., 1996; Goldberg and Schneider, 1994). Therefore, studies are in progress to evaluate more precisely the mechanism of the transcriptional induction of VEGF expression by hypoxia in BME cells in the absence and presence of dexamethasone.

In summary, the present data demonstrate that dexamethasone decreases the hypoxia-induced hyperpermeability via a reduction in VEGF transcription. These findings might be helpful to get more knowledge about molecular mechanisms to protect the blood–brain barrier from hypoxia-induced permeability changes and to reduce brain edema formation.

Acknowledgements

We would like to thank M. Wiesnet for her technical assistance.

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