Ca^{2+}/calmodulin-dependent nitric oxide synthase activity in the human cervix carcinoma cell line ME-180

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We show here that the human cervix carcinoma cell line ME-180 expresses a constitutive nitric oxide (NO) synthase, as demonstrated by formation of [^{3}H]citrulline and nitrite. The enzyme is dependent on tetrahydrobiopterin, NADPH, flavins and Ca^{2+}/calmodulin. Enzyme activity is located in the cytosol rather than in the membrane fraction and can be inhibited by N^G-monomethyl-L-arginine (NMMA). An antiserum to NO synthase purified from porcine cerebellum inhibited the enzyme activity. ME-180 cells released NO, as was shown by stimulation of guanylate cyclase (EC 4.6.1.2) in RFL-6 detector cells; this release was stimulated 8-fold by the Ca^{2+} ionophore A23187 and 2-fold by increasing the intracellular tetrahydrobiopterin levels with cytokines. This is the first characterization of a Ca^{2+}/calmodulin-dependent NO synthase activity in human epithelial-type tumour cells.

INTRODUCTION

Formation of nitric oxide (NO) in mammalian cells and tissues occurs as a result of NO synthase activity [1]. The enzyme uses L-arginine as substrate, producing L-citrulline and NO in equimolar concentrations [2]. Different types of NO synthase can be distinguished on the basis of Ca^{2+}/calmodulin requirement, intracellular distribution and inducibility by cytokines.

Both brain-type and endothelial-type NO synthases depend on Ca^{2+}/calmodulin and are constitutively expressed [3–5]. However, whereas the brain-type enzyme is found in the cytosol, more than 95% of the endothelial-type enzyme is membrane-bound [6]. A constitutive cytosolic NO synthase dependent on Ca^{2+}, but not on calmodulin, was reported in rat neutrophils [7]. In contrast, the macrophage-type enzyme is independent of Ca^{2+}/calmodulin and is expressed only upon *de novo* protein synthesis induced by endotoxin and/or a cytokine [8–10]. All NO synthases tested so far use tetrahydrobiopterin, flavins and NADPH as cofactors [4,8,11–13]. Analysis of NO synthase purified from porcine cerebellum showed that the enzyme contains tightly bound tetrahydrobiopterin, FMN and FAD [14]. Binding of FMN and FAD was also shown for the cytokine-induced NO synthase of murine macrophages [8,15].

Characterization of NO synthase has been carried out thus far with material from rodent, bovine or porcine origin. Regarding human-derived material, constitutive NO synthase was described for neutrophils, HL-60 cells [16,17] and platelets, and dependence on Ca^{2+} and NADPH was again shown [18]. Ca^{2+}/calmodulin-dependent NO synthase was purified from human cerebellum [19]. Concerning human tumour cell lines, only two colorectal adenocarcinoma cell lines (SW-480, SW-620) have so far been described to show NO synthase activity. These cell lines have a constitutive, cytosolic and NADPH-dependent NO synthase which was not influenced by changing the free Ca^{2+} concentration [20].

We describe here NO synthase activity in another epithelial-like human tumour cell line, ME-180. This NO synthase activity is constitutively expressed, is dependent on Ca^{2+}/calmodulin, tetrahydrobiopterin, NADPH and flavins, and was neutralized by an antiserum to purified NO synthase from porcine cerebellum [4]. Further, we studied whether intact ME-180 cells released NO and whether this release was influenced by cytokines or by stimulation of Ca^{2+} influx.

EXPERIMENTAL

Materials

Human recombinant interferon-γ was kindly provided by Bioferon (Laupheim, Germany) and had a specific activity of 2×10^7 units/mg of protein. Cell culture media were from Serva (Heidelberg, Germany). Fetal calf serum (FCS) was obtained from Biochrome (Berlin, Germany). Pteridines were purchased from Dr. B. Schircks Laboratories (Jona, Switzerland). L-[2,3,4,5-^{3}H]Arginine monohydrochloride (35–70 Ci/mmol) and the radioimmunoassay for cyclic GMP (cGMP) determination were from Amersham. L-[2,3,4,5-^{3}H]Arginine was purified by h.p.l.c. over a Nucleosil 10 SA column from Mackerey and Nagel (Düren, Germany) in 100 mM sodium acetate buffer, pH 4.5. Tissue culture plasticware was from Falcon (Becton-Dickinson, Plymouth, U.K.). The protein dye reagent was from Bio-Rad (Richmond, CA, U.S.A.). Calmodulin, the calcium ionophore A23187, glucose-6-phosphate dehydrogenase from yeast (EC 1.1.1.49), glucose 6-phosphate and nitrate reductase from *Aspergillus* sp. (EC 1.6.6.2) were from Serva. Lipopolysaccharide (LPS) from *Escherichia coli* 055:B5, N^G-monomethyl-L-arginine (NMMA), 2,4-diamino-6-hydroxypyrimidine (DAHP), calmidazolium (compound R24571), Dowex-50W, rabbit serum and cell culture supplements were from Sigma (Munich, Germany).

Cell culture

ME-180 cervix carcinoma cells were a gift from Dr. M. W. Taylor, Indiana University (Bloomington, IN, U.S.A.).

Abbreviations used: NO, nitric oxide; FCS, fetal calf serum; NMMA, N^G-monomethyl-L-arginine; DAHP, 2,4-diamino-6-hydroxypyrimidine; LPS, lipopolysaccharide; cGMP, cyclic GMP; SNP, sodium nitroprusside.

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Cells were grown in McCoy's 5A medium supplemented with 10% (v/v) heat-inactivated FCS, 2 mM L-glutamine, penicillin (100 units/ml) and streptomycin (0.1 mg/ml). For determination of NO synthase activity in cell homogenates, confluent cells from 10–20 75 cm^2 flasks [day 7 of culture, corresponding to $(1–2) \times 10^8$ cells] were harvested after trypsinization, washed with PBS (130 mM NaCl, 2 mM KCl, 6 mM Na_2HPO_4, 1 mM KH_2PO_4), pH 7.4, and frozen in 1 ml of distilled water at −80 °C for up to 5 weeks without loss of activity. For experiments using intact cells, confluent monolayers were pretreated with 250 μM NMMA, 5 mM-DAHP or 250 units/ml interferon-γ in combination with 1 μg/ml LPS for 24 h.

Fetal fibroblast-like RFL-6 cells derived from rat lung tissue were obtained at passage 7 from the American Type Culture Collection (Rockville, MD, U.S.A.). Cells were grown in minimum essential medium (Eagle) with Earle's salts, supplemented with 5% (v/v) FCS, 2 mM L-glutamine and antibiotics as described above. For determination of cGMP in cell monolayers upon stimulation of guanylate cyclase (EC 4.6.1.2) by NO or sodium nitroprusside (SNP) [21], RFL-6 cells between passages 9 and 12 were seeded in 6-well plates at a density of 2×10^5 cells/well in 5 ml of culture medium and grown for a further 3–5 days until confluent.

Determination of NO synthase activity

NO synthase activity in cell homogenates was determined according to a method modified from refs. [4,8]. Briefly, ME-180 cells frozen at −80 °C were thawed rapidly, centrifuged for 15 min at 10000 *g* and 4 °C, and the supernatant was freed from low-molecular-mass compounds by Sephadex G-25 chromatography. The protein fraction was eluted with 40 mM Tris/HCl, pH 8.0, containing 100 μM phenylmethanesulphonyl fluoride. Standard reaction mixtures contained 40 mM Tris/HCl, pH 8.0, 100 μM L-arginine, 25 μM FAD, 25 μM FMN, 2 mM NADPH, 5 μM 6*R*-tetrahydrobiopterin, 100 μM phenylmethanesulphonyl fluoride, 60000–80000 c.p.m. of purified L-[2,3,4,5-^{3}H]-arginine and 100 μl of cell extract (about 200 μg of total cell protein) in a final volume of 200 μl. Samples were incubated for 30 min at 37 °C and the reaction was stopped by addition of 800 μl of 200 mM sodium acetate, pH 5.0, containing 200 μM EDTA and 1 mM L-citrulline. [^{3}H]Citrulline was quantified after separation from [^{3}H]arginine by the cation exchanger Dowex 50W [2]. Enzyme activity is given as pmol of [^{3}H]citrulline formed/min per mg of protein in the cell extract.

In some experiments, the free Ca^{2+} concentration was adjusted by 0.15 mM EGTA, 0.9 mM EDTA and 2.05 mM $MgCl_2$ without $CaCl_2$ for Ca^{2+}-free conditions, and 0.15 mM EGTA, 0.9 mM EDTA, 1.78 mM $MgCl_2$ and 0.27 mM $CaCl_2$ for a free Ca^{2+} concentration of 3 μM [22]. With this method the free Ca^{2+} concentration is adjusted in the presence of a fixed Mg^{2+} concentration, taking advantage of the different affinities of Ca^{2+} and Mg^{2+} for EDTA and EGTA respectively. Stock solutions (10 mM) of the calmodulin antagonist calmidazolium (compound R24571) and of the Ca^{2+} ionophore A23187 were prepared in dimethyl sulphoxide. Neutralization of NO synthase activity in ME-180 cell extracts was performed using a rabbit antiserum to NO synthase purified from porcine cerebellum [4] in the presence of calmodulin (10 μg/ml) and 3 μM free Ca^{2+}. For controls, we used either rabbit non-immune serum or anti-(NO synthase) antiserum preincubated for 20 min on ice with 40 μg/ml purified NO synthase from porcine cerebellum for saturating the antibody.

For determination of the stoichiometry of nitrite + nitrate and citrulline, the standard reaction mixture was supplemented with an NADPH-regenerating system (0.25 unit/ml glucose-6-phosphate dehydrogenase and 20 mM glucose 6-phosphate), and the incubation time was prolonged to 3 h at 37 °C. For determination of nitrite + nitrate, nitrate was reduced by nitrate reductase (1.25 units/ml) in the presence of 2.5 mM NADPH for 1 h at room temperature.

Formation of [^{3}H]citrulline and NO was linear with time for up to 1 h in the standard assay and for up to 3 h in the assay supplemented with an NADPH-regenerating system. The assays were also linear as the amount of cell extract was increased between 30 and 300 μg of protein.

To determine whether NO synthase activity is localized in the cytosol or in the membrane fraction, the pellet from the cell extract preparation was washed twice with PBS and then treated with 20 mM CHAPS for 20 min on ice with gentle shaking. After centrifugation for 10 min at 10000 *g*, the supernatant was then purified over Sephadex G-25 and NO synthase activity was determined. Comparison of enzyme activities in the soluble fraction either treated with 20 mM CHAPS for 20 min or untreated showed no interference of CHAPS with NO synthase activity.

Protein in cell extracts was determined according to Bradford [23] using the Bio-Rad reagent and pure BSA as standard.

Determination of NO release

An assay was used to detect NO-mediated stimulation of guanylate cyclase in RFL-6 detector cells [21]. ME-180 cells were harvested by trypsinization, washed with PBS and resuspended at a density of 10^8/ml in 10 mM Hepes buffer, pH 7.5, with 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$ and 1 mM 3-isobutyl-1-methylxanthine. Confluent RFL-6 monolayers, grown in 6-well plates, were pretreated for 15 min with 900 μl of 10 mM Hepes buffer (see above) at 37 °C. ME-180 cells (1×10^7 in 100 μl) were added for a further 10 min, in some cases in the presence of A23187. ME-180 cells were then removed by aspiration, the RFL-6 monolayers were washed vigorously two times with 10 mM Hepes buffer to remove ME-180 cells, and cGMP was extracted from RFL-6 cells with 0.01 M HCl (1 ml/well) for 1 h at 4 °C. Samples were stored at −20 °C. cGMP was determined by radioimmunoassay (Amersham). Data are presented as fmol of cGMP per mg of total cell protein and are means ± S.D. of triplicate wells in one of two similar experiments. Individual well in a typical experiment yielded 300 ± 35 μg of total cell protein (mean of 30 wells ± S.D.).

H.p.l.c. analysis of pteridines and nitrite

An h.p.l.c. system, consisting of a liquid chromatograph (LC 5500; Varian, Palo Alto, CA, U.S.A.), an LS 4 fluorescence detector (Perkin–Elmer, Beaconsfield, Bucks., U.K.) and an AASP module (Varian) for solid phase on-line elution, was used for detection of pteridines in homogenates of ME-180 cells. Fresh homogenates were treated with iodine in acidic or alkaline solution according to Fukushima and Nixon [24] and then measured by h.p.l.c. with fluorescence detection as previously described [25]. Stereoisomers of tetrahydrobiopterin were detected electrochemically using an ESA Coulochem detector (Bedford, MA, U.S.A.) according to [26]. Nitrite in NO synthase reaction mixtures was determined according to Green et al. [27] using the Griess–Ilosvays reagent from Merck (Darmstadt, Germany).

RESULTS

In a 3 h assay the standard incubation yielded 110 ± 14 pmol·min^{-1}·mg^{-1} of [^{3}H]citrulline and 129 ± 15 pmol·min^{-1}·mg^{-1} of nitrite + nitrate (means ± S.D. of three incubations), indicating that L-citrulline and NO were formed in equimolar concentrations as was demonstrated previously for rat cerebellar slices [2]. NO synthase activity in CHAPS extracts of ME-180 membrane pellets was 7 ± 3 pmol·min^{-1}·mg^{-1} as compared with 113 ± 11 pmol·min^{-1}·mg^{-1} (means ± S.D. of three incubations) in the cytosol preparation. Thus more than 90% of the enzyme activity was located in the soluble fraction.

Table 1 shows NO synthase activities in the presence of various cofactors, Ca^{2+}, calmodulin and the calmodulin antagonist R24571. Whereas omission of NADPH, flavins or tetrahydrobiopterin reduced the activity by about 50%, decreasing the free Ca^{2+} concentration with EGTA/EDTA completely abolished NO synthase activity. Results from standard incubations without adjustment of the free Ca^{2+} concentration did not differ from those from incubations in which the Ca^{2+} concentration was 3 μM or in which calmodulin (50 μg/ml) was added, indicating that standard incubation mixtures contained sufficient Ca^{2+} and calmodulin in the cell extract. The calmodulin antagonist R24571 (5 μM) decreased NO synthase activity to 11% of that in untreated controls. This effect could be totally reversed by 50 μg/ml calmodulin (see Table 1) and was dose-dependent: 1 μM R24571 yielded 140 ± 14 pmol·min^{-1}·mg^{-1} (means ± S.D. of three incubations) and 25 μM R24571 completely inhibited NO synthase activity.

Table 1 Characterization of NO synthase activity in ME-180 cell extracts

ME-180 cells were harvested, disrupted and Sephadex G-25 eluates were prepared. Enzyme incubation mixtures contained all components except the omitted cofactor. For changing the Ca^{2+} concentration or testing the effects of calmodulin or R24571, EGTA/EDTA and $MgCl_2$ buffers were included [22]. Enzyme activities were determined as described in the Experimental section. Data are mean values ± S.D. of triplicate incubations from two experiments. BH_4, tetrahydrobiopterin.

Conditions	[^{3}H]Citrulline (pmol·min^{-1}·mg^{-1})
Standard	145 ± 5
NADPH omitted	72 ± 4
Flavins omitted	80 ± 4
BH_4 omitted	72 ± 5
Ca^{2+}-free	< 2
Ca^{2+} (3 μM)	145 ± 8
Ca^{2+}/calmodulin (50 μg/ml)	143 ± 7
Ca^{2+}/R23571 (5 μM)	16 ± 2
Ca^{2+}/calmodulin/R23571	147 ± 6

Table 2 Inhibition of NO synthase activity in ME-180 cell extracts by rabbit anti-(NO synthase)

A rabbit antiserum to NO synthase purified from porcine cerebellum was included in incubations of cell extracts from ME-180 cells, containing 10 μg/ml calmodulin and a free Ca^{2+} concentration of 3 μM (see the Experimental section). For controls, we used either rabbit non-immune serum or rabbit anti-(NO synthase) preincubated for 20 min on ice with purified porcine cerebellum NO synthase (40 μg/ml) to saturate the antibody. Values are means ± S.D. of triplicate incubations from one of two similar experiments and were related to the protein content of ME-180 cell extracts. *A blank run with anti-(NO synthase)/NO synthase (40 μg/ml) 1:10 was taken into account, and only the contribution of ME-180 cell extracts is shown.

Antibody and dilution	[^{3}H]Citrulline (pmol·min^{-1}·mg^{-1})
Control	112 ± 5
Anti-(NO synthase) (1:10)	13 ± 3
Anti-(NO synthase) (1:100)	56 ± 1
Anti-(NO synthase) (1:1000)	106 ± 6
Rabbit non-immune serum (1:1000)	129 ± 5
Anti-(NO synthase)/NO synthase (40 μg/ml) (1:10)	86 ± 7*

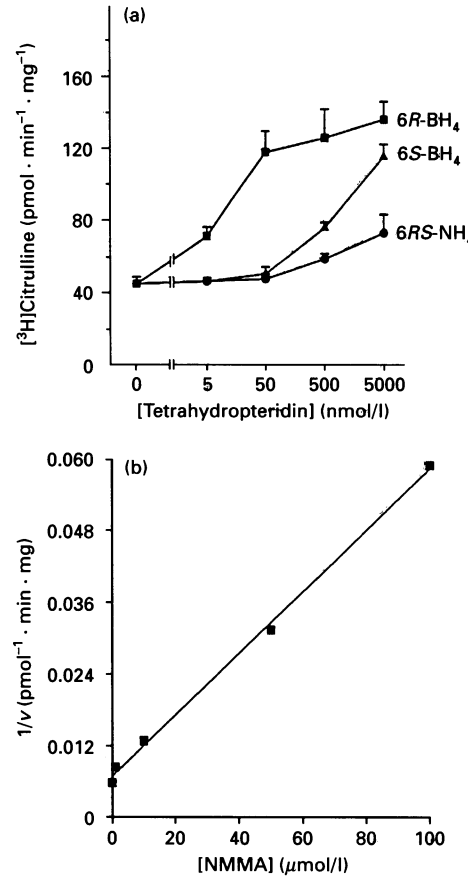

Figure 1 NO synthase activity in ME-180 cell homogenates: stimulation by pteridines (a) and inhibition by NMMA (b)

Cell extracts from ME-180 cells were prepared and an NO synthase assay was performed using standard assay conditions as detailed in the Experimental section. (**a**) 6*R*-Tetrahydrobiopterin (■), 6*S*-tetrahydrobiopterin (▲), 6*RS*-tetrahydroneopterin (●); (**b**) $1/v$ versus [NMMA]. Values are means ± S.D. of triplicate incubations from one of two similar experiments. BH_4, tetrahydrobiopterin; NH_4, tetrahydroneopterin.

As demonstrated in Table 2, a rabbit antiserum to purified NO synthase from porcine cerebellum [4] inhibited NO synthase activity in ME-180 cell extracts in a concentration-dependent manner. Antiserum diluted 1:10 decreased NO synthase activity by 88%. This effect was not seen with rabbit non-immune serum. Pretreatment of the antibody with purified NO synthase (40 μg/mg) could restore NO synthase activity in ME-180 cell extracts.

Figure 1(a) shows that NO synthase activity in ME-180 cell extracts was stimulated by the 6*R*- isomer of tetrahydrobiopterin rather than by the 6*S*- isomer. Stimulation of activity by 0.5 μM and 5 μM of the 6*S*- isomer can be explained by a contamination of the product with 3.3% of the 6*R*- isomer (corresponding to 15 and 150 nM), as detected by h.p.l.c. analysis with electrochemical

Table 3 Release of NO from intact ME-180 cells as detected by cGMP formation in RFL-6 detector cells

ME-180 cells either untreated or pretreated for 24 h with 25 μM NMMA, 5 mM DAHP or 250 units/ml interferon-γ in combination with 1 μg/ml LPS (IFN-γ/LPS) were harvested by trypsinization, washed in PBS and resuspended at a density of 10^8/ml in 10 mM Hepes buffer, pH 7.5 (see the Experimental section). RFL-6 monolayers were incubated with 10^7 ME-180 cells with or without A23187 for 10 min at 37 °C. cGMP was extracted with 0.01 M HCl and quantified by radioimmunoassay (see the Experimental section). Incubation with Hepes buffer alone was used as blank. A23187 had no direct effect on RFL-6 cells. SNP (1 mM) was used as positive control and led to formation of 38 ± 18 nmol $\cdot$ mg^{-1} cGMP, corresponding to a 40-fold increase in cGMP levels compared with untreated RFL-6 cells. Values are means $\pm$ S.D. of triplicate wells from one of two similar experiments and are given as fmol of cGMP per mg of total cell protein from RFL-6 cells. Total intracellular neopterin and biopterin were detected in extracts of ME-180 cells that were either untreated or had been treated for 24 h with NMMA, DAHP, IFN-γ/LPS or IFN-γ/LPS/DAHP and had been used for assay of NO release, upon iodine oxidation in acid media using h.p.l.c. and fluorescence detection [24,25]. Values are pmol per mg of total cell protein and are means $\pm$ S.D. of three determinations. nd, not done.

Additions	cGMP (fmol $\cdot$ mg^{-1})	Neopterin (pmol $\cdot$ mg^{-1})	Biopterin (pmol $\cdot$ mg^{-1})
Control	497 ± 143	1.0 ± 0.5	21 ± 2
+ A23187 (1 μM)	681 ± 222	nd	nd
+ A23187 (10 μM)	3462 ± 153	nd	nd
+ A23187 (50 μM)	3861 ± 662	nd	nd
NMMA (250 μM)	< 2	nd	nd
DAHP (5 mM)	208 ± 19	< 0.5	4 ± 1
IFN-γ/LPS	1195 ± 101	2.0 ± 0.8	113 ± 33
IFN-γ/LPS/DAHP	547 ± 160	< 0.5	6 ± 2

detection [26]. Tetrahydroneopterin could only slightly increase NO synthase activity. Further, we tested whether neopterin or 7,8-dihydroneopterin, which can be detected in ME-180 cell extracts (see Table 3), influenced NO synthase activity. Neopterin (0.5 μM) in combination with 5 μM 7,8-dihydroneopterin yielded 46 ± 1 pmol $\cdot$ min^{-1} $\cdot$ mg^{-1}. When neopterin (0.5 μM) plus 7,8-dihydronoepterin (5 μM) were applied together with 5 μM tetrahydrobiopterin, 132 ± 2 pmol $\cdot$ min^{-1} $\cdot$ mg^{-1} product was formed, in comparison with 135 ± 10 pmol $\cdot$ min^{-1} $\cdot$ mg^{-1} with tetrahydrobiopterin alone (means $\pm$ S.D. of three incubations).

The NO synthase activity of ME-180 cells could be inhibited by NMMA in a concentration-dependent way. A 50 % inhibition of enzyme activity was found at about 10 μM (Figure 1b), which is in accordance with previous findings for purified cerebellar NO synthase [28].

Intact ME-180 cells released NO (Table 3), which was measured as formation of cGMP in RFL-6 detector cells [21]. Pretreatment of ME-180 cells with interferon-γ plus LPS led to a significant increase in NO release ($P < 0.02$, Student's t test), whereas NMMA treatment of whole cells abolished NO release. Pretreatment of ME-180 cells with 5 mM DAHP reduced NO release by about 50 %, as compared with untreated ME-180 cells ($P < 0.05$). The same was seen for cells treated with interferon-γ plus LPS tested with or without DAHP ($P < 0.01$). Short-term stimulation of Ca^{2+} influx by the Ca^{2+} ionophore A23187 led to a concentration-dependent and up to 8-fold (at 50 μM; $P < 0.001$ compared with untreated controls) increase in NO release from intact ME-180 cells.

NO synthase activity in homogenates of ME-180 cells treated with interferon-γ plus LPS was 118 ± 2 pmol $\cdot$ min^{-1} $\cdot$ mg^{-1} in comparison with 112 ± 3 pmol $\cdot$ min^{-1} $\cdot$ mg^{-1} in untreated controls (means $\pm$ S.D. of three incubations), indicating that the increase in NO release did not occur as a result of induction of NO synthase. As for untreated control cells, the NO synthase activity in extracts of cells treated with interferon-γ plus LPS was also dependent on Ca^{2+} (< 2 pmol $\cdot$ min^{-1} $\cdot$ mg^{-1} under Ca^{2+}-free conditions). This shows that there is no switch from Ca^{2+}-dependent to Ca^{2+}-independent NO synthase activity by treatment of cells with interferon-γ plus LPS. As indicated in Table 3, intracellular pteridine levels were increased up to 5-fold by interferon-γ plus LPS, and were decreased to about 20 % by DAHP. More than 90 % of biopterin occurred in its tetrahydroform, whereas neopterin was found in its oxidized and its dihydro- form, as was determined by iodine oxidation in alkaline media [24].

DISCUSSION

Similar to a number of different human cell types [29,30], ME-180 cervix carcinoma cells show increased synthesis of pteridines, i.e. tetrahydrobiopterin and neopterin/dihydroneopterin, when treated with interferon-γ. This is due to induction of GTP cyclohydrolase I (EC 3.5.4.16), the first enzyme of pteridine synthesis [31], and we have previously reported GTP cyclohydrolase I activities and their stimulation by interferon-γ in ME-180 cells [32]. Compared with other human cell types [29,30], ME-180 cells have a high constitutive GTP cyclohydrolase I activity [32], leading to easily detectable tetrahydrobiopterin levels even in untreated control cells. We reported previously that murine fibroblasts form tetrahydrobiopterin in order to provide a limiting cofactor for NO synthesis [33], and therefore investigated whether the comparatively high GTP cyclohydrolase I activity of ME-180 cells also indicates NO synthesis. We found that ME-180 cells have a soluble, Ca^{2+}/calmodulin-dependent NO synthase activity which is present constitutively. The activity was inhibited by an antibody to NO synthase from porcine cerebellum. This antibody did not react with NO synthase from porcine aortic endothelial cells either in enzyme incubations or in Western blot analysis (B. Mayer, unpublished work). The epithelial-like tumour cell line ME-180 thus expresses an NO synthase which is antigenically closely related to the brain-type enzyme. The enzyme activity requires tetrahydrobiopterin, NADPH and flavins for full activity, like other NO synthases described so far [11–15].

In contrast to, e.g., murine cells [34], human cells form neopterin/dihydroneopterin in addition to tetrahydrobiopterin [29]. This is due to a comparatively low 6-pyruvoyl tetrahydropterin synthase activity, leading to accumulation of 7,8-dihydroneopterin triphosphate, the first intermediate of pteridine synthesis [31]. Furthermore, 7-neopterin derivatives were found in human urine, suggesting the occurrence of tetrahydroneopterin and its participation in hydroxylation-type reactions in humans [35]. We therefore tested whether neopterin or its reduced species could influence NO synthase activity. As was reported for cytokine-induced NO synthase from murine macrophages [13], tetrahydroneopterin was marginally active, whereas the oxidized or the dihydro- forms, which are normally detected in cell extracts, had no effect on the NO synthase activity of ME-180 cells, nor did those neopterin species interfere with the effects of tetrahydrobiopterin. Thus, NO synthase from human cells does not seem to differ from the NO synthases of other species with regard to pteridine requirements.

Intact ME-180 cells released NO, as indicated by formation of cGMP in detector cells. NO release could be increased 2-fold by treatment with interferon-γ plus LPS, and our data suggest that this effect is due to increasing intracellular tetrahydrobiopterin levels rather than to a direct effect on NO synthase. Thus not only cytokine-induced NO formation [33], but also the constitutive activity, seems to be modulated by cytokine-induced

pteridine synthesis. As in endothelial cells [36], stimulation of Ca^{2+} influx by A23187 strongly increased NO release from ME-180 cells.

NO exerts a variety of physiological actions. The most prominent among them are relaxation of smooth muscle, inhibition of platelet aggregation and action as a neurotransmitter and cytotoxic agent (for review see [37]). It has been suggested that the NO synthase of tumour cells regulates the metastatic potential of the cells via their different potentials to inhibit platelet aggregation, which is correlated with their NO synthase activity [20]. The inhibitory effect of NO on platelet aggregation as well as its vasodilatatory potential may also support optimal circulation within the tumour tissue and thus promote tumour growth. Furthermore, NO release has been shown to decrease leukocyte adhesion [38], which raises the possibility that it might interfere with cellular immune mechanisms. The data presented here suggest that cytokines augment NO formation in ME-180 cells by increasing the biosynthesis of pteridines. It is conceivable that this increase in NO formation participates in the observed correlation of poor prognosis with increased pteridine synthesis in the cervix carcinoma [39].

We thank Mrs. Petra Höfler for excellent technical assistance. This work was supported by the Austrian Research Funds (Zur Förderung der Wissenshaftlichen Forschung), project P 8231 and 8836.

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Received 8 May 1992/29 June 1992; accepted 13 July 1992