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Vasoactive substances produced by cultured rat brain endothelial cells

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Abstract

The vasoactive substances synthesized by primary cultures of rat brain endothelial cells were investigated and compared to those from two, immortalized cell lines, RBE4 and GP8. The vasoactivity of endothelium-derived substances was measured on isolated canine coronary artery. Vascular tone was significantly decreased by both primary and GP8, but not by RBE4 cells. Indomethacin pretreatment of primary and GP8 cells turned vasorelaxation into contraction while N^{ω}-nitro-L-arginine pretreatment decreased the vasorelaxation induced by primary, but not by GP8 cells. Eicosanoid production was determined after incubation with [^{14}C]arachidonic acid. The predominant vasoactive eicosanoid was prostaglandin E_2 in both primary and GP8 cells. RBE4 cells synthetized mainly prostaglandin $F_{2\alpha}$ and thromboxane B_2 and significantly less prostaglandin E_2 than did either primary or GP8 cells. The capacity of cerebral endothelium to regulate vascular tone by production of dilator and constrictor substances can be preserved under certain circumstances in immortalized cell lines.

Keywords: Prostaglandin; Nitric oxide (NO); Endothelial cell, brain; Vasoregulation; Blood–brain barrier

1. Introduction

Cerebral endothelial cells form the blood–brain barrier which is important for maintaining the homeostasis of the central nervous system by controlling the traffic of molecules and cells between blood and brain. The brain microvascular endothelium, like other endothelial cells, is also capable of producing several substances mediating endothelium-dependent vasorelaxation and contraction (Kontos et al., 1990). The released vasoactive agents can modulate the endothelial second messenger systems, regulate blood–brain barrier permeability (Joó and Klatzo, 1989) and play a key role in the regulation of the vascular tone of cerebral vessels.

A molecule important for the determination of vascular tone is synthetized by endothelial cells, the endothelium-derived relaxing factor, which has been identified as nitric oxide, or a closely related compound (Ignarro et al., 1987). In various cell types, a number of stimuli are known to activate phospholipase A_2, which leads to the release of free fatty acids, including arachidonic acid, the precursor of the family of eicosanoids. Prostacyclin (prostaglandin I_2) and prostaglandin E_2 are known to mediate vascular relaxation, while tromboxane A_2 and prostaglandin $F_{2\alpha}$ are implicated in vasoconstriction in various vessels (Toda, 1980; Hintze and Kaley, 1984; Van Diest et al., 1986). Previous studies revealed the eicosanoid profile of isolated cerebral microvessels (Dux et al., 1981; Joó et al., 1981; Gecse et al., 1982) and it was suggested that pure cultures of rodent cerebrovascular endothelial cells produced predominantly the vasodilator prostaglandin E_2 and prostacyclin (Moore et al., 1988; De Vries et al., 1995). The eicosanoids released in cerebral microvessels also play a role in the changes in blood–brain barrier permeability (Villacara et al., 1990), cerebral edema formation, and inflammatory reactions in the central nervous system (Chan et al., 1983). Significant differences in the type, amount, and ratio of vasoactive eicosanoids released under basal conditions or upon chemical or physical stimulation have been demonstrated, depending on the localization of the endothelium within the circulatory system.

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In the present experiments we compared the production of vasoactive agents of three different rat cerebral endothelial cell types: primary rat brain endothelial cells and two immortalized rat brain endothelial cell lines, RBE4 and GP8. These cell lines display a non-transformed, well differentiated endothelial phenotype and express several structural and pharmacological characteristics of the blood-brain–barrier (Durieu-Trautmann et al., 1993; Roux et al., 1994; Greenwood et al., 1996). A bioassay system based on the effect of cultured endothelial cell suspensions on isolated coronary artery from dogs was devised in order to detect the vasoactive action of endothelium-derived substances. The effect of the cyclooxygenase inhibitor, indomethacin, and the nitric oxide synthase inhibitor, N^{ω}-nitro-L-arginine, on the vasoactive substance producing activity was also determined. The profile of eicosanoid synthesis was also investigated.

2. Materials and methods

Materials used in the experiments were: collagenase type II, bovine serum albumin, Dulbecco's modified Eagle's medium/nutrient mixture F-12 Ham (DMEM/F-12), fetal calf serum, basic fibroblast growth factor, heparin, monoclonal anti-mouse Thy 1.1 antibody, rabbit complement serum (HLA-ABC), antibody against Factor VIII-related antigen, prostaglandin $F_{2\alpha}$, indomethacin, acetylcholine chloride, N^{ω}-nitro-L-arginine, arachidonic acid (grade I), prostaglandin E_2, prostaglandin D_2, thromboxane B_2, prostaglandin $F_{2\alpha}$, and 6-keto-prostaglandin $F_{1\alpha}$, 12-L-hydroxy-5,8,10-heptadecatrienoic acid (12-HHT), Medium 199, (Sigma, Germany), collagenase-dispase (Boehringer, Germany), Percoll (Pharmacia, Sweden), L-glutamine, penicillin–streptomycin, geneticin (Gibco, UK), plasma-derived serum (First Link, UK), 1-[^{14}C]arachidonic acid (spec. act. 2035 MBq/mM) (Amersham, UK), silica gel thin-layer plates (0.25 mm) (Merck, Germany).

2.1. Primary rat brain endothelial cell culture

Primary cultures of cerebral endothelial cells were prepared and characterized as previously described (Deli et al., 1997). Cerebral cortexes from two-week-old CFY rats were finely minced, then incubated in collagenase–dispase solution (270 U/ml collagenase, 1 mg/ml dispase, DMEM-F12 containing antibiotics) at 37°C for 1.5 h in a shaking waterbath. After this incubation 20% bovine serum albumin/DMEM-F12 (2 ml/brain) was added to the homogenate and centrifuged at $1000 \times g$ for 15 min. The pellet containing the microvessels was washed once in DMEM-F12 then further digested for another 1 h with the above mentioned enzymes at 37°C. The cell suspension was carefully layered on a continuous 33% Percoll gradient and centrifuged at $1000 \times g$ for 10 min. The band of the endothelial cell clusters was aspirated, washed twice in DMEM-F12. The cells were seeded onto rat-tail collagen-coated 35-mm plastic dishes in culture medium (DMEM-F12 containing 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamycin, 2 mM glutamine, 20% heat-inactivated plasma-derived serum and from the second day 1 ng/ml basic fibroblast growth factor). Primary cultures on the 2nd day in vitro were treated with monoclonal anti-mouse Thy 1.1 antibody followed by rabbit complement serum to eliminate the few contaminating non-endothelial cells, mainly pericytes, by selective cytolysis (Risau et al., 1990). The cultures became confluent on the 7th day in vitro and consisted of more than 98% endothelial cells (Fig. 1A), verified by positive Factor VIII-related antigen (Fig. 1B), negative glial fibrillary acidic protein and neurofilament immunohistochemistry.

2.2. Cell lines

RBE4 cells (Fig. 1C) are derived from rat brain microvessel endothelium immortalized with the plasmid pE1A-neo and characterized with respect to blood–brain barrier properties (Durieu-Trautmann et al., 1993; Roux et al., 1994). The cells were passaged twice a week in DMEM/F12, supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 1 ng/ml basic fibroblast growth factor and 300 μg/ml geneticin onto rat-tail collagen-coated dishes and used between passages 30 and 50. GP8 (Fig. 1D) is a temperature-sensitive SV40 large T immortalized rat brain capillary cell line described by Greenwood et al. (1996). These cells, used between passages 10 and 20, were cultured in 20% plasma-derived serum, 2 mM glutamine, 1 ng/ml basic fibroblast growth factor and 200 μg/ml geneticin in DMEM/F12.

2.3. Bioassay for the vasoactive metabolites of brain endothelial cells

Mongrel dogs of either sex weighing 9–15 kg were anesthetized with sodium pentobarbital (30 mg/kg i.v.) and heparinized (1000 I.U./kg i.v.). The heart was excised and placed in a Krebs–Henseleit solution of the following composition (in mM): NaCl 120, KCl 4.2, $CaCl_2$ 1.5, $NaHCO_3$ 20, $MgCl_2$ 1.2, KH_2PO_4 1.2 and glucose 11. Rings (1.1–1.9 mm o.d., 5 mm widths) from the circumflex branches of the left coronary artery were isolated. Endothelium was removed by gently rubbing the endothelial surface with a stainless-steel wire covered with a cotton swab. Rings were mounted in water-jacketed baths containing 2 ml of Krebs–Henseleit solution bubbled with 95% O_2 and 5% CO_2 gas mixture at 37°C. Isometric tension was recorded with a force-displacement transducer (Hugo Sachs Elektronic, Type F30, Germany). Rings were stretched up to 10 mN and allowed to stabilize for 45 min. This tension was readjusted to 10 mN during equilibration. The arterial rings were exposed to 25 μM prostaglandin $F_{2\alpha}$, and at the maximal amplitude of contraction, 1 μM

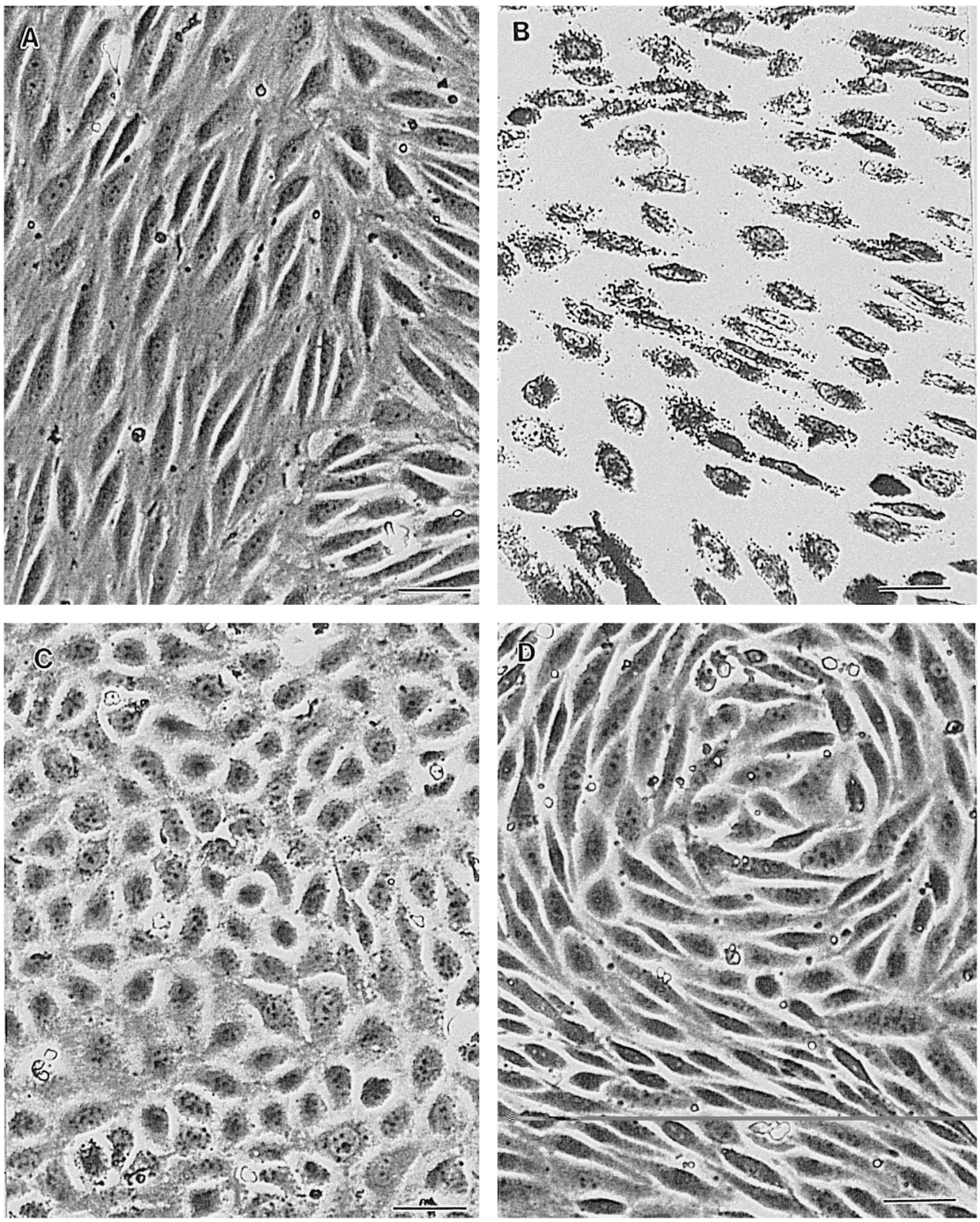

Fig. 1. (A) Uniform, confluent monolayer of primary rat brain endothelial cells on the 6th day in vitro. (B) Antibody against Factor VIII-related antigen gives a cytoplasmic dot-like staining in primary rat brain endothelial cells on the 7th day in vitro (light microscopy, 200 ×). Monolayers of RBE4 (C) and GP8 (D) cell lines. (A,C,D) Phase contrast microscopy, 200 ×, bar: 50 μm.

acetylcholine was applied. Only those arterial preparations were used for the experiments that responded with contraction after the addition of the endothelium-dependent vasodilator, acetylcholine. This protocol provided evidence for functionally de-endothelialized arterial preparations. Confluent monolayers of cultured rat brain endothelial cells were suspended in Krebs–Henseleit solution, resulting in 5×10^6 cells/ml and 5×10^5 cells/ml. The cell suspensions were divided into two parts: one was treated with solvents (control), the other either with 100 μM N^{ω}-nitro-L-arginine or with 10 μM indomethacin for 30 min at 37°C. The final concentration of indomethacin at the highest cell number in the organ bath was 0.3 μM. When the prostaglandin $F_{2\alpha}$-induced contraction of coronary rings had reached its steady state amplitude the control or treated endothelial suspensions were added cu-

mulatively. The animal experiments were performed according to the National Institute of Health Guidelines and approved by the ethical committee of the Biological Research Center of the Hungarian Academy of Sciences.

2.4. Assay of prostaglandin synthesis in rat brain endothelial cell

Confluent cultures of primary, RBE4 or GP8 cells in Petri dishes (35 mm diameter, approx. 3×10^5 cells/dish) were incubated at 37°C with the tracer substrate, 1-[^{14}C]arachidonic acid (0.172 pmol, 3.7 kBq) in 1 ml serum-free DMEM-F12. Thirty minutes later the incubation medium of the cell culture was removed and acidified to pH 3 with formic acid. According to our preliminary experiments a period of 30 min was appropriate for labelling in vitro the rat brain capillary endothelial cells. Arachidonate metabolism was determined as described previously (Mezei et al., 1997). Briefly, the arachidonate metabolites were immediately extracted from the samples with ethyl acetate (2×3 ml), and the organic phases were pooled and evaporated to dryness under nitrogen. The residues were reconstituted in 150 μl ethyl acetate and quantitatively applied to silica gel thin-layer plates. The plates were developed to a distance of 15 cm in an organic phase of ethyl acetate: acetic acid: 2,2,4-trimethylpentane: water (110:20:30:100) by means of overpressure thin-layer chromatography. The radiolabeled products of arachidonic acid were identified with unlabeled authentic standards, which were detected with anisaldehyde reagent. Each 3 mm band of the chromatograms was scraped off and the radioactivity was determined in a Packard Tri-Carb 2100TR liquid scintillation analyser, using 5 ml toluene containing 0.44% w/v 2,5-diphenyloxazole, 0.02% w/v 1,4-di-[2-(5-phenyl)-oxazole]benzene and 10% v/v ethanol.

2.5. Statistical analysis

Enhancement or reduction of arterial tone was calculated as a percent of the maximum increase (+) or decrease (−) of contractile force compared to pre-drug values. The results are expressed as means ± S.E.M. and *n* refers to the number of experiments. One-way analysis of variance (ANOVA) followed by the Newman–Keuls multiple range test or the Tukey-B multiple comparison post hoc test was used to determine the significance of differences between corresponding mean values. $P < 0.05$ was taken as statistically significant.

3. Results

3.1. Bioassay

In the first part of the experiments the vasoactive action of endothelial cell culture suspensions was determined. Fig. 2 and Table 1 demonstrates the effect of primary rat brain endothelial cells on the tone of isolated coronary artery contracted with prostaglandin $F_{2\alpha}$. The primary cells decreased the vascular tone which correlated with the cell number, and the maximal relaxation was 36% after administration of 6.2×10^5 cells/ml. RBE4 cells caused a small but not significant contraction (Fig. 3) Enhancement of arterial tone by these cells showed no cell-response relationship between $0.2–6.2 \times 10^5$ cells/ml with an average of 15–18% contraction. GP8 cells, like primary cells markedly decreased the tone of contracted arterial rings (Fig. 4) which depended on the number of cells with a maximum of $33.2 \pm 8.2\%$ ($n = 7$) decrease of prostaglandin $F_{2\alpha}$-induced tone.

The second part of the bioassay experiments concerned the possible involvement of nitric oxide as endothelium-dependent vasodilator in the changes of coronary tone. After 30-min incubation of primary endothelial cells with the inhibitor of nitric oxide synthesis, N^{ω}-nitro-L-arginine, the rat brain endothelial cell-induced vasorelaxation was decreased (Fig. 2). No significant change in the vasoactivity of the two immortalized cell lines was obtained with N^{ω}-nitro-L-arginine (Figs. 3 and 4). This suggests the absence, or a very low level, of basal nitric oxide production by constitutive endothelial nitric oxide synthase enzyme in the immortalized cell lines.

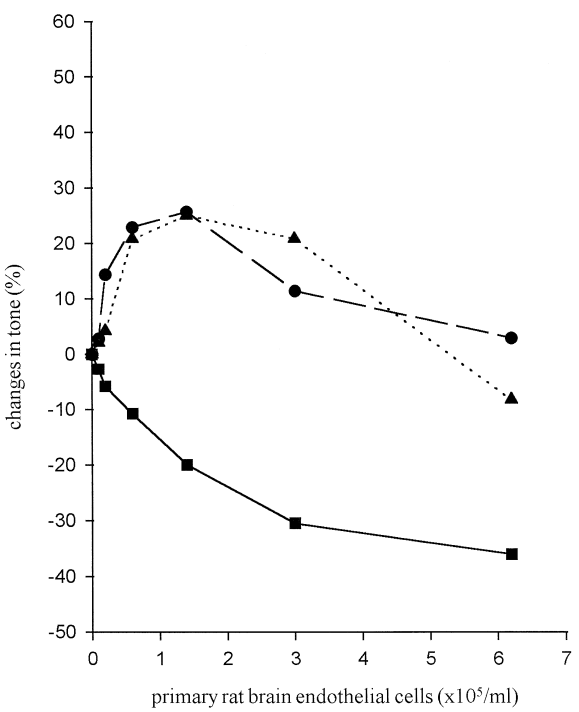

Fig. 2. Effect of primary rat brain endothelial cells on the tone of endothelium denuded coronary artery isolated from dogs after steady state contraction by prostaglandin $F_{2\alpha}$. Under control conditions (—) primary cells decreased the prostaglandin $F_{2\alpha}$-induced tone that was dependent on the number of the cells. Pretreatment of the cells with 100 μM N^{ω}-nitro-L-arginine (---) or 10 μM indomethacin (· · · · ·) for 30 min turned the vasorelaxation into a contraction. ($n = 1–3$).

Table 1
Effect of primary rat brain endothelial cells on the tone of endothelium denuded coronary artery isolated from dogs after steady state contraction by prostaglandin $F_{2\alpha}$

	Control		N^{ω}-nitro-L-arginine		Indomethacin		ANOVA
	mean ± S.E.M.	*n*	mean ± S.E.M.	*n*	mean ± S.E.M.	*n*	*F*
0.1×10^5 cells/ml	−2.7 ± 0.71	3	2.8 ± 1.76	3	2.1 ± 2.32	3	0.126
0.2×10^5 cells/ml	−5.8 ± 2.69	3	14.3 ± 5.83[a]	3	4.87 ± 1.96	3	0.029
0.6×10^5 cells/ml	−10.8 ± 3.11	3	22.9 ± 6.72[a]	3	20.8 ± 8.13[a]	3	0.016
1.4×10^5 cells/ml	−20.0 ± 7.43	3	25.7 ± 6.13[a]	3	25 ± 6.62[a]	3	0.005
3.0×10^5 cells/ml	−30.5 ± 11.11	3	11.4 ± 7.2[a]	2	20.8 ± 8.7[a]	2	0.043
6.2×10^5 cells/ml	−36.1 ± 12.6	2	2.9 ± 5.7	2	−8.3	1	0.195

Each value represents the mean ± S.E.M. in percent (%) of changes in tone of endothelium denuded coronary artery isolated from dogs after steady state contraction by prostaglandin $F_{2\alpha}$ under control conditions and after pretreatment of the cells with 100 μM N^{ω}-nitro-L-arginine or 10 μM indomethacin for 30 min. *n* means the number of experiments performed, and *F* means the *F* values of analysis of variance (ANOVA). Significant differences between primary rat brain endothelial cells and GP8 or RBE4 are indicated by [a] ($P < 0.05$).

In the third series of bioassay experiments the cells were pretreated with the cyclooxygenase inhibitor, indomethacin, for assessing the role of prostaglandins in the vasoactive potency of cerebral microvascular endothelium. The highest concentration of indomethacin (0.3 μM) by itself caused a small but significant increase of the acceptor vascular tone (from zero to 9.6 ± 1.4%, $n = 6$, $P < 0.05$). This increase reflects the known basal release of vasodilator prostaglandins from the smooth muscle of the acceptor coronary artery (Sakanashi et al., 1980). The small enhancement of tone by indomethacin was deduced from the effect of the endothelial suspension in Fig. 2. Indomethacin did not influence the contraction induced by RBE4 cells (Fig. 3) but turned the primary rat brain endothelial cell (Fig. 2) and GP8 cell (Fig. 4) -induced

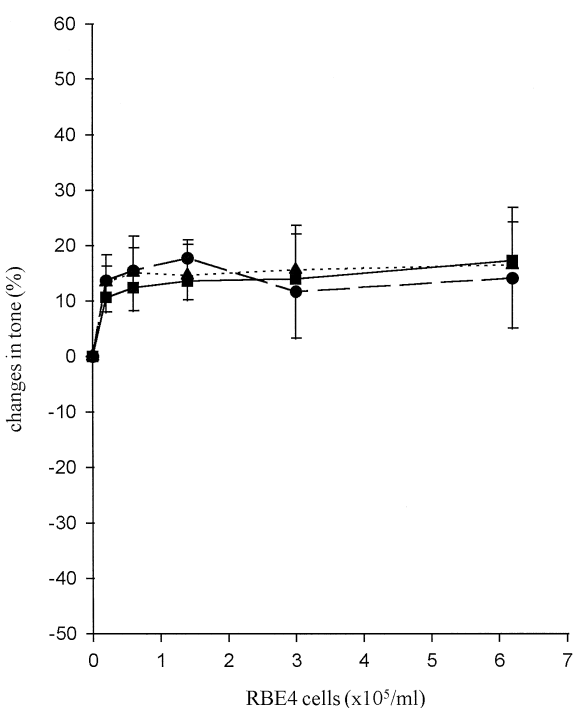

Fig. 3. Effect of RBE4 cell line on the tone of endothelium-denuded coronary artery isolated from dogs after steady state contraction by prostaglandin $F_{2\alpha}$. RBE4 cells (—) caused a small contraction of the coronary artery which was cell number-independent. A 30-min preincubation whether with 100 μM N^{ω}-nitro-L-arginine (---) or with 10 μM indomethacin (· · · · · ·) induced no change in the tone of coronary artery. Each value represents the mean ± S.E.M. ($n = 6$).

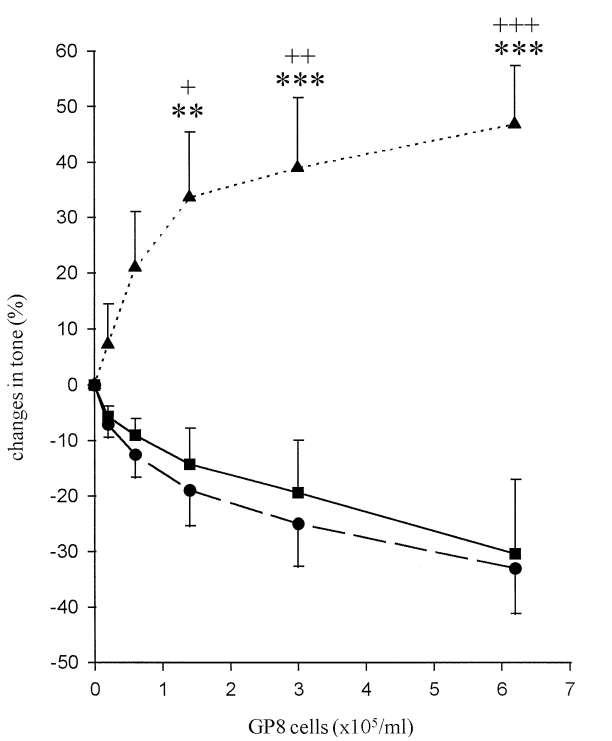

Fig. 4. Effect of GP8 cell line on the tone of endothelium-denuded coronary artery isolated from dogs. Under control conditions (—) GP8 cells decreased the prostaglandin $F_{2\alpha}$-induced tone that was dependent on the number of the cells in the organ bath. After 30-min pretreatment of endothelial cells with 100 μM N^{ω}-nitro-L-arginine (---) the vascular tone was not altered significantly as compared to the control. The vasorelaxation by GP8 cells was turned to contraction after inhibition of endothelial cyclooxygenase by 10 μM indomethacin (· · · · · ·). Each value represents the mean ± S.E.M. ($n = 7$). Significant differences between the control and indomethacin-treated cells are: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; between the N^{ω}-nitro-L-arginine and indometacin treated-cells are: $^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.001$.

Table 2
The profile of eicosanoid production by primary rat brain endothelial cells, RBE4 and GP8 immortalized cell lines

	Primary RBEC		RBE4		GP8	
	In DPM/3×10^5 cells/30 min and in percent of the total production					
	DPM	%	DPM	%	DPM	%
6-keto-$PGF_{1\alpha}$	427 ± 67	8.12	481 ± 12	16.64	635 ± 17	8.48
PGE_2	1500 ± 17	28.51	271 ± 41^{a}	9.38	$3186 \pm 41^{b,c}$	42.56
PGD_2	734 ± 15	13.95	368 ± 16	12.73	408 ± 73	5.45
$PGF_{2\alpha}$	626 ± 84	11.90	735 ± 74	25.43	711 ± 15	9.50
TxB_2	1166 ± 28	22.16	705 ± 74	24.39	1476 ± 14^{c}	19.72
12-HHT	889 ± 13	16.90	338 ± 12	11.70	1086 ± 31	14.51
TOTAL	5261 ± 60	100.00	2890 ± 33^{a}	100.00	$7486 \pm 91^{b,c}$	100.00

Prostanoid production was measured for the incubation solution of 3×10^5 cells at 37°C 30 min after the administration of 1-[^{14}C]arachidonic acid (0.172 pmol). Each value represents the mean ± S.E.M. ($n = 6$). Significant difference between primary rat brain endothelial cells and RBE4 is indicated by [a]($P < 0.05$), between primary rat brain endothelial cells and GP8 by [b]($P < 0.05$) and between RBE4 and GP8 by [c]($P < 0.05$). Abbreviations: 6-keto-$PGF_{1\alpha}$, 6-keto-prostaglandin $F_{1\alpha}$ (the stable metabolite of prostacyclin), PGE_2, prostaglandin E_2, PGD_2, prostaglandin D_2, $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$, TxB_2, thromboxane B_2 (the stable metabolite of thromboxane A_2), 12-HHT, 12-L-hydroxy-5,8,10-heptadecatrienoic acid, TOTAL, amount of all prostanoids measured.

vasorelaxation into contraction. These results suggest a higher activity of cyclooxygenase enzyme in primary rat brain endothelial cells and GP8 cells.

3.2. Production of arachidonic acid metabolites in cerebral endothelial cells

The cyclooxygenase pathway of the arachidonate cascade, 6-keto-prostaglandin $F_{1\alpha}$ (the stable metabolite of prostacyclin), prostaglandin D_2, prostaglandin E_2, prostaglandin $F_{2\alpha}$, thromboxane B_2 (the stable metabolite of thromboxane A_2) and 12-L-hydroxy-5,8,10-heptadecatrienoic acid were identified in the incubation medium of three different types of non-stimulated rat brain endothelial cell cultures. The profile of eicosanoid production by primary rat brain endothelial cells, RBE4 and GP8 cells is shown in Table 2.

Each type of rat brain endothelial cell was found to be capable of synthesizing prostaglandins. Significant ($P < 0.05$) differences in total cyclooxygenase metabolite release were found, with synthesis rates in increasing order, as follows: RBE4 < primary cells < GP8 cell line. The predominant vasodilator substance was prostaglandin E_2 in both primary cells and GP8 cell line, being 28 and 43% of the total eicosanoid production, respectively. The prostaglandin E_2 release (9% of total), from RBE4 cells was significantly ($P < 0.05$) lower than that from the other cells, and the main metabolites formed by RBE4 cells were prostaglandin $F_{2\alpha}$ and thromboxane B_2, (25 and 24%), respectively. While primary and GP8 cells released more than three times more prostaglandin E_2 than prostacyclin, the prostaglandin E_2:prostacyclin ratio produced by the RBE4 cell line was approximately 1:2. The percentage distribution of thromboxane B_2 and 12-L-hydroxy-5,8,10-heptadecatrienoic acid was in a similar range in all three cell types. A possible explanation for RBE4 cells' lower eicosanoid release could be the different way of immortalization and the higher passage number, which might have led to a somewhat less differentiated phenotype than that of the other two types of cerebral endothelial cells used in the study.

4. Discussion

Primary cultures of cerebral microvessels, as an in vitro blood–brain barrier model system, are widely used for morphological, functional and pharmacological studies (for review see Joó, 1992, 1996). Immortalized cell lines present several advantages: the time-consuming and expensive procedure of preparing primary cultures can be avoided, and it is easy to produce large amount of cells for genetic, immunological or biochemical experiments. Besides their use in basic research, cerebral endothelial cell lines are likely to find a new area of application in gene therapy. These cells are good candidates for gene delivery to the nervous system because of their normal localization at the blood-parenchyma interface and of their ability to proliferate in vivo and in vitro. It has been reported recently that RBE4 cells modified to express β-galactosidase reporter can be stably engrafted to growing gliomas in rats (Johnston et al., 1996). Altered RBE4 cells expressing both β-galactosidase and human fibroblast growth factor-1 gene survived following implantation to neonatal and adult rat brain (Lal et al., 1994).

The in vitro vasoactive properties of three types of rat brain endothelial cells, such as primary cells, as well as two immortalized cell lines, RBE4 and GP8, were investigated. A bioassay system was developed to analyse the composition of the paracrin signal derived from these cells in the absence of chemical stimulation. The effect of vasoactive substances released by rat brain endothelial cells was investigated on the isometric tone of an endothe-

lium-denuded canine coronary arterial in vitro preparation. The smooth muscle of this blood vessel is known to be very sensitive to prostaglandins (Hyman et al., 1978), and also has an active soluble guanylate cyclase enzyme for detecting nitric oxide in vitro (Hintze and Kaley, 1984; Feletou et al., 1989). Prostaglandin E_2 and prostacyclin have been found to be the main cyclooxygenase metabolites released by rat brain endothelial cells in vitro (Moore et al., 1988; De Vries et al., 1995). Our findings obtained from both the bioassay experiments and determination of arachidonic acid metabolites are in accordance with the previous observations. The vasorelaxant effect of primary cells and the GP8 cell line corresponds well with the greater prostaglandin E_2 release. Furthermore, the abundance of the dilator type of prostaglandin (prostaglandin E_2 and prostacyclin) released by primary rat brain endothelial cells and GP8 cells in relation to the amount of the vasoconstrictor type of cyclooxygenase metabolites, such as prostaglandin $F_{2\alpha}$ (Miller et al., 1989), prostaglandin D_2 (Sakanashi et al., 1980) and thromboxane A_2 (Toda et al., 1986), also explains the vasorelaxant effect. The release of vasoactive eicosanoids from RBE4 was limited, which was consistent with the low-profile functional effect induced by these cells in the bioassay.

The presence of functionally relevant quantities of arachidonic acid metabolites released from primary and GP8 cells suggests that eicosanoids of cerebral endothelium may have physiological and/or pathophysiological significance in vivo. It has been demonstrated recently that vasodilator prostanoids derived from small cerebral blood vessels play roles in the regulation of the cerebral circulation (Anzai et al., 1995; Satoh et al., 1995). Endogenous vasodilator prostaglandins (prostaglandin E_2 and prostacyclin) have been found to inhibit cerebral vasoconstriction induced by experimental hematoma (Yakubu et al., 1995), while exogenous prostacyclin was successfully used to protect against endothelial dysfunction during chronic cerebral vasospasm (Egemen et al., 1995). Cultured brain microvascular endothelium was shown to produce less vasodilator prostaglandins than did the endothelium of a large vessel (Satoh et al., 1995). It is interesting that the higher prostaglandin E_2: prostacyclin ratio found in primary cells and the GP8 cell line was similar to that seen in the endothelium of microvessels, while the predominance of prostacyclin measured in the RBE4 cell line resembles the eicosanoid profile of larger vessels (Renzi and Flynn, 1992). In our experiments, the endothelial cell-induced vasodilation of contracted canine coronary artery was turned to vasoconstriction when primary and GP8 endothelial cells were preincubated with indomethacin. We do not know which mediators are responsible for the in vitro contractile effect, but indomethacin may shift the arachidonate cascade to the lipoxygenase pathway releasing vasoconstrictor leukotriene C_4, leukotriene D_4 and leukotriene E_4 (Woodman and Dusting, 1982; Yakubu et al., 1995). In addition indomethacin could enhance the vasoconstrictor effects of endothelin-1 on porcine pial arterioles in vivo (Yakubu et al., 1995).

Both primary cells and the GP8 cell line significantly decreased coronary vascular tone, while the RBE4 cell line was ineffective. In a previous study only the inducible type of nitric oxide synthase was detectable in RBE4 cells (Durieu-Trautmann et al., 1993). In accordance with these findings, our recent preliminary results indicated the expression of mRNA of constitutive, endothelial nitric oxide synthase in cultured primary rat brain endothelial cells and the GP8 cell line, which was missing in RBE4 cells (unpublished data, Krizbai et al.). However, the nitric oxide synthase inhibitor, N^{ω}-nitro-L-arginine, applied to the organ bath, decreased the vasorelaxation of canine coronary artery caused by primary cells, but failed to do so with GP8 cells. The ineffectiveness of N^{ω}-nitro-L-arginine on the GP8 cell line might be explained by the fact that, compared to the eicosanoids, nitric oxide plays only a minor role in the vasorelaxation observed. We cannot exclude an ineffective basal release of nitric oxide in GP8 cells, at least up to the concentration of 1.2×10^6 cells in a 2-ml volume. It is possible that this number of endothelial cells released insufficient amounts of nitric oxide for producing relaxation. The volume of Krebs–Henseleit solution was four orders of magnitude greater than the greatest estimated endothelial cell volume (approx. 150 μm^3/cell) and the nitric oxide molecules produced, having a half-life shorter than a second, could lose activity before reaching the effector cells. On the other hand, free radicals produced by GP8 cells might inactivate nitric oxide (Rubanyi and Vanhoutte, 1986). The major source of free radicals in cerebral endothelial cells is the cyclooxygenase pathway of the arachidonate cascade (Kontos et al., 1985), and our results showed that cyclooxygenase was more active in GP8 cells than in primary cells. These data might also explain our findings in the bioassay experiments, where we failed to demonstrate a biologically significant nitric oxide production of GP8 cells on isolated dog coronary artery.

In the present study we used primary cerebral endothelial cells as well as RBE4 and GP8 immortalized cell lines in order to determine their bioactivity and the production of vasoactive eicosanoids. We conclude that the eicosanoid profile of the GP8 cell line is closer to that of primary cerebral endothelial cells than to that of RBE4 cells, which was also reflected by the results of the bioassay. Nevertheless, both cell lines may be useful tools for biotechnological and therapeutic applications in the near future (Lal et al., 1994; Johnston et al., 1996).

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