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Applying blood-brain barrier *in vitro* models to study the influence of drugs on endothelial cells – effects of selected COX-inhibitors

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The influence of three cyclooxygenase (COX) inhibitors (indometacin, lornoxicam and celecoxib) with different COX-1/COX-2 profiles on endothelial cells using *in vitro* blood-brain barrier (BBB) models was investigated. For the experiments two BBB mimicking cell lines (PBMEC/C1-2 and ECV304) and primary human umbilical vein endothelial cells (HUVEC) were used. In preliminary tests the two cell lines and HUVEC were characterized by cell ELISA in respect of the presence of the tight junction proteins occludin and zonula occludens protein-1 (ZO-1), the adhesion molecules ICAM-1 and VCAM-1 and the endothelial marker von Willebrand factor (vWF). Then, the influence of indometacin, lornoxicam and celecoxib on the expression of occludin, ZO-1, ICAM-1 and vWF of the two cell lines and HUVEC was analysed by cell ELISA. The COX inhibitors caused an effect on PBMEC/C1-2 and HUVEC but no influence was observed on ECV304. The results of PBMEC/C1-2 and HUVEC indicated that in comparable therapeutical concentrations celecoxib had a higher potential to impair endothelial cells and to decrease the expression of occludin, ZO-1 and ICAM-1 than indometacin and lornoxicam.

1. Introduction

The blood-brain barrier (BBB) is a physical and metabolic barrier between the central nervous system (CNS) and the systemic circulation, which maintains the homeostasis of the microenvironment of the brain. The BBB consists of brain microvascular endothelial cells. These cells are connected by tight junctions, which results in high transendothelial electrical resistance and decreased paracellular permeability. Impairment of the BBB integrity can cause serious effects on the CNS.

Extensive work about BBB models has been published (Garberg et al. 2005; Roux and Couraud 2005; Rubin et al. 1991), many of the studies all investigative in principle but only very few of them consider factors such as robustness and validity of the models. Modern drug research requires assessment of ADME (absorption, distribution, metabolism, excretion) parameters already during the drug discovery phase, therefore we evaluated BBB models in respect of their qualification to study the impact of the drugs on endothelial cells.

Triggered by the findings that cyclooxygenase (COX)-2 inhibitors increase the cardiovascular risk in patients (Chen and Ashcroft 2007), we chose selected non steroidal anti inflammatory drugs (NSAID) for a first series of experiments to investigate the effect of drugs on endothelial cells *in vitro*. Three NSAID with known and differing COX-1/COX-2 profile were applied: indometacin (COX-1 inhibitor), lornoxicam (COX-1 and -2 inhibitor) and celecoxib (COX-2 selective inhibitor).

The COX enzymes catalyse the conversion of arachidonic acid to prostacyclin, thromboxane and various prostaglandins. COX exists in two isoforms, COX-1 and COX-2. COX-1 isoenzyme is constitutive and essential for maintenance and function of many tissues. In contrast, COX-2 is in most cases induced by various inflammatory stimuli. Unselective COX inhibitors are known to cause gastric side effects and bleeding due to COX-1 inhibition. COX-2 selective inhibitors cause these side effects to a lesser extent, but chronic treatment with COX-2 inhibitors causes cardiovascular side effects (Krötz et al. 2005; Mukherjee et al. 2001). Physiological mechanisms, which are influenced by COX-2 inhibitors, are jointly responsible for the cardiovascular side effects. The most important cause for physiological impairment by COX-2 inhibitors is the imbalance between prostacyclin and thromboxane resulting from selective COX-2 inhibition. Another reason for side effects, due to COX-2 inhibition, is the increased metabolism of arachidonic acid through the lipoxygenase pathway. This results in a higher amount of leukotrienes, which in turn attracts neutrophil leucocytes. Moreover, COX-2 expression is also induced by laminar shear stress (Topper et al. 1996) which further leads to an increased prostacyclin synthesis which again results in vasodilatation and inhibition of platelet aggregation. A selective inhibition of COX-2 can therefore cause a loss of the described mechanism and contribute to side effects.

The experiments were carried out with two BBB mimicking cell lines (ECV304 and PBMEC/C1-2) together with primary human umbilical vein endothelial cells (HUVEC).

ECV304 (Takahashi et al. 1990) and PBMEC/C1-2 (Teifel and Friedl 1996) were cultured in an astrocyte conditioned medium (ACM) to mimic BBB conditions. Astrocytes are responsible for the induction of characteristic BBB features such as tight junction formation, a high activity of BBB specific enzymes and a reduction of the permeability (Easton and Abbott 2002; Hurst and Fritz 1996; Janzer and Raff 1987). Primary HUVEC were included in the investigations to compare the response of the BBB cell lines with primary endothelial cells. First, the two cell lines and HUVEC were characterized with a cell enzyme-linked immunosorbent assay (ELISA) in respect of the presence of the tight junction proteins occludin and zonula occludens protein-1 (ZO-1), the adhesion molecules intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), and the endothelial marker von Willebrand factor (vWF). Then, the concentration dependent impact of the three COX inhibitors onto the expression of occludin, ZO-1, ICAM-1 and vWF was studied. The COX inhibitors were used in concentrations related to their therapeutic maximum plasma concentrations (C_{max}). The experiments were analysed by cell ELISA and Bradford assay.

2. Investigations and results

The expression of occludin, ZO-1, ICAM-1 and vWF was verified on PBMEC/C1-2 and ECV304, whereas VCAM-1 was not detectable in both cell lines. The expression pattern of HUVEC was slightly different. The presence of occludin, ICAM-1 and vWF could be verified, but evidence for ZO-1 and VCAM-1 expression could not be provided. (These results were also obtained by western blotting or fluorescence microscopy using the same primary antibodies (data not shown)).

The results of PBMEC/C1-2 obtained from Bradford assay (Fig. 1a) showed that celecoxib decreased the whole protein amount of the cells in the highest applied concentration (10x C_{max}). By screening these cell layers with light microscope it was evident that a great amount of the cells were detached and dead. This was considered as a toxic effect. For all other substances and concentrations no noticeable effect was detected.

Results of the cell ELISA indicated that the influence of indometacin, lornoxicam and celecoxib on occludin (Fig. 1b), ZO-1, ICAM-1 (Fig. 1c) and vWF (Fig. 1d) of PBMEC/C1-2 was quite similar. That celecoxib was toxic in 10-fold plasma concentration was confirmed by the cell ELISA results. Furthermore, cell ELISA showed that applied concentrations of indometacin and lornoxicam did not significantly decrease the expression of the investigated proteins of PBMEC/C1-2, whereas the influence of celecoxib was dose-dependent. The 10-fold plasma concentration of celecoxib was toxic. The 5-fold plasma concentration of celecoxib caused a significant reduction of ICAM-1 ($11.2 \pm 2.9\%$) and ZO-1 ($18.7 \pm 3.7\%$) expression, but it had no significant effect on expression of occludin and vWF. The two lower celecoxib concentrations did not significantly affect the expression of any of the investigated proteins of PBMEC/C1-2.

For the ECV304 cell line no definite effect of the three COX inhibitors on the protein amount was detected by Bradford assay (Fig. 2a). The results of the cell ELISA did not prove a clear impact of indometacin, lornoxicam and celecoxib on occludin (Fig. 2b), ZO-1, ICAM-1 (Fig. 2c) and vWF (Fig. 2d) of ECV304 at the applied concentrations.

Applied on HUVEC Bradford assay (Fig. 3a) showed a significant reduction of the protein amount of $17.9 \pm 1.4\%$ with the 10-fold plasma concentration of

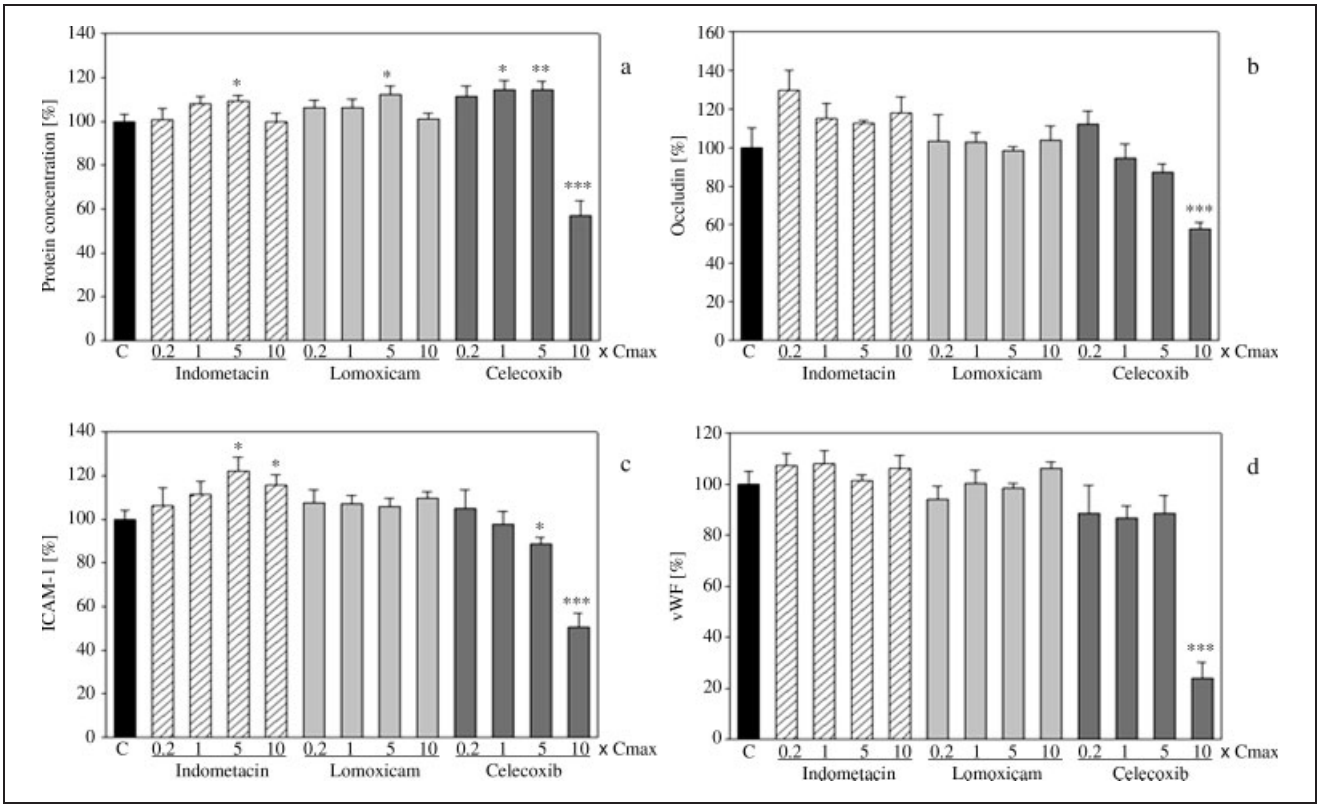

Fig. 1: Effect of indometacin, lornoxicam and celecoxib on PBMEC/C1-2. Impact on the whole protein amount of cells analysed by Bradford assay (n = 14–15) (a) and on the expression of occludin (n = 5–9) (b), ICAM-1 (n = 5–12) (c) and vWF (n = 3) (d) obtained by cell ELISA. The data represents mean ± S.E.M. For statistical comparison with the control (C) the student's t-test was used (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$)

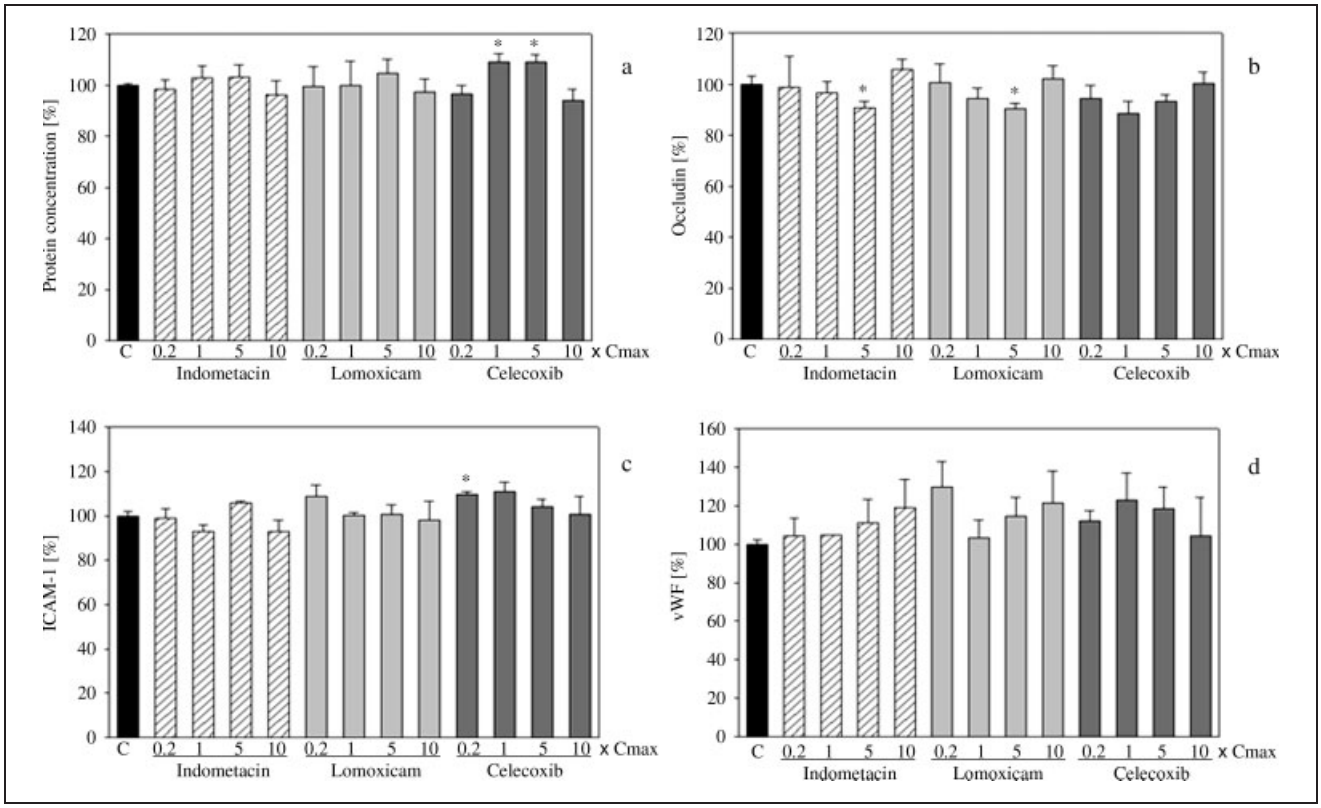

Fig. 2: Effect of indometacin, lornoxicam and celecoxib on ECV304. Impact on the whole protein amount of cells analysed by Bradford assay (n = 6) (a) and on the expression of occludin (n = 5–6) (b), ICAM-1 (n = 3) (c) and vWF (n = 3) (d). The data represents mean ± S.E.M. For statistical comparison with the control (C) the student's t-test was used (* $p < 0.05$)

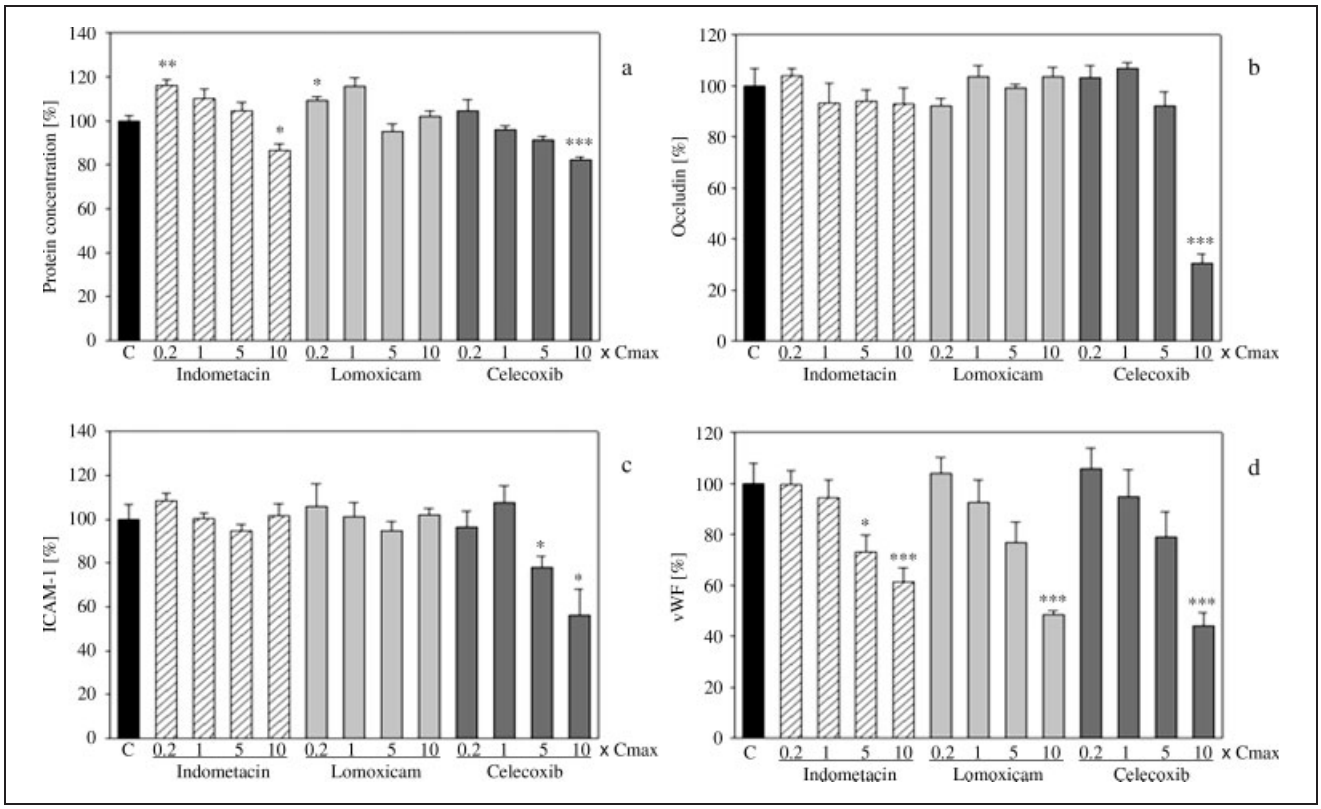

Fig. 3: Effect of indometacin, lornoxicam and celecoxib on HUVEC. Impact on the whole protein amount of cells analysed by Bradford assay (n = 6) (a) and on the expression of Occludin (n = 3–6) (b), ICAM-1 (n = 3–6) (c) and vWF (n = 8–12) (d). The data represents mean ± S.E.M. For statistical comparison with the control (C) the student's t-test was used (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$)

Celecoxib and also a significant reduction of $13.3 \pm 2.8\%$ with the 10-fold plasma concentration of indometacin. Light microscopy confirmed the toxicity of 10-fold plasma concentration of celecoxib, whereas no effects of highest indometacin concentration were seen.

The results of cell ELISA for occludin (Fig. 3b) verified the toxic effect of the highest concentration of celecoxib and did not indicate any other influence of the COX inhibitors on the expression of occludin. A significant reduction of ICAM-1 expression (Fig. 3c) was shown by cell ELISA with the 10-fold plasma concentration of celecoxib probable due to its toxicity. The 5-fold plasma concentration of celecoxib caused a significant reduction of the ICAM-1 expression of $22.1 \pm 5.2\%$. Interestingly, the influence of the applied substances on vWF (Fig. 3d) was significantly different in comparison to all other, rather consistent effects. Indometacin, Iornoxicam and celecoxib had a dose-dependent effect on the expression of vWF. The higher the applied concentration of COX inhibitors, the lower was the detected vWF amount.

3. Discussion

Every drug transported by blood is in contact with the endothelium and the BBB. The BBB integrity is crucial for neuronal activity and function. Therefore, it is of high importance to ensure that drugs transported by blood do not influence the BBB integrity. *In vitro* BBB models have been widely used to study the transport of metabolites and drugs into the brain (Deli et al. 2005). However, very little information is available about the effect of drugs on the endothelial cells of the BBB. The aim of this study was to assess, whether cell lines used for *in vitro* BBB models (Hurst and Fritz 1996; Lauer et al. 2004; Neuhaus et al. 2006) could also be useful to study the impact of drugs on the BBB.

Thus, the effects of indometacin, lornoxicam and celecoxib on the expression of occludin, ZO-1, ICAM-1 and vWF of two BBB mimicking cell lines and primary HUVEC were investigated. A significant drawback of the application of PBMEC/C1-2 and HUVEC was given by the fact that these cells detached from the substrate, when they became over confluent. Therefore, the duration of the described experiments was limited to 72 h. Since drug effects on the BBB include up- and down-regulation of protein expression (particularly with respect to tight junction proteins and adhesion molecules), which might become significant only after a longer time period of application, effects with delayed onset might not be seen in the experiments. Applying the drugs not only in therapeutical plasma concentrations but also in 5- and 10-times higher concentrations, will not only mimic the situation of overdosing, but could also enhance the "late onset effects" to be more easily detectable during the short time experiments.

We were able to observe effects on PBMEC/C1-2 and on HUVEC but no influence on ECV304 was found. In other words, ECV304 were less sensitive than PBMEC/C1-2 and HUVEC to the applied COX inhibitors under the described conditions. According to the fact that ECV304 were introduced as spontaneously transformed HUVEC (Takahashi et al. 1990), it seems that ECV304 had lost or gained a certain mechanism which is responsible for the robustness of these cells against indometacin, lornoxicam and celecoxib. The results of another report also proved that the effects of celecoxib on two different kinds of cells were diverse. Niederberger et al. (2004) have found that celecoxib inhibited the proliferation of HUVEC in a dose dependent manner, whereas vascular smooth muscle cells were not affected. Except the results of vWF cell ELISA of HUVEC the findings of PBMEC/C1-2 and HUVEC were quite consistent. To describe the results in more detail, indometacin and lornoxicam did not have any effect on the expression of occludin, ZO-1, ICAM-1 and vWF of PBMEC/C1-2 and occludin and ICAM-1 of HUVEC. The influence of celecoxib on the cells was dose related. Celecoxib was toxic in a 10-fold therapeutical plasma concentration, whereas indometacin and lornoxicam were not. The 5-fold plasma concentration of celecoxib significantly decreased certain protein amount but indometacin and lornoxicam did not. As already mentioned, the results of the vWF cell ELISA of HUVEC were different. In this case, indometacin, lornoxicam and celecoxib showed a dose dependent effect on vWF. It should be considered that HUVEC expressed a 4- to 6-times higher amount of vWF than the investigated cell lines. This could mean that in case of HUVEC which are primary cells, vWF was stronger expressed and also more sensitive to the applied drugs.

The results of this investigation demonstrate that celecoxib, a selective inhibitor for COX-2, has in comparable concentrations a higher potential to affect endothelial cells and to decrease occludin, ZO-1 and ICAM-1 than indometacin, which is a COX-1 inhibitor, and lornoxicam, which is an unselective COX inhibitor. It seems advisable to continue the exploration of the applicability of validated *in vitro* BBB models, and their cell lines, not only for transport studies, but – in an add on option – also to study the impact of the drugs on the endothelial cells of the BBB itself.

4. Experimental

4.1. Materials

Isocove's modified Dulbecco's medium (IMDM), Ham's F12, newborn calf serum (NCS), L-glutamine, penicillin/streptomycin, trypsin/EDTA and PBS were bought from Invitrogen Corporation (Gibco™, CA, USA). Transferrin, amphotericin B, gelatine, tetramethylbenzidine (TMB) and protease inhibitor cocktail were purchased from Sigma (MO, USA). Heparin was obtained from MP Biomedicals Inc. (OH, USA). Medium 199 was from Cambrex Bio Science (BioWhittaker™, Belgium). Fetal bovine serum (FBS) was purchased from Pan Biotech (Germany) and endothelial cell growth supplement (ECGS) from Technoclone (Austria). Indometacin was bought from Fagron (Germany). The goat polyclonal antibodies: occludin (sc-8145), ZO-1 (sc-8146), the mouse monoclonal antibodies: ICAM-1 (sc-8439), VCAM-1 (sc-13160), vWF (sc-21784) and the horse radish peroxidase conjugated secondary antibodies: donkey anti-goat IgG (sc-2020) and goat anti-mouse IgG (sc-2005) were all purchased from Santa Cruz Biotechnology Inc. (CA, USA). Lornoxicam was a kind gift from Dr. Mayrhofer (AGES PharmMed, Austria). Cell culture flasks and 96-well plates were obtained from Greiner bio-one (Austria).

4.2. Cell culture

PBMEC/C1-2 were a kind gift from Teifel and Friedl (1996). C6 cells derived from a rat glioma (Benda et al. 1968) were obtained from the German Cancer Research Center Heidelberg (DKFZ, Heidelberg, Germany) and cultured in C6 medium. The C6 medium is a 1 : 1 mixture of Ham's F12 and IMDM supplemented with 7.5 % (v/v) NCS, 7 mM L-glutamine, 5 μg/ml transferrin, 0.5 U/ml heparin, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B. In order to obtain ACM the supernatant was collected every day. PBMEC/C1-2 were cultured in PBMEC medium (50 % C6 medium, 50 % ACM) at passages 63–73.

The ECV304 cell line was obtained from the European Collection of Animal Cell Cultures (ECACC, Wiltshire, UK) and cultured in PBMEC medium. Cells from passages 152–174 were used for the described experiments.

Detailed culture conditions were reported earlier (Lauer et al. 2004). No differences were found between early and late passages of PBMEC/C1-2 and ECV304 (Suda et al. 2001; Teifel and Friedl 1996).

HUVEC were isolated according to a previously published method (Gimbrone et al. 1974) and cultured in Medium 199 containing 16.25 % (v/v) FBS, 32.5 μg/ml ECGS, 1.6 mM L-glutamine, 4.9 U/ml heparin, 81.2 U/ml penicillin, 81.2 μg/ml streptomycin and 0.2 μg/ml amphotericin B. Cells were amplified

from passage 1 to 3 in order to get the amount of cells necessary for the experiments. Passages 4 and 5 of HUVEC were used for the described assays.
All cells were cultured at 37 °C, 5 % CO_2 and 95 % humidity. For subcultivation a Trypsin/EDTA solution was used.

4.3. Application of COX inhibitors

Cells were seeded on gelatine-coated 96-well plates in a concentration of 80 000 cells/cm^2. Three hours after seeding, COX inhibitors were added to the culture medium. Indometacin, lornoxicam and celecoxib were used in four concentrations related to therapeutical applications (0.2, 1, 5 and 10-fold C_{max}). The maximum plasma concentrations of the three COX-inhibitors were 5.03 μM for indometacin (Sandoz Pharmaceuticals, Germany, 2003), 2.69 μM for lornoxicam (Nycomed Pharma, Austria, 2001) and 2.62 μM for celecoxib (Davies et al. 2000). The COX inhibitors were dissolved in DMSO. DMSO concentration in culture wells was lower than 0.5 % (v/v) and has been previously assured not to disturb the experimental setup. Control samples were treated in the same way without the addition of COX inhibitors. After 72 h incubation, the influence of COX inhibitors on certain cell molecules was analysed by Bradford assay and cell ELISA.

4.4. Cell lysis and Bradford assay

Cell layers were washed twice with PBS and then treated with ice cold lysis puffer (8 M urea, 2 M thiourea, 0.5 % (v/v) TritonX-100, 2 % (w/v) CHAPS, 5 mM EDTA, 5 mg/ml dithiothreitol, 10 μl/ml protease inhibitor cocktail). The obtained samples were centrifuged (15 000 rpm, 4 °C, 10 min) and analysed by Bradford assay (Bradford 1976).
Therefore samples were mixed with Bradford reagent (0.01 % (w/v) coomassie brilliant blue G250, 5 % (v/v) ethanol, 10 % (v/v) phosphoric acid), incubated for 5 min at room temperature and measured at 595 nm on a spectrophotometer (UV-1700 Pharma Spec, Shimadzu, Austria). The protein amount of the samples was calculated by means of a bovine serum albumin calibration curve.

4.5. Cell ELISA

Cell layers were washed twice with PBS and then fixed with 0.1 % (v/v) glutaraldehyde for 10 minutes at room temperature. Cells were again washed twice with PBS and then incubated with the primary antibody (1 μg/ml PBS containing 1 % milk powder) for 90 min at 37 °C and afterwards with the horse radish peroxidase conjugated secondary antibody (0.08 μg/ml PBS containing 1% milk powder) for 60 min at 37 °C. After each incubation step cells were washed three times with PBS containing 0.1 % milk powder. Next, the peroxidase substrat TMB was applied. Enzyme reaction was stopped after 15 min by the addition of 2 M HCl. Optical density was measured with a microplate reader (polarstar galaxy, BMG Labtech, Germany) at a wavelength of 450 nm.

4.6. Statistical analysis

Data are presented as mean ± standard error of the mean (S.E.M.) and for statistical analysis the student's t-test was used (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$).

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