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Hydrocortisone Reinforces the Blood–Brain Barrier Properties in a Serum Free Cell Culture System

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The increasing number of newly developed drugs demands for functional *in vitro* models of the blood-brain barrier to determine their brain uptake. Cultured cerebral capillary endothelial cells are considered to be such a model, however in serum containing media they exhibit low electrical resistances and high permeabilities compared to the *in vivo* situation. Here we report the establishment of a serum-free cell culture model. Withdrawal of serum already caused a twofold increase of transendothelial resistance (TER), which in presence of serum is about 100-150 $\Omega \cdot cm^2$. We tested several supplements and found that hydrocortisone is a potent stimulator for the formation of barrier properties. TERs up to 1000 $\Omega \cdot cm^2$ were measured in the presence of physiological relevant hydrocortisone concentrations. In correspondence to the TER increase hydrocortisone decreased cell monolayer permeability for sucrose down to $5 \cdot 10^{-7}$ cm/s, which is close to the *in vivo* value of $1.2 \cdot 10^{-7}$ cm/s and by a factor of five lower compared to cultures without hydrocortisone and in presence of serum.

Combinatorial chemistry is able to yield high numbers of compounds of pharmaceutical interest. An important aspect for a new drug is to know its availability in the nervous system, which means the ability to cross the blood-brain barrier (BBB). Thus the permeability of thousands of compounds will have to be screened in the near future. This will not be possible *in vivo*, so that powerful *in vitro* models of the BBB are demanded, that mimic the differentiated BBB at least with respect to its barrier properties.

In the last years preparation methods and cell culture systems of brain capillary endothelial cells (BCEC), which build up the BBB, were established and *in vitro* models of the barrier were developed [1, 2, 3]. Many of these *in vitro* models are able to mimic the *in vivo* situation very well, but for the best cell culture systems it was until now necessary to grow BCEC in co-cultures with astrocytes [2] or to apply a combination of astrocyte-conditioned medium and agents that elevate intracellular cAMP [3]. All of them used serum in the cell culture medium, which deteriorates the reproducibility of experiments and the analysis of permeabilities.

Here we report the establishment of a cell culture system for BCEC with low sucrose permeability and high electrical resistance, which is not dependent on a co-culture with astrocytes or astrocyte-conditioned medium and does not require the use of serum. Hydrocortisone supplementation was found to be essential and sufficient to induce barrier properties *in vitro*. Maintenance of the barrier lasts for several days, which is long enough for pharmaceutical screening.

METHODS

The cells were isolated from freshly slaughtered pigs by several enzymatic digestion and centrifugation steps according to a modified method of Bowman et al. [4], as described previously [5]. In short, after removal of the meninges and the secretory areas, the grey and white matter of the brain cortex was minced using a sterile cutter with staggered rolling blades. The material was suspended in DMEM/Ham's F12 (Biochrom, Berlin, Germany) and incubated with dry powdered Dispase II from bacillus polymyxa (1% (w/v); Boehringer, Mannheim, Germany) for about 3 h at 37°C. A dextran solution (mw ~ 162000, 18% (w/v); Sigma, Deisenhofen, Germany) was added to get a final 10.8% (w/v) suspension, and the suspension was centrifuged at 6800 g for 10 min at 4°C. To separate larger vessels the pellet was resuspended and filtered through a 180 μm nylon sieve (ZBF, Zürich, Switzerland). The capillaries were incubated with 0.1% (w/v) collagenase/dispase II (vibrio alginolyticus/bacillus polymyxa; Sigma, Deisenhofen, Germany) at 37°C for 2-3 h under gentle stirring by a hanging magnetic stirrer. After collecting the released cell aggregates by low spin centrifugation (140 g, 10 min, 20°C) they were further purified by density gradient centrifugation. For this step the yield of 1-2 brains was resuspended in 10 ml M199 and centrifuged on a discontinuous Percoll-gradient (15 ml 1.07 g/cm^3; 20 ml 1.03 g/cm^3; Sigma, Deisenhofen, Germany) at 1300 g for 10 min in a swinging bucket rotor. The cell clusters of the PBCEC, which are gathered at the interface, were washed in DMEM/Ham's F12 and centrifuged at 140 g for 10 min. Cell clusters from one brain

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were sown on 450 cm^2 collagen G (Seromed, Berlin, Germany) coated culture surface.

Cultured BCEC were characterized by their typical spindle shaped morphology, their expression of von Willebrand factor and their high specific activity of alkaline phosphatase and γ-glutamyl-transpeptidase. They were grown in DMEM/Ham's F12 containing 4 mM glutamine (Seromed, Berlin, Germany), 10% (v/v) ox serum (PAA, Linz, Austria), penicillin/streptomycin (100 μg/ml) and gentamicin (100 μg/ml) (both Sigma, Deisenhofen, Germany). In order to prepare confluent cell monolayers, primary cultured BCEC were subcultivated after three days *in vitro* by sowing them again on rat tail collagen coated filter inserts ($1.5 \cdot 10^5$ cells/cm^2). After another day *in vitro* medium was exchanged by DMEM/Ham's F12 containing different supplements. The BBB-features of cultured cell monolayers were scrutinized by determination of transendothelial electrical resistance (TER) with AC impedance analysis [6] and by measuring the permeability coefficient of [^{14}C]-sucrose (Amersham, Buckinghamshire, UK).

RESULTS

In order to improve the barrier characteristics of PBCEC monolayers the influence of hormones or growth factors like insulin, EGF and hydrocortisone was studied. Normal culture medium containing serum was exchanged one day after subcultivation by fresh medium, which contained hormones or growth factors (incubation medium). In general PBCEC were able to build up dense cell monolayers with electrical resistances two days after subcultivation (Fig. 1). Highest barriers properties were detected four to five days after subcultivation, which is 7-8 days *in vitro* (DIV). In DMEM/Ham's F12 with all three supplements TER increased considerably to up to 1000 $\Omega \cdot cm^2$ at 7-8 DIV. Therefore, further permeability studies or analysis of transendothelial electrical resistance (TER) were performed at 7-8 DIV.

EGF between 2-8 nM showed no effect on TER. Insulin up to 700 pM slightly improved TER, but for a significant effect it had to be added in an unphysiological high concentration up to 70 μM, which increased the TER by a factor of two. However since these concentrations were more than 10.000 times higher than the physiological concentration in the human bloodstream (70-700 pM [7]) insulin has to be considered as non effective with respect to barrier formation.

Addition of hydrocortisone to the incubation medium caused drastic increase of the TER of PBCEC monolayers shown in Fig. 1. Under serum free culture conditions the TER of PBCEC monolayers, which were incubated with hydrocortisone, was up to 2-3 times higher than TER in hydrocortisone free medium. The maximal effect of hydrocortisone was reached at a concentration of 70 nM or more (Fig. 2), which is well in the physiological concentration range of hydrocortisone in the human bloodstream between 70-550 nM [7]. In the following experiments we thus used 550 nM hydrocortisone in the incubation medium to mimic the *in vivo* situation as close as possible.

In the experiments shown in Fig. 2 PBCEC monolayer developed a maximum TER of about 700 $\Omega \cdot cm^2$. This is a typical preparation with a reliable and clearly reproducible effect. The absolute maximum TER values we observed varied and depended on the primary culture of PBCEC. Some preparations of PBCEC yielded cells, which were able to build up dense cell monolayers with TER values up to 1000 $\Omega \cdot cm^2$ after incubation with 550 nM hydrocortisone (e.g. Fig. 1). On the other hand we were confronted with some primary cultures that only developed TERs of 300-500 $\Omega \cdot cm^2$, which is still a good result. Combinations of hydrocortisone, EGF and Insulin were not different in their effect compared to hydrocortisone alone.

Fig. 3 summarizes the effect of serum and hydrocortisone on TER and [^{14}C]-sucrose permeability of PBCEC in culture. Relative values are given considering the broad distribution of initial TERs obtained during different preparations. Cell monolayers in the absence of serum and without hydrocortisone were taken as reference and set to 100% (column 3 in Fig. 3). Serum reduced the TER to 50% compared to the reference and increased the permeability of sucrose by about a factor of 3 (column 1 in Fig. 3). If hydrocortisone was added to the medium in presence of ox serum (column 2 in Fig. 3) the TER-reduction was only 30% and the permeability increase of sucrose only came up to 185 % of the reference value without serum and hydrocortisone. In absence of serum hydrocortisone increased the resistance to 250% and permeability of sucrose was decreased correspondingly to 30% of the reference value. Typical permeability values were $1.8 \cdot 10^{-6}$ cm $\cdot s^{-1}$ in the absence of serum and hydrocortisone.

4.0×10^{-6} cm $\cdot s^{-1}$ in the presence of serum and with hydrocortisone and 0.5×10^{-6} cm $\cdot s^{-1}$ after supplementation of serum free medium by 550 nM hydrocortisone.

DISCUSSION

We were able to establish a primary endothelial cell culture system, which is a simple and reproducible model for the BBB and closely mimics the *in vivo* situation. In the absence of serum and in the presence of hydrocortisone the permeability of sucrose across a PBCEC monolayer was found to be only 0.5-1 $\times$ 10^{-6} cm $\cdot s^{-1}$ and their TER values reached 1000 $\Omega \cdot cm^2$. These TER values are comparable to the TER of brain capillaries *in vivo,* which was estimated to 1900 $\Omega \cdot cm^2$ [8]. One outstanding feature of our cell culture model is the possibility to perform experiments under serum free conditions. Serum free culture conditions are the basis for reproducible transport studies and facilitates investigations regarding cell-cell interaction at the BBB. The identification of BBB-inducing agents, e.g. in co-cultures, will now be enabled. Therefore, our *in vitro* model will be a good basis to determine molecules like intercellular messengers, which possibly are secreted

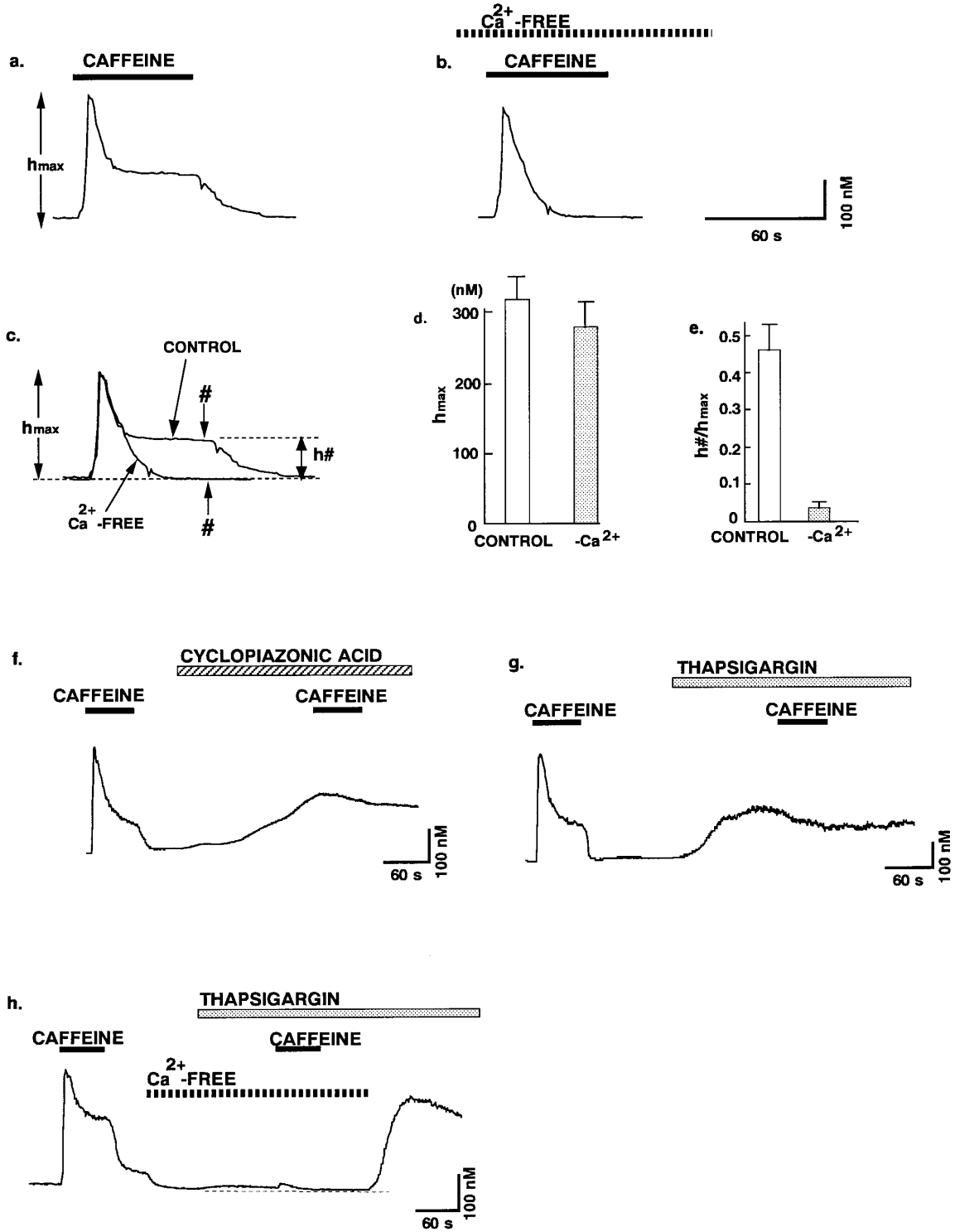

FIG. 1. TER of PBCEC monolayers during the course of culture time. ■, TER of PBCEC monolayers, which were cultured without serum but with 550 nM hydrocortisone. ●, TER of PBCEC monolayers, which were cultured with 10% (v/v) ox serum but without hydrocortisone. DIV: days *in vitro.* Data are given as mean ± se (n = 5).

from astrocytes or pericytes. To our knowledge this is the first time that a negative effect of serum was observed on the barrier properties of an endothelial cell monolayer. This seems to be feasible since some years ago a decrease of the electrical resistance had been observed at cell monolayers of an epithelial cell

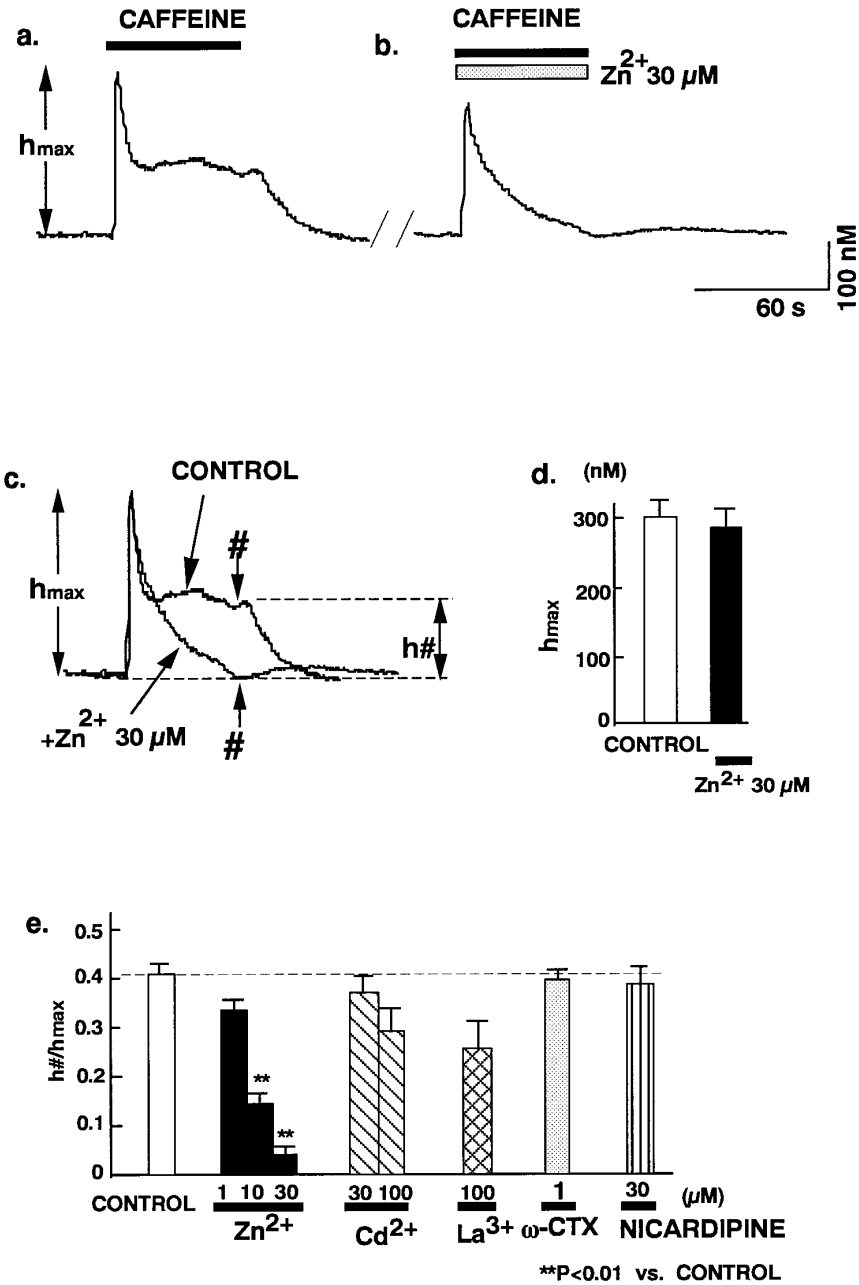

FIG. 2. Influence of hydrocortisone on the TER of PBCEC monolayers. Analysis of the TER was performed after 7 days *in vitro*. Data are given as mean ± se (n = 5).

line, the MDCK cells, under the influence of serum [9, 10]. The opening of tight junctions by serum has also been observed in retinal epithelial cells [11]. The negative effect of serum on BBB features could be explained by the presence of serum growth factors. These agents inducing proliferation are not further needed in confluent cell culture, and they could rather hinder final differentiation of the cells. Cytokines or hormones, which are always present in the blood of the donor animal, could be another possibility of the barrier weakening effect of serum. The molecular structure of these serum factors however has to be determinated.

Hydrocortisone was the only supplement found so far to improve barrier properties. The serum free cell culture model reported here could be improved in such a way, that TER and sucrose permeability came close to the corresponding parameters *in vivo*. With respect to the *in vivo* situation it is important to note that hydrocortisone was added to the cell culture in a physi-

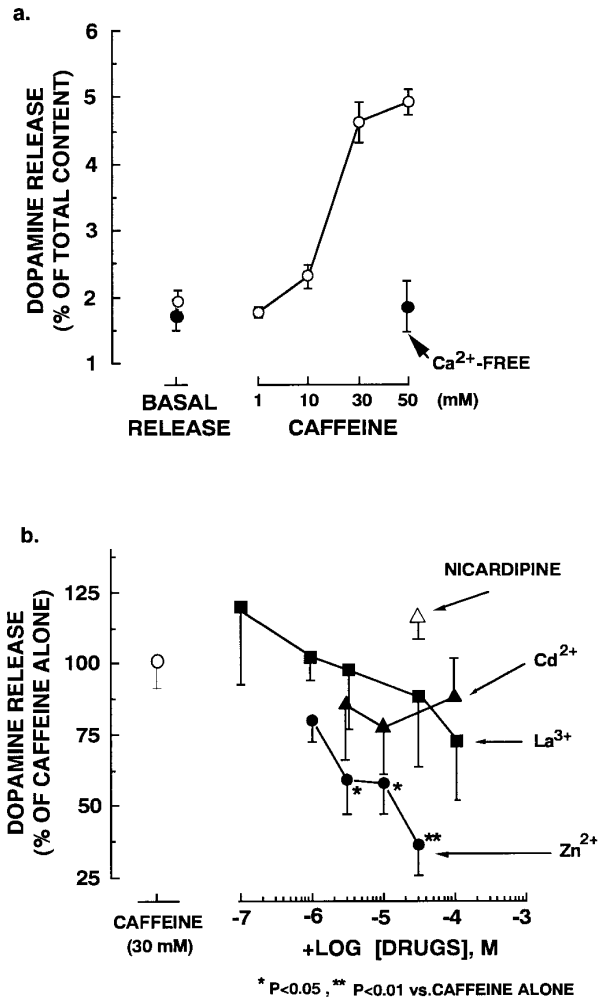

FIG. 3. Influence of hydrocortisone and ox serum on the barrier properties of PBCEC monolayers. Experiments were performed after 7 days *in vitro*. Incubation medium: 1, with 10% (v/v) ox serum, without hydrocortisone; 2, with 10% (v/v) ox serum, with 550 nM hydrocortisone; 3, without serum, without hydrocortisone; 4, without serum, with 550 nM hydrocortisone. A: Analysis of the TER. Relative data are given as mean ± se (n = 36). B: Sucrose-permeability. Relative data are given as mean ± se (n = 8).

ological concentration. Hydrocortisone now is a glucocorticoid and influences the metabolism of many cell types preferentially in the catabolic pathways. Beside this an anti inflammatory effect was described [7]. Moreover, Zettl et al. [12] were able to show that dexamethasone, a chemically modified hydrocortisone, improved TER of monolayers of 31EG4 cells. Forthcoming analysis of the intracellular signal transduction pathway of hydrocortisone will provide new insights in the mechanisms regulating the structural organisation of tight junctions.

In summary with help of hydrocortisone we were able to establish an *in vitro* model of the BBB, which is independent of serum, astrocyte conditioned medium or any other undefined additives. Our model is close to the *in vivo* situation and could therefore be the basis for serial permeability studies of pharmaceutical active substances under defined conditions.

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