TIMP-3: A novel target for glucocorticoid signaling at the blood–brain barrier

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ABSTRACT

Glucocorticoids (GCs) are used in the treatment of neuroinflammatory diseases such as multiple sclerosis. Several studies have demonstrated the beneficial effect of GCs on the balance between matrix metalloproteinases (MMPs) and their endogenous inhibitors, the TIMPs (tissue inhibitors of metalloproteinases). We could demonstrate that all four known TIMPs are present at the blood–brain barrier (BBB) endothelium. Hydrocortisone (HC) selectively upregulates TIMP-3 while TIMP-1, TIMP-2 and TIMP-4 were downregulated on the mRNA-level. This effect could be completely reversed by the glucocorticoid receptor inhibitor mifepristone (Mife). On the protein-level all TIMPs could be detected in the apical supernatants whereas in the isolated extracellular matrix (ECM) only TIMP-3 was found. The application of HC led to a strong enrichment of TIMP-3 in the ECM. Our findings demonstrate that HC directly targets TIMP-3 at the BBB assuming a protective role against matrix disruption and thus to guarantee the barrier integrity.

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Materials and methods

Materials

Cell culture. All media were obtained from Biochrom (Germany) except for Dulbecco’s modified Eagle’s medium Ham’s F-12, which was purchased from Sigma (Germany). Biochrom was the supplier of l-glutamine, antibiotics, trypsin, and collagen G. The enzymes for isolating porcine brain capillary endothelial cells (PBCECs) were purchased from Roche (Germany). New born calf serum (NCS) was obtained from PAA (Austria). Culture dishes were obtained from Nunc (Denmark).

RNA isolation and PCR. RNA was isolated via the RNeasy Mini Kit (Qiagen, Germany); the RT-PCR was performed via the Reverse Transcription Core Kit (Eurogentec, Belgium). Quantitative real-time PCR (qRT-PCR) was conducted using the Power SYBR® Green PCR Master Mix (Applied Biosystems, Germany) and the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Germany).

Protein biochemistry. CellLytic™ M buffer was obtained from Sigma (Germany), complete™ protease inhibitor from Roche (Germany). Concentration of protein-samples was performed in microcon (YM-3, Millipore, Germany). For Western blotting nitrocellulose membranes and enhanced chemiluminescence technique (ECL) from GE Healthcare (Germany) were used.

Antibodies and ELISA-kits. Antibodies against the different TIMPs were obtained from following companies: mouse anti-TIMP-1, mouse anti-TIMP-2 (Calbiochem, Germany) and rabbit anti-TIMP-3, rabbit anti-TIMP-4 (Chemicon, Germany). POD-labelled goat anti-mouse (Sigma, Germany) and anti-rabbit IgG (Chemicon, Germany) were applied. TIMP-ELISA-kits were purchased from following companies: TIMP-1, TIMP-2 (Calbiochem, Germany), TIMP-3 (R&D systems, Minneapolis, USA) and TIMP-4 (Quantikine, Minneapolis, USA).

Cells

Porcine brain capillary endothelial cells (PBCEC) were isolated and cultured according to [15], day of isolation: day 1. The determination of the phenotype is shown in [16].

Sample preparation and ECM purification

RNA from PBCECs grown on 75 cm²-flasks was isolated according to the manufacturer’s instructions. Whole-cell-protein was isolated from PBCECs grown on 75 cm²-flasks. The cell-layer was washed twice with PBS. Lysates were scrapped off using 1 ml of lysis buffer (CellLytic™) containing complete™ protease inhibitor, centrifuged (1000g, 10 min) and the pellet was discarded. Cell-culture supernatants were centrifuged to remove cell debris.

To isolate the ECM, a protocol according to [16] was applied. For Western blot analysis the ECM was scraped off according to the manufacturer’s instructions. Concentration of samples was performed via centrifugation in microcon (nominal molecular weight limit: 3 kDa).

Quantitative real-time PCR

The RT-PCR (200 ng RNA per sample) and the qRT-PCR were performed according to the manufacturer’s instructions. Primers for qRT-PCR were chosen using the software Primer Express (version 2.0; Applied Biosystems). TIMP-1 forward: CAAAACTCAGGGTGATCGT reverse: CCGACCCAGGATTTCTCAT. TIMP-2 forward: CAGCTACAGATGGGCTGTGA, reverse: ACTCGTCCGGAGA GGAGATGTAG. TIMP-3 forward: GTACCGAGGCTTCAC, reverse: GC TTCTGTGAGGATATAC. TIMP-4 forward: TCTCTCAGGAGGGAGGT, reverse: TCTCTGCAAAGGATAGGTTCTC. β-Actin forward: TTC AGAGGCCTCTTCGA, reverse: CGCAGCTATGACGAGTGTG. For all primers the melting temperature constituted 60°C. The cDNA-quantities were measured as critical thresholds (Ct), which were normalized using simultaneously measured β-actin levels (ΔCt). The ΔCt-values of the control (without HC) were subtracted from each condition resulting in ΔΔCt-values.

Gel electrophoresis and immunoblotting

Western blots were performed according to [16]. The acrylamide-concentration of the stacking gel was set to 4% acrylamide and the separating gel to 12%. Per lane 10 µg of protein were loaded onto the gel. The antibodies were applied in following concentrations: 0.5 µg/ml (mouse anti TIMP-1 and mouse anti TIMP-2), 2 µg/ml (rabbit anti TIMP-3, rabbit anti TIMP-4 2 µg/ml), 0.25 µg/ml (anti-mouse POD, anti-rabbit POD).

Enzyme-linked immunosorbsent-assay (ELISA)

In order to quantify the TIMP expression in the supernatants we took advantage of the ELISA technique which was performed according to the manufacturer’s instructions. Equal volumes of supernatants were applied per condition.

Immunocytochemistry

For the detection of ECM-bound TIMP-3 we seeded PBCECs on 8-Well-Labteks (Permanox™) and purified the extracellular matrix on day 7 as described before. The protocol is shown in [16]. Antibodies were applied in following concentrations: 10 µg/ml (TIMP-3 antibody), 2 µg/ml (FITC-labelled secondary anti-rabbit antibody). The 8-well-channel was cut off and mounted in Aqua Poly/Mount (Polysciences, Warrington, PA, USA) and covered with coverglass slides.

Statistical analysis

Each experiment was carried out with different PBCEC-preparations (n equals the number of preparation). Per condition double to quadrupel determinations have been performed. All data are presented via the mean and the standard deviation of the mean. The statistical analysis was conducted as follows: data-sets were checked for normality distribution and for equal variances. When both preconditions were fulfilled, parametric tests (t-test for two samples, F-test and Tukey-test (ANOVA) for multiple comparisons) were carried out. When one of both preconditions could not be fulfilled, non-parametric tests (Mann–Whitney-U-test for two samples, Kruskal–Wallis-H-test and Dunn-test (ANOVA) for multiple comparisons) were performed. Presentation of data was done using Origin5.0 (Microcal Software, Northampton, USA). Statistical calculations were performed via GraphPad InStat version 3.06 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

Results

Glucocorticoids have been shown to alter the expression levels of TIMPs at the BBB [13]. In this study we used the well-established porcine in vitro model of the BBB to examine the impact of HC on the expression levels of all known TIMPs, with a special focus on the release into the ECM or into the supernatants. All TIMP-determinations were performed on day 7 in vitro since on this day the
lowest permeability (highest electrical resistance) of PBCECs was recorded. HC was added at 550 nM on day 5 in vitro [17].

**HC regulation of TIMPs on mRNA and whole protein-level**

We investigated whether glucocorticoid mediated signaling would have a specific effect on the TIMP-mRNA-levels in PBCECs. TIMP-3 turned out to be the only TIMP which was strongly upregulated via HC (Fig. 1A). On the contrary, we discovered significantly lowered mRNA-levels of TIMP-1, TIMP-2 and TIMP-4. Addition of mifepristone (5 μM) led to a nearly complete reversal of this effect. The cycle values of the different TIMPs indicate that TIMP-4 (ΔCT = 9.9 ± 0.9 cycles – control condition) is much lower expressed than TIMP-1 (ΔCT = 4.8 ± 0.6), TIMP-2 (ΔCT = 4.4 ± 0.2) and TIMP-3 (ΔCT = 2.3 ± 0.2). β-Actin expression as a control was not changed by the presence of HC (Ct(HC) = 15.7 ± 0.5, Ct(control) = 15.5 ± 0.7). The HC-mediated upregulation of TIMP-3 could be confirmed on the protein-level via Western blotting of the whole cell lysate (Fig. 1B). Again, this effect was turned down completely by mifepristone. Western blots of the whole cell lysate did not reveal the unbound form of TIMP-1, TIMP-2 and TIMP-4 (data not shown). In contrast to this, the unbound unglycolized and glycolized forms of TIMP-3 were clearly evident in the cytoplasm and in the ECM of PBCECs and both were significantly upregulated by HC.

**HC-impact on TIMPs in the supernatants of PBCECs**

For the quantification of the secreted TIMPs in the supernatants sandwich ELISAs were conducted. Fig. 2 shows that HC reduces the release of TIMP-1, TIMP-2 and TIMP-4 into the supernatants, whereas TIMP-3 was secreted at slightly higher levels compared to control conditions. For the detection of TIMP-1 and TIMP-2 non-concentrated PBCEC supernatants have been used, whereas for TIMP-3 10 times diluted and for TIMP-4, 5 times concentrated supernatants were applied. Under control conditions (absence of HC) TIMP-3 was detected at 4.6 ± 1.2 ng/ml, TIMP-2 at 17.4 ± 1.6 ng/ml, TIMP-3 at 4.3 ± 0.3 ng/ml and TIMP-4 at 55.2 ± 2.4 pg/ml. The ratio between the TIMPs confirms that TIMP-2 is the most prominent TIMP in the cell-culture supernatants of PBCECs followed by almost equal amounts of TIMP-1 and TIMP-3. TIMP-4 seems to play a minor role at the blood–brain barrier.

**HC effect on TIMP-3 in the ECM**

The purified ECMS were taken off the culture dish, 10 times concentrated and applied to Western blotting analysis. TIMP-1, TIMP-2 and TIMP-4 were nearly undetectable in the ECM whereas the HC-induced upregulation of TIMP-3 was obvious, both for the glycolized and the unglycolized form. Mifepristone antagonized this effect (Fig. 3A). We could confirm this finding by immunofluorescence studies on the isolated ECMS which demonstrate that HC directly alters the composition of the PBCEC–ECM (Fig. 3B).

**Discussion**

The breakdown of the BBB is a key feature in many neuroinflammatory conditions which are induced by infection, autoimmune reactions and hypoxia/ischemia. Since MMPs attack the ECM and TJ-proteins, their actions are a common pathway for opening the BBB with the consequence that cells enter the CNS and attack the origin of the inflammation. The balance between MMPs and TIMPs determines proteolytic damage [18,5].

The beneficial impact of GCs on the MMP–TIMP balance was demonstrated by Rosenberg et al. [14], who detected markedly lowered levels of MMP-9 and higher levels of TIMP-3 in the CSF of MS-patients after the treatment with high-dose methylprednisolone. At the BBB the effect of GCs on MMPs and TIMPs has been examined by Harkness et al. [12] and Forster et al. [13] in vitro. However, TIMP-3 and TIMP-4 were not included in both in vitro studies. TIMP-3 is especially important because it plays a major role in regulating inflammatory processes.

Our study is to the best of our knowledge the first report to demonstrate that HC influences all known TIMPs at the BBB. TIMP-3 was selectively upregulated whereas the levels of TIMP-1, TIMP-2 and TIMP-4 were notably reduced. The observed effects

![Fig. 1. (A) Summary of qPCRs against TIMPs (n = 5 for TIMP-1–3, n = 3 for TIMP-4). Higher mRNA-expression led to higher −ΔΔCT-values; due to the normalization, ΔΔCT-values for the control were set to zero. The data was significantly different from control and from +HC-condition (**P < 0.01, ***P < 0.001). (B) Western blot against TIMP-3 on whole cell lysates (n = 4). The glycolized and unglycolized forms of TIMP-3 are highlighted.](#)

![Fig. 2. Results of ELISAs against different TIMPs in PBEC supernatants (n = 3) (*P < 0.05, **P < 0.01, ***P < 0.001).](#)
show a clear correlation with another characteristic of the four TIMPs, which is their secretion pattern: TIMP-1, TIMP-2 and TIMP-4 act primarily as secreted proteins and could thus not be detected in the ECM. On the contrary TIMP-3 is secreted at lower levels such as AP-1-signaling.

The finding that the administration of the anti-inflammatory HC led to a downregulation of three TIMPs can be explained by the hypothesis of Bugno et al. [19]. They suggested that the TNFα-induced upregulation of TIMP-1 serves as a protective mechanism against uncontrolled proteolysis. Thus, the HC-induced lowering of TIMP-1-, TIMP-2- and TIMP-4-levels should be due to an interference of the activated GC-receptor with signaling pathways which are activated under inflammatory conditions and which are present in cultured PBCECs at lower levels such as AP-1-signaling.

The reversal of the HC-mediated effect on the TIMPs via mifepristone was here demonstrated on the mRNA and the protein-level, for the latter including whole cell lysate and the ECM. Nevertheless, the secretion of TIMPs into the supernatants of PBCECs was even lower in the presence of mifepristone compared to the control condition (TIMP-3) or compared to the HC treated conditions (TIMP-1 and TIMP-4, data not shown). Only the HC-impact on the secretion of TIMP-2 was nearly completely inhibited by mifepristone. Thus, we presume that mifepristone, apart from interfering with the glucocorticoid receptor, acts individually on the secretory activity of PBCECs.

Our study demonstrates that HC specifically favours the upregulation of the most potent form of the known TIMPs, TIMP-3. TIMP-3 not only inhibits a broad range of secreted MMPs but also the membrane bound MT-MMPs and ADAMs. Especially the inhibition of ADAM-17 (TACE) can lead to lowered inflammatory responses of the BBB-endothelium towards TNFα [6,9,10]. Since MMPs can be released from cerebral cells under inflammatory conditions [21] we hypothesize that HC stabilizes the BBB-endothelium via rescuing the integrity of the ECM and via lowering the response to TNFα when the main inflammatory attack towards the BBB originates from the cerebral interstitium. Furthermore, higher TIMP-3-levels in the ECM can act as a second line of defence which can hinder blood-borne immune cells from entering the brain parenchyma.

Addition of HC to our culture system resulted in a strong increase in barrier function [17]. Harkness et al. [12] and Forster et al. [13] found that glucocorticoid signaling impeded MMP-9 expression induced by inflammatory cytokines. We could also detect that HC was capable to reduce the level of MMP-9 and MMP-activity in the supernatants of PBCECs isolated under puromycin-free conditions (data not shown). Puromycin, an agent which selectively removes the contaminating pericytes from PBCEC-preparations was recently shown to deplete the secretion of MMP-9 by PBCECs [16]. Accordingly, MMP-9 expression and secretion appears to be a general target for GCs even if pericyte–PBCEC-interactions [16] activate other signaling pathways than pro-inflammatory cytokines.

Taken together the BBB-endothelium reveals to be an important source for MMPs and TIMPs. It is moreover a key target for GC-mediated signaling in order to impede inflammation-induced alteration of the MMP–TIMP balance which in turn prevents the breakdown of the BBB. Our conclusions basically agree with the findings of Forster et al. [13]. However, we found that TIMP-3 is the main target of GC-signaling. This apparently seems to be the more effective way of counteracting inflammatory processes since TIMP-3 is a very potent and versatile tool in order to impede these disorders. In addition, our study provides fundamental molecular basics about the anti-inflammatory consequences of GC-signaling at the BBB.

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References