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# 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.



## Introduction

The blood–brain barrier (BBB) limits the transfer of drugs and blood-borne substances from the blood into the cerebral interstitium, and hence guarantees a constant chemical environment in the central nervous system (CNS) that is a prerequisite for neuronal transmission. This barrier is formed by highly specialized endothelial cells that line the cerebral blood vessels. These brain capillary endothelial cells (BCECs) are coupled via tight junctions and are surrounded by a common extracellular matrix (ECM) which connects the BCECs to the local microenvironment [1].

In many neuroinflammatory and neurodegenerative processes such as multiple sclerosis (MS), bacterial meningitis but also in stroke, BBB- and ECM-disruption are coincident, with the consequence that solutes, plasma and leucocytes can enter the brain parenchyma [2,3]. Main mediators of these processes are the matrix metalloproteinases (MMPs), which are zinc-dependent endopeptidases directed against extracellular matrix (ECM) molecules [4] but also against Tight Junction-proteins [5]. Active MMPs can be blocked via endogenous MMP-inhibitors, the tissue inhibitors of metalloproteinases (TIMPs). TIMPs are secreted proteins, which form non-covalent 1:1 complexes with MMPs and thus limit MMP-mediated ECM-degradation. Up to date, four different TIMPs are known, which revealed to be similar in their quaternary structure [3,6]. They can be distinguished via their affinity to different MMPs, distinct structural features and a differentially regulated expression. TIMP-1 (28–34 kDa), TIMP-2 (21 kDa) and TIMP-4 (23 kDa) act predominantly as soluble proteins, TIMP-3 (unglycolized: 24 kDa, glycolized: 27–30 kDa) is the only TIMP which strongly binds to the ECM [7,8]). TIMP-3 and to some extent TIMP-1 inhibit members of the ADAM-family (adamalysin-like proteinases, [9]). ADAM-17 (TACE, TNFα converting enzyme), the activator of the key inflammatory cytokine TNFα, is a major target for TIMP-3 [10]. Since knockouts of TIMP-1–3 revealed an altered phenotype only for the elimination of TIMP-3, it is suspected to be a major regulator of MMP-activity in vivo [11].

Glucocorticoids (GCs) are generally used as an impulse therapy in MS to cure inflammatory symptoms but their exact mode of action still remains to be clarified. In-vitro studies revealed the cerebral endothelium as an important regulator for the balance between TIMPs and MMPs which is affected by GC-mediated signaling [12,13]. However, no study actually exists that reveals the effects of GC-signaling on all four different TIMPs where TIMP-3 is especially important since Rosenberg et al. detected a significant increase of this molecule in the cerebrospinal fluid (CSF) of MS-patients [14]. Therefore, we examined the impact of HC on the TIMP expression, their secretion into the supernatants and their deposition in the ECM using a well-established in vitro model of the BBB [15]. We could demonstrate that HC strongly upregulates the expression of TIMP-3 in the ECM, whereas the other TIMPs are downregulated.

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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). Cell 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.* CelLytic™ M buffer was obtained from Sigma (Germany), complete™ protease inhibitor from Roche (Germany). Concentration of protein-samples was performed in microcons (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) and the FITC-labelled anti-rabbit IgG (Invitrogen, 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 $cm^2$-flasks was isolated according to the manufacturer's instructions.

Whole-cell-protein was isolated from PBCECs grown on 75 $cm^2$-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 (1000*g*, 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 whole-cell-protein preparation.

Concentration of samples was performed via centrifugation in microcons (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: CAAAACTGCA GGTGGTGATGTG reverse: CGCAGCCAGGAGTTTCTCAT. TIMP-2 forward: CAGGTACCAGATGGGCTGTGA, reverse: ACTCGTCCGGAGA GGAGATGTAG. TIMP-3 forward: GTACCGAGGCTTCAC, reverse: GC TTCTGTGTGGATATAC. TIMP-4 forward: TCCTCAGCGACGGAAAGGT, reverse: TCTCTCTGCAAAAAGGATAGGTTCTC. β-Actin forward: TCC AGAGGCGCTCTTCCA, reverse: CGCACTTCATGATCGAGTTGA. For all primers the melting temperature constituted 60 °C. The cDNA-quantities were measured as critical thresholds ($C_T$), which were normalized using simultaneously measured β-actin levels ($\Delta C_T$). The $\Delta C_T$-values of the control (without HC) were subtracted from each condition resulting in $\Delta\Delta C_T$-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 immunosorbent-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-chamber 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 quadruple 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