University of Szeged
Faculty of Science and Informatics
Doctoral School in Biology

Anti-inflammatory effects of α-melanocyte stimulating hormone on culture models of biological barriers

Ph.D. thesis

András Harazin

Supervisors:

Dr. Mária Deli, scientific adviser

HAS Biological Research Centre

Institute of Biophysics

Dr. Vilmos Tubak, scientific director

Creative Laboratory Ltd.

Szeged

2018

1. Introduction

Multicellular organisms have different epithelial barriers covering the surface of the body and body cavities. The biggest outer barrier is the intestinal mucosa, which prevents the entry of xenobiotics, harmful agents, gut bacteria or pathogens into the body, and provides a surface for digestion and food uptake. One of our most important blood-tissue barrier is the blood-brain barrier, which insures the ionic homeostasis and nutrient supply of the central nervous system. The anatomical basis of both barriers consists of intercellular tight junctions (**Table 1.**), which connect barrier cells, physically preventing the entry of various pathogens and compounds, and add polarity to these cells, providing a regulated transport of molecules.

Table 1. Comparison of the intestinal and the blood-brain barrier (Deli, 2009).

	Gut epithelia	**Blood-brain barrier endothelia**
Barrier	outer	inner
Apical/basal appendix	**microvilli**, glycocalyx	glycocalyx
Tight junction (TJ) forming proteins	occludin, tricellulin	occludin, tricellulin
	claudin-1, **-2**, -3, **-4**, **-7**, -15	claudin-1, -3, **-5**, -15
	JAM-A, -B, -C, CAR	JAM-A, -B, -C, CAR, **ESAM**
Adherens junction (AJ) forming proteins	**E**-cadherin	**VE**-cadherin
	nectins	nectins, **PECAM-1**

The maintainence of homeostasis and protection of the organism afforded by the dynamic interface of barriers are important not only under physiological conditions, but in diseases too. In most forms of pathology local or systemic inflammation occurs, which influences barrier functions. Among inflammatory cytokines TNF-α and IL-1β are the most studied molecules. Both of them participate in inflammatory bowel diseases and in neuroinflammation inducing barrier impairment and an increase in permeability. Therefore we have chosen these two cytokines for our experiments.

The α-melanocyte stimulating hormone (α-MSH) is an endogenous peptide from the group of melanocortins. The main and most abundant receptor of α-MSH among the melanocortin receptors (MCR) is MC1R (**Table 2.**), a G-protein coupled molecule, which exerts its effects through 3',5'-cyclic adenosine monophosphate (cAMP) secondary messenger. The α-MSH peptide is known to determine skin and hair colour. Besides, protective effects of the hormone in allergic and inflammatory diseases are also widely investigated. The protective effects of α-MSH were reported in rat models of acute and

chronic colitis, and in instestinal ischemia. Moreover, neuroprotective effects of the peptide were observed in brain inflammation induced by bacterial lipopolisaccharide, in brain ischemia, and in kainic acid induced or traumatic brain injury, too.

Table 2. Properties of MCR receptors (Brzoska et al, 2008; Lasaga et al, 2008).

Receptor	Binding affinity	Tissue expression	Cellular expression	Biological function
MC1R	α-MSH = ACTH = β-MSH >> γ-MSH	skin, **brain**, **gut**, testis, placenta, lung, liver	melanocyte, **endothelial cell,** astrocyte, **intestinal epithelia**, immune cells	pigment production, **inflammation**
MC2R	ACTH	adrenal gland, testis, skin	adipocyte, keratinocyte	steroid synthesis
MC3R	γ-MSH = ACTH= β-MSH ≥ α-MSH	**brain**, heart, skeletal muscle	**intestinal epithelia**, immune cells	energy homeostasis, **inflammation**
MC4R	α-MSH = ACTH = β-MSH >> γ-MSH	**brain**, skin, skeletal muscle	dendritic cell	energy homeostasis, erection, **inflammation**
MC5R	α-MSH ≥ β-MSH = ACTH > γ-MSH	skeletal muscle, **brain**, skin, exocrine glands, lung, heart	adipocyte, secretory epithelia, **intestinal epithelia**	exocrine secretion

2. Aims

Several studies, mainly animal experiments, proved the anti-inflammatory and protective effects of α-MSH. However, the effects of the peptide on biological barrier functions have not been investigated yet. Therefore our aims were: (i) to demonstrate the expression of MC1R in culture models of the intestinal epithelia and the blood-brain barrier, (ii) to test the direct effects of α-MSH on the viability of brain endothelial cells, (iii) to investigate the effects of the hormone on the cytokine induced increase in permeability of biological barriers and on morphological changes of intercellular junctions, (iv) to examine whether α-MSH inhibits NF-κB nuclear translocation and reactive oxygen species production in cultured brain endothelial cells.

3. Materials and methods

3.1 Cell cultures

3.1.1. Human Caco-2 intestinal epithelial cell line

Human Caco-2 intestinal epithelial cells were purchased from ECACC (#86010202; European Collection of Authenticated Cell Cultures, UK). The cell line was cultured in Dulbecco's Minimum Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and gentamycin (50 μg/ml). Surfaces of the culture dish were coated with rat tail collagen to promote cell adhesion and proliferation.

To investigate the barrier function of intestinal epithelial cells, monolayers were grown on permeable polycarbonate inserts (0.4 μm pore size, 0.33 cm^2 surface, Transwell, Corning Costar, USA).

3.1.2. *In vitro* model of the blood-brain barrier

For modelling the blood-brain barrier, primary rat brain endothelial cell monolayer was used. For permeability studies and for immunostaining of intercellular junctional proteins, a triple co-culture model was used, where primary rat brain endothelial cells, glial cells and pericytes were cultured together. The isolation of the different cell types and the set-up of the models were published by our team in several papers (Nakagawa et al, 2009; Veszelka et al, 2013; Walter etl al, 2015).

For co-culture models, pericytes were subcultivated to the bottom, so called abluminal side of the polyester culture insert (0.4 μm pore size, 1.12 cm^2 surface, Transwell, Corning Costar, USA), then the cells could attach to it for 3 hours at 37 °C. After that the inserts were transfered above glial cells grown in 12-well plates, then endothelial cells were pipetted onto the top, so called luminal side of the membrane. The three cell types were co-cultured for 3-5 days.

3.2. Treatments

The sensitivity of different cell types was taken into account in our experiments. Therefore, the human Caco-2 cell line was treated with α-MSH at concentrations of 10^{-16} M (100 aM), 10^{-12} M (1 pM), 10^{-8} M (10 nM) and 10^{-4} M (100 μM). In experiments for studying cytokine induced damage IL-1β was applied at a concentration of 25 ng/ml and TNF-α at 50 ng/ml (Van De Walle et al, 2010), while from α-MSH was administered at its most effective 10^{-8} M (10 nM) concentration.

In the case of rat brain endothelial cells, α-MSH was used at concentrations of 10^{-12} M (1 pM), 10^{-11} M (10 pM), 10^{-10} M (100 pM), 10^{-8} M (10 nM) és 10^{-6} M (1 μM). The cytokine damage on brain endothelial cells was performed in different combinations starting with the concentrations used for the Caco-2 cell line: 25 ng/ml IL-1β and 50 ng/ml TNF-α, 25 ng/ml IL-1β and 25 ng/ml TNF-α, 10 ng/ml IL-1β and 25 ng/ml TNF-α, 10 ng/ml IL-1β and 10 ng/ml TNF-α.. For further experiments the most effective 10^{-12} M (1 pM) and 10^{-11} M (10 pM) α-MSH, and the lowest 10 ng/ml IL-1β + 10 ng/ml TNF-α concentrations were used.

The control group received culture medium only without any treatment.

3.3. Reverse transcription polymerase chain reaction

To investigate the gene activity of *Mc1r* brain microvessels were isolated from Wistar rats and rat brain endothelial cells were cultured in 6 cm Petri-dishes (Corning Costar, USA). After washing with PBS tissue and cell culture samples were homogenized in TRI Reagent (Molecular Research Center, USA). Then total RNA was isolated by chloroform according to the manufacturer's instructions. One microgram of total RNA was treated with DNase enzyme (ThermoFisher, USA), then transcribed to cDNA by a cDNA synthesis kit (Thermo Fisher, USA) according to the manufacturer's instructions.

3.4. Cell viability measurements

To test α-MSH effects on cell viability a colorimetric (MTT) and a real-time cell electronic sensing (RTCA-SP) method were used. In the MTT assay brain endothelial cells were grown in 96-well plates until confluency. Then cells were treated with α-MSH at different concentrations for 24 hours. After treatment cells were incubated with MTT solution (0.5 mg/ml) for 3 h in a CO_2 incubator. The formazan crystals produced from the dye were dissolved in dimethyl sulfoxide, then formazan concentrations were determined by absorbance measurement with a multiwell plate reader.

For the impedance-based cell analysis (RTCA-SP device, ACEA Biosciences, USA) experiments were performed under physiological conditions with continuous monitoring. E-plates, special 96-well plates with built in gold electrodes, were coated with collagen type IV (100 μg/ml) and fibronectin (100 μg/ml) for brain endothelial cells, dried under UV light and air-flow, then the background resistance was registered. Then rat brain endothelial cell suspension (5×10^3 cells/well) was distributed in each well and they was cultured at 37 °C in a CO_2 incubator until confluency. When the impedance did not increase further (plateau phase) the cells were treated either with the α-MSH peptide (10^{-12}-10^{-6} M), or with IL-1β and

TNF-α (10-10 ng/ml) after 1 hour of α-MSH pretreatment (10^{-12}-10^{-11} M). The device registered the cell index in every 10 min for 24 hours. The impedance values were normalized to the timepoint just before the treatment and the calculated values were represented in function of the time.

3.5. Functional analysis of biological barriers

3.5.1. Measurement of the transepithelial electrical resistance

The formation of tight paracellular barriers in Caco-2 layers was monitored by the measuring the transepithelial electrical resistance (TEER) with a Millicell-ERS volt-ohm meter (Millipore, USA) using chopstick electrodes. The measured resistance values represented the entire surface of the Transwell filters (Ω × cm2) covered with epithelial cell. The TEER of cell-free inserts was subtracted from these values, and the average of the TEER values of epithelial cells was calculated.

3.5.2. Permeability measurements

The permeability measurements of barrier models were performed with marker molecules of different sizes. Sodium fluorescein (SF, 376 Da) was used to determine the paracellular permeability on human Caco-2 intestinal epithelial cells, while in the rat blood-brain barrier co-culture model fluorescein isothiocyanate-labeled dextran (FITC-dextran, 4.4 kDa) was used. The other marker molecule was Evans blue-labeled albumin (EBA, 67 kDa).

Caco-2 intestinal epithelial cells were treated for 24 hours with IL-1β and TNF-α cytokines without or with α-MSH. The primary rat blood-brain barrier co-culture model was treated for 1 hour with pro-inflammatory cytokines without or with α-MSH. The concentrations of the marker molecules in samples from the upper and lower compartments were determined by a fluorescence multiwell plate reader. The results were given as apparent permeability coefficient values (P_{app}).

3.6. Reactive oxygen species production

In brain endothelial cells reactive oxygen species (ROS) production was detected by chloromethyl-dichloro-dihydro-fluorescein diacetate (DCFDA; Life Technologies, USA). Confluent brain endothelial cells were cultured in black 96-well plates with glass bottoms (Corning Costar, Corning, NY, USA). After 1 h of treatment cells were incubated with Ringer-Hepes buffer containing 2 μM DCFDA for 1 h at 37°C. Fluorescence was measured in every 5 min during 1 hour. Hydrogen peroxide (100 μM) was used as a reference compound.

3.7. Immunohistochemistry

For MC1R receptor, claudin-4 integrant membrane TJ protein, ZO-1 linker protein and NF-κB transcription factor immunolabelings Caco-2 epithelial cell cultures were fixed after washing them in PBS. Non-specific binding sites were blocked with 3% BSA. Primary antibodies rabbit anti-human MC1R (1 μg/ml), rabbit anti-human ZO-1 and rabbit anti-human claudin-4 (2,5 μg/ml; Thermo Fisher Scientific, USA), and rabbit anti-human NF-κB antibodies (2,5 μg/ml; Santa Cruz Biotechnology, USA) were used overnight at 4 °C. The appropriate secondary antibodies were Alexa Fluor 488 and Cy3 conjugated anti-rabbit antibodies (2 μg/ml), which were used with propidium iodide (1 μM) or H33342 (1 μg/ml) for staining cell nuclei. After mounting (Fluoromount-G, Southern Biotech, USA) samples were examined with an Olympus FV1000 (Olympus Corporation, Japan) and a Leica SP5 confocal microscope (Leica Camera AG, Germany).

Rat brain endothelial cells co-cultured with pericytes and glial cells and freshly isolated brain microvessels were fixed after washing them in PBS non-specific binding sites were blocked, then the samples were incubated with rabbit anti-human MC1R (5 μg/ml) primary antibody overnight at 4 °C. Alexa Fluor 488 conjugated anti-rabbit secondary antibody (2 μg/ml) was used for fluorescence labeling and cell nuclei were stained with ethidium homodimer (1 μM).

The immunostaining of junctional proteins and NF-κB was performed also on rat brain endothelial cells co-cultured with pericytes and glial cells. The endothelial cells were washed with PBS and fixed after the treatments, then non-specific binding sites were blocked. The samples were incubated with rabbit anti-rat β-catenin, rabbit anti-rat claudin-5 and rabbit anti-human NF-κB primary antibodies (2,5 μg/ml; Santa Cruz Biotechnology, USA) overnight at 4 °C. The next day Cy3 conjugated anti-rabbit secondary antibodies (1 μg/ml) were used for 1 hour at room temperature. Cell nuclei were stained with H33342 (1 μg/ml). After mounting (Fluoromount-G, Southern Biotech, USA) samples were analyzed with a Leica SP5 confocal microscope (Leica Camera AG, Germany).

The quantitative changes of rat brain endothelial immunostainings were analyzed with MATLAB program (MathWorks, USA), while changes of the NF-κB staining intensity were analyzed with ZEN 2012 v.1.1.0.0. software (Carl Zeiss AG, Germany) in the case of Caco-2 cell line and with the ImageJ program (National Institute of Health, USA) in the case of rat brain endothelial cells.

3.8. Statistical analysis

Statistical analysis was performed using the GraphPad Prism 5.0 software (GraphPad Software Inc, USA). Data are presented as means ± SEM.

Data were analyzed using one-way analysis of variance and the comparison of groups was performed with Dunnett or Bonferroni tests in cell the viability experiments, in quantifications of the immunostaining intensities and in ROS production measurements. In transepithelial electrical resistance and in permeability measurements the statistical analysis of data was performed using two-way ANOVA followed by Bonferroni test.

Differences were considered significant at $P<0.05$. Experiments were repeated at least three times and the treatments were triplicated in each experiment.

4. Results

4.1. The expression of MC1R in intestinal epithelial and brain endothelial cells

The primary receptor for mediating the effects of the α-MSH peptide is MC1R. Its expression was detected in both culture models of biological barriers.

In Caco-2 intestinal epithelial cells the MC1R expression was detected on both the apical and basal membranes using immunocytochemistry. The fluorescent immunolabeling was more pronounced on the apical side, as compared to the staining at the basal surface. This difference between the staining intensity was also visualized by imaging a horizontal section of a representative confluent Caco-2 cell monolayer.

The expression of the MC1R gene was detected in freshly isolated rat brain microvessels and also in confluent rat brain endothelial cell cultures with reverse transcription followed by PCR amplification. This result was strengthened by immunohistochemistry for MC1R both in cultured brain endothelial cells and in endothelial cells of isolated brain microvessels showing immunoreactive sites.

4.2. The effect of α-MSH on brain endothelial cell cultures

The effects of α-MSH on the cell viability of rat brain endothelial cells were investigated by impedance based real-time cell electronic sensing analysis and MTT assay based endpoint analysis. Treating the cells with α-MSH peptide alone at different concentrations (1 pM – 1 μM) did not have any significant effect on cell viability. The kinetic curves of treated brain endothelial cells revealed by real-time cell electronic sensing ran similarly to those obtained in untreated control samples (**Fig. 1A**) indicating good cell viability, attachement and barrier integrity. In addition, the colorimetric MTT assay did not

detect any metabolic change either in cells after 24 hour treatment with α-MSH as compared to controls (**Fig. 1B**). For further experiments 10^{-12} M (1 pM) and 10^{-11} M (10 pM) concentrations of α-MSH were selected, which correspond to the physiological range of the neurohormone in the blood (Kovács et al, 2001; Magnoni et al, 2003).

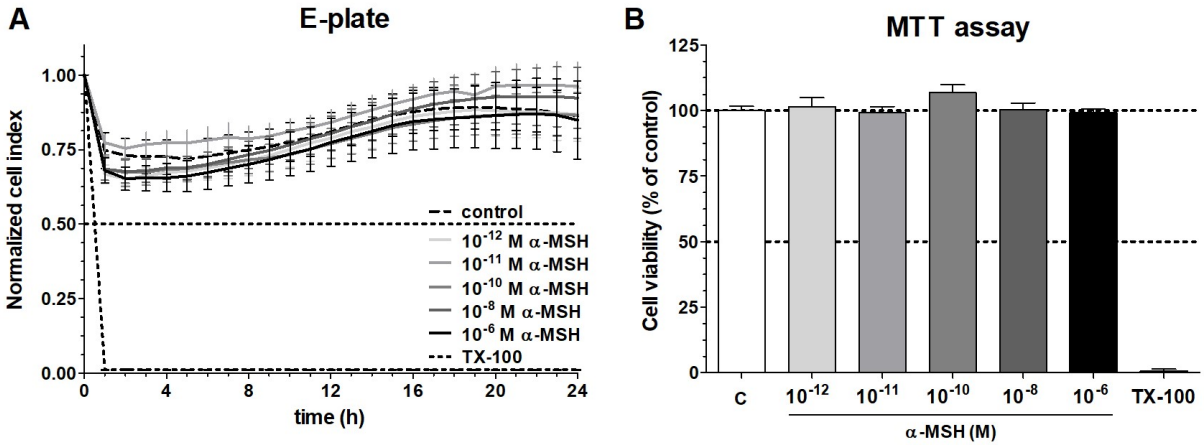

Figure 1. The effect of different concentrations of α-MSH on the cell viability of rat brain endothelial cells. **A)** Real-time cell electronic sensing. **B)** MTT test. Statistics: one-way ANOVA and Bonferroni test.

4.3. The effect of cytokines and α-MSH on cell viability of brain endothelial cell cultures

To determine the concentrations of TNF-α and IL-1β to induce brain endothelial damage, four different combinations of cytokine concentrations were tested. Starting from the highest, which was used on Caco-2 cells, 50 ng/ml TNF-α and 25 ng/ml IL-1β through lower 25+25 ng/ml treatment concentrations till the lowest 10 ng/ml TNF-α and 25 ng/ml IL-1β and 10+10 ng/ml concentrations (**Fig. 2**) were used.

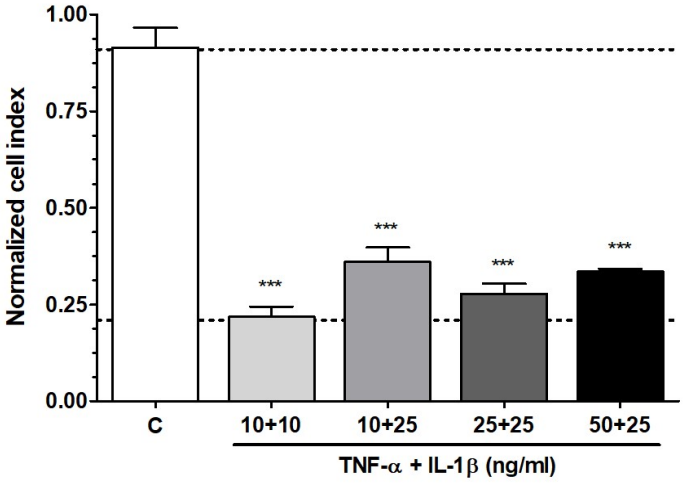

Figure 2. The effect of different concentrations of cytokine cocktails on cell viability of cultured rat brain endothelial cells. Statistics: ***, P < 0,001 compared to control group, one-way ANOVA and Bonferroni test.

After a 24 hour treatment all cytokine combinations resulted in a significant decrease of brain endothelial cell index measured by real-time cell electronic sensing. There was no statistically significant difference between the four cytokine treatment groups. For further experiments the smallest, 10+10 ng/ml concentrations of the cytokines were selected.

The treatment of rat brain endothelial cells with 10+10 ng/ml TNF-α and IL-1β induced a decrease in the cell index by more than 50% at 6 h timepoint, which was attenuated by α-MSH treatment (**Fig. 3**). The α-MSH peptide at low concentration (1 pM) significantly protected the cells against the cytokine-induced decrease in cell viability (**Fig. 3**). There was no statistically significant difference between the two cytokine and α-MSH treatment groups.

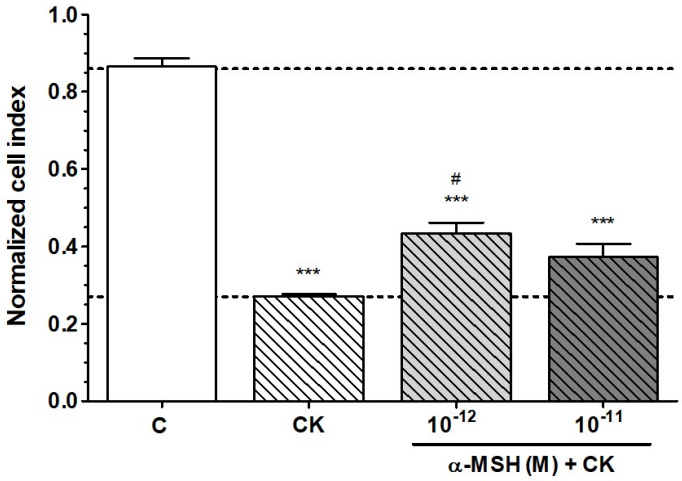

Figure 3. The effect of α-MSH on the cell viability of cytokine treated rat brain endothelial cells. Real-time cell electronic sensing after 24 hour treatment CK: 10 ng/ml TNF-α + 10 ng/ml IL-1β. Statistics: ***, $P < 0{,}001$ compared to control group; #, $P < 0{,}05$ compared to cytokine treated group, one-way ANOVA and Bonferroni test.

4.4. Effects of cytokines and α-MSH on the integrity of culture models of biological barriers

4.4.1. Transepithelial electronic resistance

The tightness of biological barrier forming epithelial and endothelial cell layers is characterized by high resistance between the two sides of the cell layer. Its phycial basis consists of intercellular TJ proteins which prevent the free paracellular flow of ions. There was a decrease in the resistance of Caco-2 monolayers treated with cytokines (**Fig. 4**). A concentration dependent effect of α-MSH was observed: the higher (10^{-4} and 10^{-8} M) concentrations protected against the cytokine-induced barrier disruption, while no effect was detected at smaller (10^{-12} and 10^{-16} M) α-MSH concentrations.. For further experiments on

Caco-2 intestinal epithelial cells the most effective, 10^{-8} M (10 nM) concentration of α-MSH was selected.

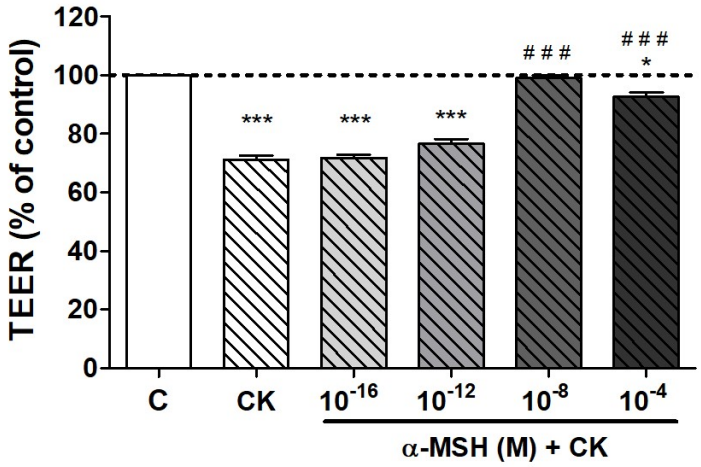

Figure 4. The effect of α-MSH on transepithelial electrical resistance (TEER) of cytokine treated Caco-2 intestinal cell line. CK: 50 ng/ml TNF-α + 25 ng/ml IL-1β. Statistics: ***, $P < 0{,}001$ compared to control group; ###, $P < 0{,}001$ compared to cytokine treated group, one-way ANOVA and Bonferroni test.

4.4.2. Permeability measurements

The integrity of epithelial and endothelial monolayers was tested by measuring its permeability for marker molecules of different sizes to monitor the proper function of these barrier models.

A low permeability for both the small molecule marker sodium fluorescein and the large biomolecule albumin was measured on the epithelial monolayers (**Fig. 5A**). Cytokine treatments significantly enhanced the permeability of the Caco-2 cell layers for both markers. The α-MSH peptide significantly blocked the barrier opening effect of the cytokines measured with sodium fluorescein, while in the case of albumin this effect was not statistically significant.

We measured the same effects on hte blood-brain barrier model too. In the model of rat brain endothelial cells co-cultured with brain pericytes and glial cells a low permeability for both dextran and albumin was measured (**Fig. 5B**). Cytokine treatments significantly increased the permeability of the co-cultures for both markers. The lower, 10^{-12} M (1 pM) concentration of α-MSH peptide significantly blocked the barrier opening effect of the cytokines for the small biomolecule FITC-dextran and the large biomolecule albumin, too. The α-MSH peptide at a concentration of 10^{-11} M (10 pM) significantly strengthened the cellular barrier for the bigger marker molecule.

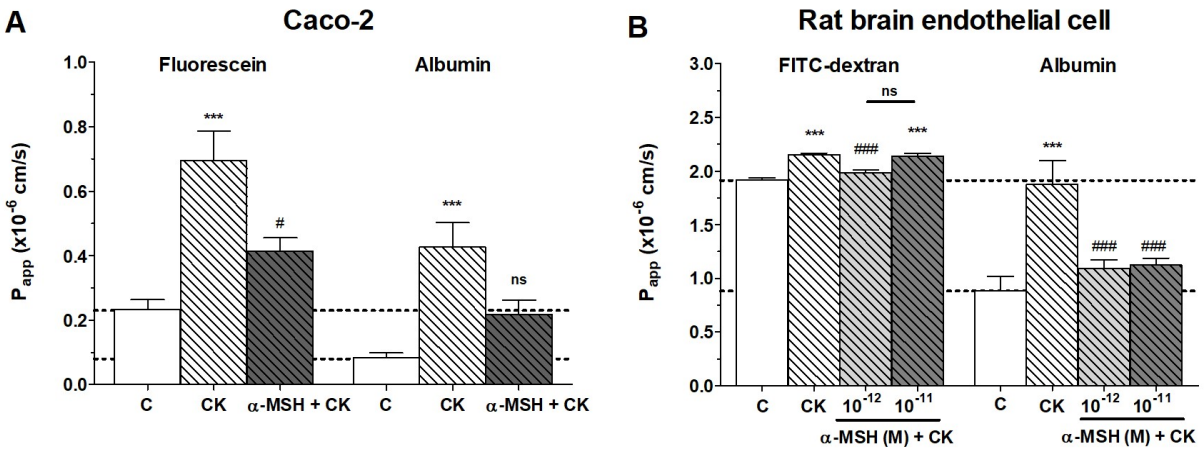

Figure 5. The effect of α-MSH on integrity of cytokine treated epithelial and endothelial cell cultures for different marker molecules. **A)** The permeability of Caco-2 intestinal epithelial cell layer for fluorescein and albumin. CK: 50 ng/ml TNF-α + 25 ng/ml IL-1β. **B)** The permeability of rat brain endothelial cell layer for 4 kDa FITC-dextran and albumin. CK: 10 ng/ml TNF-α + 10 ng/ml IL-1β. Statistics: ***, $P < 0,001$ compared to control group; #, $P < 0,05$, ###, $P < 0,001$ compared to cytokine treated groups; ns, no statistically significant difference compared to cytokine treated group, and between the two marked groups, one-way ANOVA and Bonferroni test.

4.5. Effects of cytokines and α-MSH on reactive oxygen species production of brain endothelial cells

Treatment with TNF-α and IL-1β resulted in a significant increase of ROS production in brain endothelial cells compared to the basal ROS production of the control group (**Fig. 6**). Low concentrations of α-MSH peptide alone did not change the basal ROS production, while it induced a statistically significant decrease in the cytokine-induced ROS production in cultured brain endothelial cells. Hydrogen peroxide treatment was used as a reference.

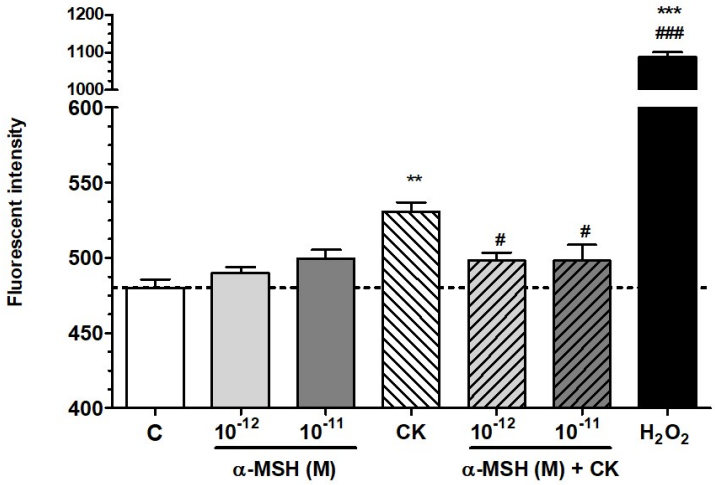

Figure 6. The effect of α-MSH treatment on the reactive oxygen species production in cytokine treated rat brain endothelial cells. CK: 10 ng/ml TNF-α + 10 ng/ml IL-1β. Statistics: **, $P < 0,01$, ***, $P < 0,001$ compared to control group; #, $P < 0,05$, ###, $P < 0,001$ compared to cytokine treated group, one-way ANOVA and Bonferroni test.

4.6. Effects of cytokines and α-MSH on junctional proteins of intestinal epithelial and brain endothelial cells

4.6.1. Claudin-4 and ZO-1 immunohistochemistry in Caco-2 cells

The intercellular junctional proteins provide the physical basis of intestinal epithelial barrier function. The integral membrane TJ protein claudin-4 and cytoplasmic linker protein ZO-1 appeared at the cell-cell borders in a continuous, belt-like manner. In cytokine treated cells the staining pattern was different: intercellular gaps, fragmented junctional staining and cytoplasmic redistribution of junctional proteins were observed. The immunostaining pattern of TJ proteins in epithelial cells treated with α-MSH was similar to that seen in the control group.

4.6.2. Claudin-5 and β-catenin immunohistochemistry in rat brain endothelial cells

In rat brain endothelial cells tight paracellular barrier forming claudin-5 and adherens junction protein β-catenin were stained and showed a strong, continuous pattern. In the cytokine treated group gaps, fragmented junctional staining and cytoplasmic redistribution of junctional proteins were observed. In cells treated with both α-MSH and cytokine the staining of TJ proteins was more similar to the staining observed in the control group.

The changes in immunostainings were quantified by image analysis software in the case of claudin-5 and β-catenin, too. Due to cytokine treatments the object number on the immunostained pictures increased significantly as compared to the control, while with α-MSH the object number decreased significantly as compared to the cytokine treated groups.

4.7. Effects of cytokines and α-MSH on nuclear translocation of NF-κB in intestinal epithelial and brain endothelial cells

The translocation of the p65 NF-κB subunit into cell nuclei is a reliable marker of inflammatory reactions at cellular level.

In Caco-2 intestinal epithelial cells in the control group the p65 subunit was observed in the cytoplasm, and the fluorescence intensity of the nucleus/cytosol ratio was low. Inflammatory cytokines induced the appearance of green-stained p65 within nuclei, which caused a significant elevation of the intensity ratio. The α-MSH treatment resulted in a significant decrease in the intensity ratio, which means that it inhibited the nuclear translocation of the p65 NF-κB subunit.

The treatment with inflammatory cytokines induced the nuclear translocation of p65 NF-κB subunit in rat brain endothelial cells too, which was also quantified with image

analysis. The α-MSH treatment of brain endothelial cells inhibited the nuclear translocation of p65 NF-κB subunit, which was verified by the statistical analysis of the fluorescence staining intensity, showing a significant decrease of nuclear staining of the protein compared to cytokine treated group.

5. Summary

The protection of biological barriers is crucial in the case of living organisms. The damage of these barriers can be observed in different inflammatory diseases, where the secreted pro-inflammatory cytokines, like TNF-α and IL-1β, impair the biological barriers. In both intestinal and central nervous system damages these cytokines play central roles contributing to the redistribution of intercellular TJ proteins, decreasing barrier resistance and increasing its permeability. The oxidative stress also plays a role in the harmful effects of pro-inflammatory cytokines.

Anti-inflammatory effects of the α-MSH peptide were described previously in several animal models and cell types, but there were no data regarding Caco-2 intestinal epithelial cells and brain endothelial cells. In the present work we proved that MC1R, the main receptor of α-MSH, is expressed both in the human Caco-2 intestinal cell line and in rat brain endothelial cells. The peptide does not impair rat brain endothelial cells even in a wide range of concentrations.

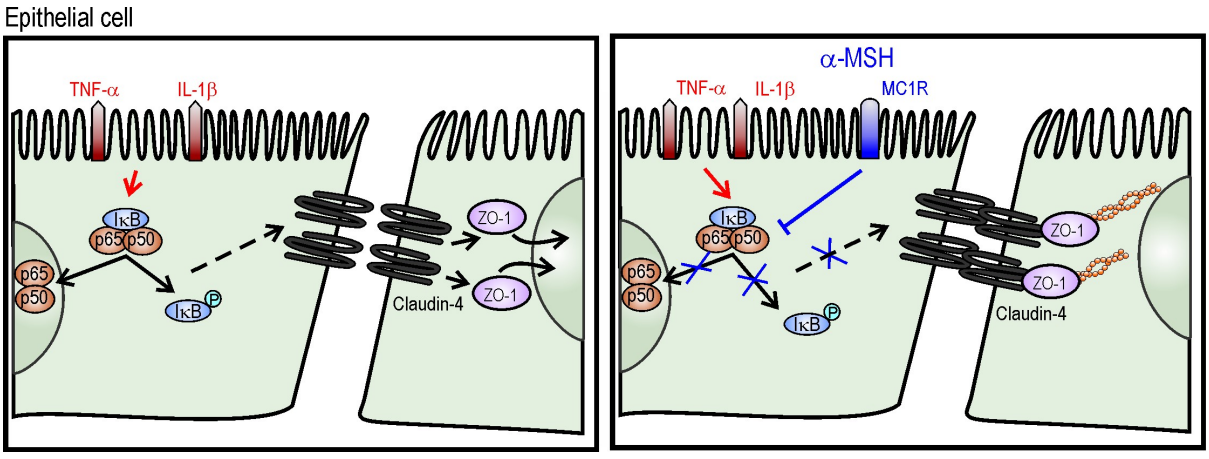

Figure 7. Anti-inflammatory effect of α-MSH on intestinal epithelial cells: inhibition of NF-κB transcription factor activation, facilitation of expression and localization of intercellular junctional proteins, by this decrease of elevated permeability.

In inflammatory conditions we demonstrated that α-MSH is protective both in culture models of the gut epithelium (**Fig. 7**) and of the blood-brain barrier (**Fig. 8**). The α-MSH

peptide reduced the production of reactive oxygen species in brain endothelial cells by inhibiting the NF-κB signaling pathway. The α-MSH peptide attenuated the cytokine induced increases in permeability and changes in the immunostaining pattern of cellular junctional proteins, occurring parallely. In our culture models of two biological barriers α-MSH, at least partly, insures the recovery of junctional protein expressions and restores appropriate barrier functions in cytokine induced damage by inhibition of the nuclear translocation of NF-κB transcription factor.

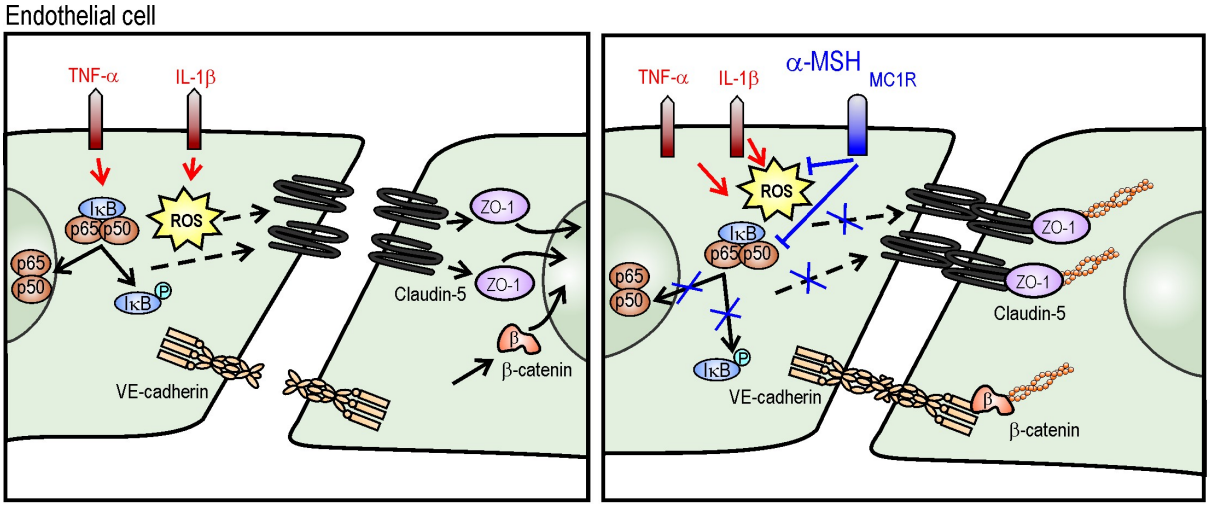

Figure 8. Anti-inflammatory effect of α-MSH on brain endothelial cells: inhibition of ROS production and NF-κB transcription factor activation, facilitation of expression and localization of intercellular junctional proteins, by this decrease of elevated permeability.

All these results strengthen the beneficial effects of α-MSH contributing to the protection of biological barriers.

Publication list:

MTMT ID: 10052432

Impact factor: 4,983

Publications related to the subject of the thesis

I. Váradi J, **Harazin A,** Fenyvesi F, Réti-Nagy K, Gogolák P, Vámosi G, Bácskay I, Fehér P, Ujhelyi Z, Vasvári G, Róka E, Haines D, Deli MA, Vecsernyés M.
Alpha-melanocyte stimulating hormone protects against cytokine-induced barrier damage in Caco-2 intestinal epithelial monolayers.
PLoS One. 12:e0170537 (2017)
IF: 2,806

II. **Harazin A,** Bocsik A, Barna L, Kincses A, Váradi J, Fenyvesi F, Tubak V, Deli MA, Vecsernyés M.
Protection of cultured brain endothelial cells from cytokine-induced damage by alpha-melanocyte stimulating hormone.
PeerJ, 6:e4774 (2018)
IF: 2,177

Other publications

III. Lénárt N, Walter FR, Bocsik A, Sántha P, Tóth ME, **Harazin A,** Tóth AE, Vizler C, Török Z, Pilbat AM, Vígh L, Puskás LG, Sántha M, Deli MA.
Cultured cells of the blood-brain barrier from apolipoprotein B-100 transgenic mice: effects of oxidized low-density lipoprotein treatment.
Fluids and Barriers of the CNS. 12:17 (2015)
IF: -

Acknowledgement

First of all I would like to thank my supervisors Dr. Mária Deli and Dr. Vilmos Tubak for their leadership and endless support during my work.

I would like to thank our cooperating partners who made possible the common publications. Thanks to Dr. Miklós Vecsernyés and the collegues of Department of Pharmaceutical Technology at the University of Debrecen for the partnership, joint work and professional support.

I say thanks to all current and former collegues of the Biological Barriers Research Group: Dr. Alexandra Bocsik, Dr. Zsófia Hoyk, Dr. Fruzsina Walter, Lilla Barna, Judit Vigh, Dr. Szilvia Veszelka, Mária Mészáros, Ilona Gróf, Ana Raquel Pato Santa Maria, Dr. Petra Sántha, Dr. Lóránd Kiss, Dr. Andrea Tóth, and our students, Balázs Sütöri and Armand Bálint. I'm thankful for the friendly atmosphere and the professional support, help.

Special thanks to the employee of Creative Laboratory Ltd.: Balázs Váczi, Rita Kovács, Judit Kereső and Anikó Simon. I would like to thank for their friendship and support during my university studies.

I am grateful to Dr. Pál Ormos and Dr. Ferenc Nagy, general directors of HAS BRC, to Dr. László Zimányi, director of Institute of Biophysics, and to Dr. László Siklós, head of Molecular Neurobiology Research Unit, for let me work in the institute. I would like to thank all the collegues of the research unit, Institute of Biophysics and the whole research centre. Special thanks to Mária Tóth, Dr. Györgyi Ferenc, András Kincses, Zsófia Melczer, Beáta Verebes, Dr. Csaba Vizler, Katalin Jósvay, Dr. Annamária Marton and Andrea Buhala for their friendship and support.

I am thankful to Dr. Csongor Ábrahám for his unique professional support and attention.

I am ineffably grateful to my friends who supported me, gave me strength during my university and doctoral studies.

Finally I feel profound gratitude to my Grandmother and my Mother for their loving guide, to my Father, my Brother, my Uncle, his Wife and their Daughter for their unconditional support and love.