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Melanocortins are comparable to corticosteroids as inhibitors of traumatic ocular inflammation in rabbits

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Abstract *Background:* Melanocyte-stimulating hormone (MSH) is a known anti-inflammatory agent and we investigated whether it reduces the inflammatory reaction following ocular surgery. *Methods:* Rabbits received a perforating corneo-limbal cut. Treatment involved topical (10^{-8}M) or intramuscular (50 mg/kg/day) MSH, topical steroids or saline. The parameters studied were hyperemia, edema, aqueous protein levels and the number of inflammatory cells in the aqueous, as well as their number determined histologically in the injured cornea. Each parameter was assessed at 24 h post injury. *Results:* Topically and systematically applied MSH reduced edema to levels 60% and 76%, respectively, of those observed in operated saline-treated eyes ($P<0.001$), and their efficacy was comparable to that of topical steroids. The decrease in hyperemia brought about by MSH was more pronounced than that produced by steroids. Aqueous protein levels were reduced by a similar degree in the steroid- and MSH-treated eyes as in the saline group ($P<0.001$ for each treatment group); In MSH- and steroid-treated eyes the number of inflammatory cells in the aqueous was reduced by 80% and 50%, respectively. *Conclusion:* We demonstrated that MSH reduced the clinical signs of ocular inflammation, curtailed blood–aqueous barrier (BAB) disruption, and reduced aqueous inflammatory cell number. The efficacy of MSH applied systemically or topically was similar to that of steroids in reducing clinical signs of trauma, but MSH efficacy in maintaining BAB integrity surpassed that of steroids. We suggest that the melanocortins might be a useful anti-inflammatory agents in ocular trauma.

Introduction

α-Melanocyte-stimulating hormone (MSH) is a basic tricapeptide whose amino acid sequence is identical to the 1–13 (N-terminal) amino acid sequence of adrenocorticotrophic hormone (ACTH). Pro-opio-melanocortin (POMC), found in the pituitary, brain, inflammatory cells, skin, and at other sites, is the precursor of ACTH, MSH (α-, β- and γ-MSH), lipotrophins, and endorphins, which constitute a family of biologically active peptides termed melanocortins. MSH, named for its effect on pigmentation in amphibian skin, is widely known as an anti-inflammatory and antipyretic agent with greater potency than paracetamol [4, 7, 18, 31, 32]. The peptide inhibits chronic [12, 33] and acute inflammation mediated by various cytokines [7] and alleviates allergic reactions [4, 18, 29, 32].

The anti-inflammatory effect of MSH takes place through a central and peripheral mechanisms [7, 31] and is related to its ability to counteract the effects and the production of pro inflammatory cytokines such as nitric oxide [36], tumor necrosis factor-α (TNF-α), and interleukin-1 (IL-1) [9, 30]. In addition, the chemotactic activity of macrophages and neutrophils is inhibited by

these peptides [5, 9, 34, 36]. Studies conducted two decades ago showed that MSH mediated changes in the blood–aqueous barrier (BAB) and in intraocular pressure [8, 11, 17, 19, 27]. In these studies crude extracts of the peptide were used in high nonphysiological doses. Recently MSH was detected in the normal aqueous of humans, mice, and rabbits [26, 38] and was demonstrated to play a role in activation of regulatory T cells in the aqueous [38]. It also modulates the melanocyte response in the retina–choroid [15].

Despite the fact that MSH takes part in anterior segment immune reactions and BAB permeability changes, its role in ocular inflammation has not been studied. In the present study the anti-inflammatory activity of MSH in trauma-induced ocular inflammation was investigated using physiological doses [1, 20] administered topically or systemically, and their effect was compared to that of topical corticosteroids.

Materials and methods

Animals

Pigmented rabbits of either sex, weighing 2.5–3 kg, were used. The rabbits were maintained in standard cages with free access to food and water, in a temperature controlled room, and were exposed to 12-h light–dark cycles.

Animal treatment followed the Association for Research in Vision in Ophthalmology resolution on the use of animals in research.

MSH drug preparation

MSH (acetate salt; Sigma, St. Louis, Mo. USA), was dissolved in sterile saline (0.9% NaCl).The stock solution containing 250 µg/ml (10^{-4} M) was stored at –20 °C. The stock solution was diluted with phosphate-buffered saline (PBS) to achieve doses for topical application (10^{-7} to 10^{-10} M). For intramuscular (i.m.) injections, a solution containing 50 µg/0.2 ml was used.

Surgical procedure

The right eye of each animal received a 6-mm-long nonperforating corneo-limbal cut, followed by paracentesis, and aspiration of 150 µl of aqueous using a 25-gauge needle. Twenty-four hours later, when the anterior chamber was reformed, the aqueous was aspirated again and protein and cells were determined.

Protein and inflammatory cell count

These parameters were determined in the secondary aqueous. Protein was measured using the Lowry technique, while inflammatory cells were counted using a Coulter cell counter.

Histopathology

At 24 h post trauma, after secondary aqueous had been withdrawn, a triangle-shaped piece of tissue was excised at the middle of the corneo-limbal cut. The sample contained sclera, cornea, and the iris ciliary body. The tissue was fixated with glutaraldehyde 2%. After a drying process the cornea was cut and stained with Giemsa.

Morphometric evaluation

The inflammatory cellular infiltrate at the traumatized corneo-limbal area was evaluated by checking 10 fields in each section. The fields were magnified by 60. projected and the inflammatory cells were counted using a computerized digitizer. The image-processing system includes a digitizing pad (Summer Graphics, Seymour, Conn., USA.) a personal computer and custom-designed software. A handle with a mirror tilted 45 deg was built to fit the projecting microscope. This allowed images of the stained section to be projected onto the digitizing pad. Limbal thickness was calculated using the same computerized digitizer, measuring seven consecutive points at the middle of each section.

Clinical assessment

Conjunctival hyperemia, edema, hemorrhages, and discharge, as well as corneal changes 24 h following ocular trauma, were assessed using the operating microscope (Wild Leica AG, Heebrugg, Switzerland). Discharge and corneal changes were negligible and therefore were not included in the clinical evaluation. Additional evaluation of hyperemia was done morphometrically using colored photos (Nikon F601 camera, Nikon, Tokyo, Japan) taken during clinical examination. Slides were coded, projected, and hemorrhages as well as congested blood vessels were quantified morphometrically as described. Hyperemia was the most reproducible clinical sign and therefore its analysis is depicted in Results.

Experimental design

The study involved five groups of rabbits (14 animals in each group), divided according to protocol: Control group 1: untreated intact rabbits; group 2: operated rabbits treated daily by topical application of 0.45% saline q.i.d., group 3: operated animals treated daily with dexamethasone 2% q.i.d.; groups 4 and 5: operated animals treated with α-MSH applied topically (MSH 10^{-8}, q.i.d.) or given systemically (i.m.) (25 µg/kg).

Treatment in-groups 2–5 was started 1 h prior to trauma.

Experimental procedure

Fully anesthetized animals underwent the surgical procedure as described. Within 1 h prior to surgery, treatment was started according to protocol. Any particular topical treatment involved application of a 50-µl drop in the lower cul de sac. Systemic treatment was applied i.m. (as described). Clinical examination of anesthetized animals, performed through an operating microscope at 24 h after trauma, included assessment of the following parameters: conjunctival hyperemia and edema, corneal damage, and aqueous flare using a score from 0 to 4. Following clinical examination, photographs of the surgical site were taken for computerized evaluation of hyperemia (as described). At the end of examination the animals were killed and secondary aqueous was withdrawn for protein and inflammatory cell determination. In addition, corneo-limbal tissue sample at the trauma site was excised for histology (as described).

Statistical analysis

Statistical analysis of the clinical observations was carried out using two-way analysis of variance (ANOVA). Other results were analyzed using Student's *t*-test.

Results

Eyes subjected to trauma developed edema and hyperemia, but no corneal damage. Discharge from the operated eyes was watery and mild and its amount varied, and therefore discharge was not included in the analysis.

Eyes treated with topical MSH (Table 1) experienced a significant reduction in edema compared with the saline-treated group ($P<0.05$). Steroids and systemically administered MSH curtailed edema similarly, ($P<0.05$ for each medication vs saline) but were less effective than the topical hormone (Table 1). Hyperemia was reduced significantly in both MSH-treated groups ($P<0.001$ for each medication vs saline), and to a lesser degree by steroids.

Computerized evaluation (Table 2) showed that MSH applied topically reduced hyperemia to levels 53% of those in operated saline-treated eyes ($P<0.001$ vs saline) being more efficacious than systemic MSH or steroids (a reduction to 62% and 75% of baseline, respectively, $P<0.05$, vs saline). Hyperemia was not influenced by the length of the surgical cut, which was similar for each group.

Protein levels were 2.8 times greater in the operated saline-treated group than in intact eyes (Table 3). In the three treated groups, protein levels (Table 3) were similarly reduced (approximately by 50%) when compared to the saline group ($P<0.001$ for each of the drugs).

The number of inflammatory cells in the aqueous (Table 3) was reduced by 80% in the two MSH groups, while a 50% decrease was noted in the steroid-treated eyes ($P<0.001$ for each drug vs saline).

Neither MSH nor steroids significantly affected the number of inflammatory cells found at the injury site (Fig. 1). However, edema at the trauma site, as manifested by limbal thickness, was significantly reduced by topical and systemic MSH, ($P<0.001$ for both vs saline) (Fig. 2), while steroids were less effective. ($P<0.05$ vs. saline).

Table 1 MSH anti-inflammatory activity: clinical assessment

Treatment group	Edema[a]	Hyperemia[a]
Saline i.m. 50 µg/kg/day	100±28	100
MSH i.m. 50 µg/kg/day	76*±18	78*±15
MSH topically 10^{-8} M	60*±15	77*±13
Steroid topically 2%	79*±14	87**±20

[a] Data given as the percentage of the ratio between the mean of each treated MSH group/ mean of saline control
* $P<0.001$; ** $P<0.05$

Table 2 Hyperemia evaluated by morphometry

Group	Corneal cut (mm)[a]	Hyperemia (% of saline group)
Saline i.m. 50 µg/kg/day	97±17	100
MSH i.m. 50 µg/kg/day	102±20	62**
MSH topically 10^{-8} M	95±15	53**
Steroid topically 2%	93±18	75**

[a] A 6-mm corneal cut was considered as 100%; measurements were made under the operating microscope
** $P<0.05$ compared to saline group

Table 3 Inflammatory cells and protein levels in the aqueous

Group	Number of cells in 0.1 ml aqueous	Protein levels (µg/ml)
Control	Undetectable	88±10
Saline i.m 50 µg/kg/day	30,000±2,500	250±9
MSH i.m. 50 µg/kg/day	6,000±750*	120±10*
MSH topically 10^{-8} M	5,700±650*	128±20*
Steroid topically 2%	15,000±1200*	150±11*

* $P=0001$ using two-way analysis, performed for each parameter separately

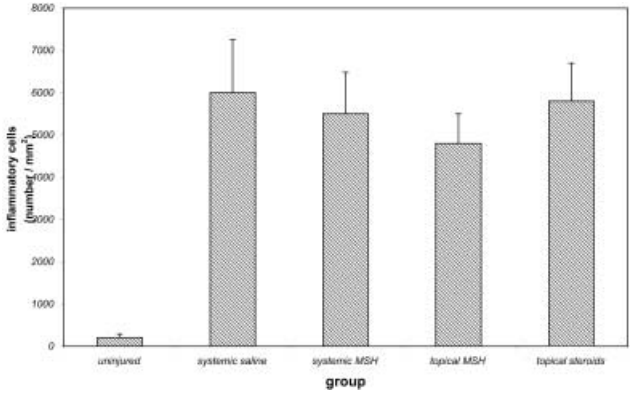

Fig. 1 MSH inhibits cellular infiltration at the injury site

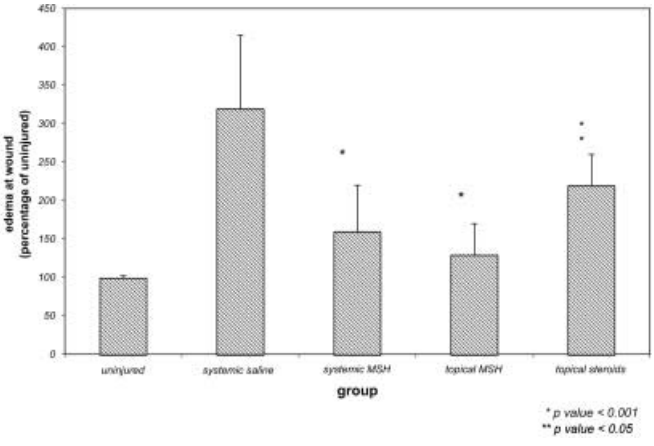

Fig. 2 MSH inhibits edema at the injury site

Discussion

Our study demonstrated for the first time that MSH, applied either topically or systemically, alleviated hyper-

emia and edema, clinical signs associated with surgical induced inflammation of the eyes. In addition, the peptide reduced BAB failure in operated eyes and the infiltration of inflammatory cells into the aqueous following the surgical trauma. The efficacy of topically applied MSH in reducing edema was slightly greater than that of systemic MSH or topical steroids. MSH treatment (topical and systemic) caused a significantly more pronounced reduction of hyperemia than was found with steroid therapy.

In our study, inflammatory (polymorphonuclear, PMN) cells infiltrated the aqueous of the operated eyes. PMN cell number at the corneal surgery site was also increased, and a failure of the BAB was manifested by leakage of protein into the aqueous.

Our findings in eyes subjected to corneo-limbal cut and perforation (paracentesis) are in agreement with well-established data on paracentesis-induced BAB failure in primates [3, 24] and with other experimental models in which inflammation was induced by intraocular injection of leukotriene B4 (LTB4) [37], combined injection of TNF-αand IL-6 [9, 13], or TNF alone [10, 16].

In our study, the reduction in the number of PMN in the aqueous by MSH administered either topically or systemically was 2 times greater than that observed in the steroid-treated group.

Protein levels in the aqueous of operated eyes in our study were reduced by 50% in all treated groups, and no statistically significant difference existed between steroid-treated and or MSH-treated eyes. Steroids are known to reduce aqueous protein levels in traumatized eyes: protein levels were diminished by steroids treatment following perforating corneal injury [37], or cataract surgery [27].

In our study, PMN cell number in the cornea at the injury site was unaffected by either drug. This is in accordance with a report that the number of PMN cells in iris and ciliary bodies in the aqueous of uveitic eyes was not reduced by steroids [38]. In another report steroids did not inhibit LTB4-induced aqueous inflammatory cell infiltration [35].

Our demonstration of an MSH-related anti-inflammatory activity following ocular surgery is in agreement with the melanocortins' well-established inhibitory effect on inflammation in many organs [4, 19] The mechanism underlying the anti-inflammatory action of MSH is attributable to its ability to counteract the pro-inflammatory effects of the cytokines TNF-α, IL-1, IL-6 and nitric oxide [8, 31, 32]. In addition, chemotaxis of macrophages and neutrophils is inhibited by MSH through receptor-specific mechanisms [8, 22, 28, 31].

Steroid-related reduction of PMN number in the aqueous might possibly be exerted through down regulation of TNF and IL-6 [2, 16] and trough inhibition of prostaglandins and a reduced expression of mRNA cyclooxygenase 2(COX-2) [23, 25].

MSH in our study was used in a physiological dose, in contrast to the studies carried out two decades ago [11, 12, 17, 20, 21, 26]. At that time MSH was known for its role in disrupting the BAB because crude MSH preparations in high nonphysiological doses were used [11, 12, 20] It was also given in amounts exceeding normal values by a factor one thousand to one million times greater than the doses used by us. Therefore these data are irrelevant to our study [17, 26].

Recent data on melanocortins showed that MSH derivatives applied systemically to humans [14, 18, 30, 36] or rats [6, 19] caused no ocular side effects.

In summary, we have demonstrated for the first time that MSH, applied topically or given systemically, reduces ocular inflammation, curtails BAB disruption and reduces PMN infiltration into the aqueous in rabbits' eyes subjected to perforating surgery. The peptide's efficacy in curtailing the inflammatory-related clinical signs and BAB failure was comparable to that of steroids. MSH was more effective than steroids in reducing hyperemia and the number of inflammatory cells in the aqueous. We suggest that the melanocortins might be considered as future novel anti-inflammatory agents.

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