Alpha-Melanocyte Stimulating Hormone Reduces Colonic Damage in Rat Model of Inflammatory Bowel Disease*

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The aim of the study was to investigate the dose-dependent in vivo effect of alpha-melanocyte stimulating hormone (α-MSH) in a rat model of inflammatory bowel disease induced by 2,4,6-trinitrobenzene sulphonic acid (TNBS). Laboratory animals (male Wistar rats weighing 200–250 g) were given 3 doses of α-MSH (0.5 mg/kg, 1 mg/kg, 2 mg/kg) intraperitoneally 1 hour prior to inducing colitis with a TNBS enema. Control animals received saline solution i.p. Rats were sacrificed after 72 hours and the area of mucosal lesions, involving the distal 10 cm of colon, was determined in mm² by means of image analysis software. α-MSH structure was analyzed by means of NMR spectroscopy. The area of colonic damage was significantly reduced following pretreatment with a single dose of 1 mg/kg α-MSH, as compared to control animals (p = 0.0147). Higher and lower doses had no significant effects. A single dose of 1 mg/kg α-MSH provided strong, statistically significant and pharmacologically relevant cytoprotection. The results point to α-MSH effectiveness in controlling inflammation and imply that further in vitro and in vivo experiments should be carried out to judge the importance and relevance of α-MSH in the control of inflammatory bowel disease. NMR data support a hairpin loop conformation of α-MSH in water solution, which includes conserved message sequence.

Keywords inflammatory bowel disease α-MSH NMR TNBS colitis cytoprotection

INTRODUCTION

Inflammatory bowel disease results from a deregulated immune response to yet undefined stimulus/stimuli that lead to mucosal inflammation characterized by rectal bleeding, diarrhoea and abdominal pain.1–3 Thus far, there is no unique effective treatment for the disorder. Therapy is directed toward controlling inflammation (5-aminosalicylic acid, antibiotics, and some diets), the immune response (azathioprin, cyclosporine, metotrexate, 6-mercaptopurine, and Infliximab) or both (corticosteroids).4 The search for more effective agents continues.3,5

One of the possible therapeutic agents is alpha-melanocyte stimulating hormone, i.e., alpha-melanotropin (α-MSH), a 13-amino acid peptide derivate of corticotropin (ACTH). There is a growing body of evidence

* Dedicated to Dr. Edward C. Kirby on the occasion of his 70th birthday with special thanks for promoting interdisciplinarity in scientific research.
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that endogenous α-MSH is part of the host reaction to infectious and inflammatory stimuli in humans.6–10 Observations indicate that there is generally a compensatory increase in neuropeptide in the presence of inflammation.7,11 It likewise reduces synthesis of proinflammatory cytokines in human disease,8,12,13 suggesting that whenever the increase in endogenous α-MSH is not adequate, the disease process is more severe. In such situations, treatment with α-MSH could be beneficial and preclinical studies have identified potential therapeutic targets, including allergic inflammation, uveoretinitis, brain inflammation, rheumatoid arthritis, septic shock and asthma.7,11–16 Furthermore, α-MSH and its receptor have been isolated from the gut, implicating its contribution to local inflammatory and anti-inflammatory processes.11,13 Some data confirming the beneficial effect of α-MSH on lesions in the digestive system already exist.13,17–20

The present study was designed to evaluate the dose-dependent protective effect of α-MSH in a classical rat model of inflammatory bowel disease and to obtain additional information on the structure and conformation of the α-MSH molecule.

EXPERIMENTAL

TNBS Colitis

Experimental animals were male Wistar rats bred at the School of Medicine, Zagreb, Croatia, weighing 200–250 g, randomized in five experimental and one control group (six rats per group). The animals were kept in a room with constant temperature (22 ± 1 °C) and a dark-light cycle (12 h/12 h), housed in cages, maximum 6 rats per cage. They were fed standard laboratory rodent pellets and given water ad libitum. Animals were food-fastened with free access to water 12 h prior to inducing colitis and sacrificing.

The tested substance α-MSH (SYSEHFRWGBKPV, >97 % purity; CBI, USA), was administered in 3 progressive doses of 0.5 mg/kg (A), 1 mg/kg (B) and 2 mg/kg (C) dissolved in 0.9 % NaCl up to the volume of 1 mL. This volume was given intraperitoneally (i.p.) 1 h prior to the induction of colitis. Pure saline solution was used in the control group to correct the influence of volume load.

After light ether anesthesia, colitis was induced by applying 1 mL of a solution containing 30 mg of 2,4,6-trinitrobenzene sulphonic acid (TNBS) (Sigma, USA) dissolved in 40 % ethanol, via rectal catheter. Rats were sacrificed 72 hours after the TNBS installation, when maximal extent of colonic damage was expected.21,22 The last 10 cm of the colon was examined. Lesions (hemorrhagic and necrotic areas) were determined in mm² by means of image analysis software (GIMP and ImageJ programs).

Statistical analysis and comparison of control and treated groups was performed with ANOVA and the Tukey HSD test.

## NMR Measurements

The structure and conformation of α-MSH were analyzed by one- and two-dimensional homo- and hetero-nuclear 1H and 13C NMR spectroscopy. The spectra of α-MSH were recorded with a Bruker AV-600 spectrometer, operating at 600.133 MHz and 150.917 MHz for the 1H and 13C nuclei, respectively. The concentration of samples was ca. 2–5 mmol dm⁻³. Samples were dissolved in water with the ratio D₂O : H₂O = 1:10.

### Table I. The 1H chemical shifts (δ/ppm)(a) of residues in α-MSH

<table>
<thead>
<tr>
<th>Residue</th>
<th>NH</th>
<th>CαH</th>
<th>CβH</th>
<th>Other H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ser1 (S)</td>
<td>8.25</td>
<td>4.60</td>
<td>3.9</td>
<td>3.95</td>
</tr>
<tr>
<td>Tyr2 (Y)</td>
<td>8.25</td>
<td>4.60</td>
<td>3.00</td>
<td>7.15</td>
</tr>
<tr>
<td>Ser3 (S)</td>
<td>8.22</td>
<td>4.60</td>
<td>3.72</td>
<td>3.95</td>
</tr>
<tr>
<td>Met4 (M)</td>
<td>8.26</td>
<td>4.50</td>
<td>2.10</td>
<td>2.61; 2.63</td>
</tr>
<tr>
<td>His6 (H)</td>
<td>8.56</td>
<td>3.22</td>
<td>3.41</td>
<td>7.11</td>
</tr>
<tr>
<td>Phe7 (F)</td>
<td>8.30</td>
<td>4.65</td>
<td>3.20</td>
<td>2.30</td>
</tr>
<tr>
<td>Arg8 (R)</td>
<td>8.25</td>
<td>4.40</td>
<td>1.83</td>
<td>1.45; 3.05</td>
</tr>
<tr>
<td>Trp10 (W)</td>
<td>8.25</td>
<td>4.60</td>
<td>3.11</td>
<td>7.25; 10.63</td>
</tr>
<tr>
<td>Gly10 (G)</td>
<td>8.15</td>
<td>4.03</td>
<td>4.05</td>
<td></td>
</tr>
<tr>
<td>Lys11 (K)</td>
<td>8.18</td>
<td>4.31</td>
<td>1.62</td>
<td>1.75; 1.47; 1.43</td>
</tr>
<tr>
<td>Pro12 (P)</td>
<td>4.50</td>
<td>2.00</td>
<td>2.00; 2.12</td>
<td></td>
</tr>
<tr>
<td>Val13 (V)</td>
<td>8.05</td>
<td>4.22</td>
<td>2.15</td>
<td>0.90; 0.92</td>
</tr>
</tbody>
</table>

(a)Measured in water, ratio D₂O : H₂O = 1:10.
field gradient mode (z-gradient). The COSY spectrum was recorded with 2048 points in F2 dimension and 512 increments in F1 dimension. The latter was subsequently zero-filled to 1024 points. Every increment was obtained by 2 scans with spectral width 6127.45 Hz. The FID resolution was 2.99 Hz/point and 11.96 Hz/point in F2 and F1 dimensions, respectively. NOESY spectra were measured with mixing times 0.2–1.0 s and 8 scans per each increment. ROESY spectra were recorded with ROE mixing time 0.15–0.5 s. The spectral width in NOESY and ROESY was 6127.45 Hz. Numbers of points used were 2048 in F2 dimension (2.99 Hz/point) and 512 in F1 dimension, subsequently zero-filled to 1024 points and in F2 (11.96 Hz/point). TOCSY spectra were collected with a mixing time 60–80 ms using MLEV-17 pulses for spin-locking.

Sequential resonance assignments were performed on the basis of TOCSY and NOESY spectral data. Backbone assignment were completed from NOE connectivities, while side-chain assignments from TOCSY connectivities. The $^1$H chemical shift assignment is presented in Table I.

RESULTS AND DISCUSSION

Areas of hemorrhagic and necrotic damage were analyzed separately and together (as total lesions); only results for the total area of lesions are presented, since no major differences between groups were observed in dependence on the type of lesions. Severe colitis following intracolonic administration of TNBS in ethanol was found in all control untreated animals, as compared to normal rat colonic mucosa. No changes in the microscopic damage score between different groups were noted, only the total area of lesions assessed in mm$^2$ differed (Table II).

Depending on the type of pretreatment, different levels of colonic damage were observed on the third day after inducing colitis. ANOVA and subsequent post hoc analysis with the Tukey HSD test show that TNBS-induced mucosal damage was significantly reduced only in the group pretreated with a 1 mg/kg dose $\alpha$-MSH (Table II, Figure 1). The results of the measurements implicate a dose-related modulatory effect of $\alpha$-MSH on the colonic mucosal damage: protective effect arises when increasing the dose from 0.5 mg/kg, peaks at 1 mg/kg, and weakens with further dose increase to 2 mg/kg (Figure 1 and Table II).

The experimental model of TNBS colitis is a simple, inexpensive and popular animal model of the human inflammatory bowel disease owing to its high reproducibility (90 %), and dose-dependent tissue lesions. This robust model is suitable for the screening of cytoprotective agents and drugs. Measuring the area of colonic lesions is an exact and precise way to determine the extent of colonic damage and it is more reliable than semi-quantitative methods using macroscopical scoring systems.

The purity of $\alpha$-MSH was analyzed by Konjevoda et al. to exclude the possibility of cytoprotective effects of the substance being due to possible impurities. In the central region of $\alpha$-MSH, a NOE between His6 and Phe7 and a strong NOE between Phe7 and Arg8 were observed (Table I). These NOEs support a turn conformation consisting of His6, Phe7 and Arg8. Detection of NOE between Glu5 and Trp9 supports the hairpin loop conformation that includes conserved message sequence. Our NMR analyses confirm that $\alpha$-MSH region from His6 to Trp9 (HFRW, Table I) constitutes an important part of the molecule, responsible for the melanocortin receptor binding and receptor subtype selectivity.

The melanocortin receptor family consists of five subtypes of melanocortin receptors (MC1R-MC5R). The ACTH melanocortin receptor (MC2R) requires for ligand binding not only the conserved HFRW sequence of $\alpha$-MSH but also a highly basic motif present solely in the ACTH molecule. Other melanocortin receptor subtypes are receptors for melanocyte-stimulating hormones.

Mechanisms of $\alpha$-MSH protection in the colon are not fully understood. TNBS exhibits hapten-like behavior and induces an immune response with inflammation that is characterized by neutrophil infiltration, in-

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Mean ± S.D.</th>
<th>Min.</th>
<th>Max.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (0.9 % NaCl)</td>
<td>754.7 ± 408.7</td>
<td>356.0</td>
<td>1414.7</td>
</tr>
<tr>
<td>$\alpha$-MSH 0.5 mg/kg</td>
<td>368.9 ± 179.4</td>
<td>176.0</td>
<td>578.0</td>
</tr>
<tr>
<td>$\alpha$-MSH 1 mg/kg</td>
<td>225.8 ± 148.4</td>
<td>16.1</td>
<td>366.5</td>
</tr>
<tr>
<td>$\alpha$-MSH 2 mg/kg</td>
<td>336.4 ± 270.8</td>
<td>34.0</td>
<td>672.5</td>
</tr>
</tbody>
</table>

ANOVA: F = 4.32, p = 0.0166, Tukey HSD test: p = 0.0147 control vs. $\alpha$-MSH 1 mg/kg.
volvement of prostaglandins, leukotriens, tromboxane, platelet activating factor, tumor necrosis factor-α, interleukin-1 cytokines, and the presence of Th1 lymphocyte subpopulation followed by B-cell response (and interleukin-2 activation in chronic disease model). Therefore, anti-inflammatory effects of α-MSH could modulate many of these features. In experiments concerning the gut, treatment with α-MSH influences the release of tumor necrosis factor-α, nitric oxide and interleukin-6 from bowel tissue, and there is indirect evidence suggesting that prostaglandins participate in the salutary effect on colonic mucosa. However, it is likely that many more molecules involved in the colonic inflammation are affected by α-MSH, as a consequence of reduced translocation of the nuclear factor kappa-B to the nucleus following peptide binding to its receptors. Therefore, α-MSH is indirect evidence suggesting that prostaglandins participate in the salutary effect on colonic mucosa. However, it is likely that many more molecules involved in the colonic inflammation are affected by α-MSH, as a consequence of reduced translocation of the nuclear factor kappa-B to the nucleus following peptide binding to melanocortin receptor MC1R. Therefore, anti-inflammatory and immunomodulatory effects of α-MSH are broad, which makes it attractive for potential treatment of inflammatory bowel disease at this point, when no etiologic factors or specific mediators of the disease have been recognized.

Our data identified the dose of 1 mg/kg of α-MSH to be effective in the rat model of inflammatory bowel disease. The same dose was found optimal in studies of gastric cytoprotection. However, lower and higher doses were not effective in our study. The dose/effect curve has a U-shape (Figure 1). Other neuropeptides exhibit a similar dose-related effect. This can be explained by additive effects of the peptide itself and its metabolites in different concentrations, or by different receptor affinities. On the other hand, a hypothetical auto-regulatory mechanism has been suggested that would protect the host from excessive effects of the endogenously produced or administered α-MSH. Therefore, it seems essential to apply a proper dose in order to achieve optimal protective effects.

During the i.p. administration of α-MSH no signs of acute toxicity in rats were observed. Even higher doses of α-MSH were applied with no signs of toxicity. This peptide is not accumulated in the body, which reduces the probability of tolerance and toxicity and allows a good control of pharmacological effects. Another advantage is that α-MSH does not affect production of inflammatory mediators in resting conditions, nor does it reduce microbial killing activity of the neutrophils, as currently used anti-inflammatory drugs, in particular corticosteroids do. All these features are in favor of further investigations concerning α-MSH as a potential agent for the treatment of inflammatory bowel disease.

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REFERENCES


SAŽETAK

Alfa-hormon koji stimulira melanocite smanjuje oštećenje sluznice debeloga crijeva u štakorskom modelu upalne bolesti crijeva

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Svrha istraživanja bila je kvantifikacija in vivo učinaka alfa-hormona koji stimulira melanocite (α-MSH) na štakorskom modelu upalne bolesti crijeva inducirane 2,4,6-trinitrosulfonbenzenskom kiselinom (TNBS). Laboratorijskim životinjama (mužjaci Wistar soja štakora tjelesne mase 200–250 g) dane su tri doze α-MSH (0.5 mg/kg, 1 mg/kg i 2 mg/kg) intraperitonealno 1 sat prije indukcije kolitisa s TNBS klizmom. Kontrolne su životinje primile fiziološku otopinu intraperitonealno. Nakon 72 sata štakori su žrtvovani, te je pomoću programa za analizu slike procijenjena površina mukoznih lezija (mm²) unutar 10 distalnih centimetara kolona. Struktura α-MSH je analizirana uporabom NMR spektroskopije. Površina oštećenja debeloga crijeva statistički je značajno smanjena kod pretretmana s jednom dozom 1 mg/kg α-MSH, u odnosu na kontrolne životinje (p = 0.0147). Više i niže doze nisu imale značajne učinke. Pojedinačna doza od 1 mg/kg α-MSH uzrokovala je statistički značajnu i farmakološku relevantnu citoprotekciju. Rezultati ukazuju na učinkovitost α-MSH peptida u kontroliranju upale i upućuju na uporabu daljnjih in vitro i in vivo istraživanja kako bi se procijenio značaj i učinkovitost α-MSH u kontroli upalne bolesti crijeva. NMR mjerenja u vodenoj otopini podupiru pretpostavku o konformaciji α-MSH i njegove konzervirane sekvencije u obliku ukosnice.