Protective Effects of Met-enkephalin on Alcohol Induced Gastric Lesions

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The model of ethanol induced gastric lesions is useful in the evaluation of gastric cytoprotection. We used it to measure cytoprotective effects of two neuropeptides, Met-enkephalin (Peptid-M, LUPEX®) and α-melanocyte stimulating hormone (α-MSH). Both peptides exhibited significant and synergistic cytoprotective effects. The best therapeutic efficacy was obtained with Met-enkephalin and α-MSH mixture 3–5 : 1. Significant cytoprotective action on ethanol induced lesions was also observed by means of two thymus peptide immunomodulators, Thymus Peptide C® and JAN 50®. Thymus Peptide C® exhibited a more significant synergistic effect with Met-enkephalin than JAN 50®. In addition to the cytoprotection of rat gastric mucosa, Met-enkephalin also induced statistically sig-
significant and dose dependent Straub tail response versus control in mice. Haloperidol, naloxone and Peptide-D antagonised its effects. NMR spectroscopic data supported molecular interaction of Met-enkephalin and haloperidol, while neutralization of Met-enkephalin induced Straub tail response by means of Peptide-D indicated the presence of new non-opioid (naloxone independent) receptor system containing Peptide-D sequence (calpastatin 201–205 aa).

Key words: Met-enkephalin, LUPEX®, α-MSH, ethanol, gastric lesions, peptide-D, cytoprotection, Straub phenomenon, haloperidol, NMR, Thymus Peptide C®, JAN 50®.

INTRODUCTION

Inflammatory diseases are still among great and unsolved problems of modern medicine because of insufficient efficacy and many side effects of antiinflammatory drugs. The most frequent side effects of antiinflammatory drugs are gastric lesions. Ethanol induced gastric lesions constitute a useful model for the evaluation of gastric cytoprotection. In this study, we have evaluated cytoprotective effects of two neuropeptides, Met-enkephalin (Peptid-M, LUPEX®) and α-melanocyte stimulating hormone (α-MSH). Both peptides exhibited significant cytoprotective effects in several experimental models, including arthritis, encephalomyelitis, graft versus host reaction, ischaemia and inflammatory bowel disease.

Synergistic action of Met-enkephalin and two thymus immunomodulating peptides (Thymus Peptide C® and JAN 50®) was also investigated on the model of ethanol induced gastric lesions in rat, since Peptid-M (LUPEX®) represents the syntethic analogue of thymus Met-enkephalin.

Additionally, in order to evaluate pharmacologically relevant interactions of Met-enkephalin, we evaluated the effects of its potential antagonists (naloxone, Peptide-D, haloperidol) on Straub tail reaction and gastric cytoprotection.

MATERIAL AND METHODS

Experimental Animals

Gastric cytoprotection was evaluated in male Wistar rats, 150–200 g, bred by the Department of Pharmacology, Zagreb University School of Medicine. The size of the experimental groups was 8. Straub tail reaction was measured in Male albino mice, NMRI, 25–30 g, bred by the Department of Pharmacology, Zagreb University School of Medicine. The size of the experimental groups was 10.
Measurement and Analysis of Cytoprotection

After sacrifice, hemorrhagic gastric area was assessed in mm², using a digital camera (JVC) and an image analysis software (SFORM, VAMS, Zagreb, Croatia). Statistical analysis was made using Kruskal-Wallis and Mann-Whitney tests, with statistical significance at $p < 0.05$.

Straub Tail Reaction and Scoring

W. Straub described this phenomenon, characterised by the sigmoid curving of the tail over the back. The Straub tail reaction is believed to be produced by the contraction of the sacroccygeus dorsalis muscle. It is the prime model for opioid effects assessment in vivo. The Straub tail reaction is recorded every 5 minutes for 50 minutes after 0.9% NaCl or Met-enkephalin application. Arithmetic mean for each animal is calculated and different groups are compared using the Analysis of Variance and Tukey HSD test. The tail reaction is scored as follows:

<table>
<thead>
<tr>
<th>Score</th>
<th>Degrees of erection</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>&lt; 45</td>
</tr>
<tr>
<td>1</td>
<td>45</td>
</tr>
<tr>
<td>1.5</td>
<td>45–90</td>
</tr>
<tr>
<td>2</td>
<td>90</td>
</tr>
<tr>
<td>2.5</td>
<td>90–180</td>
</tr>
<tr>
<td>3</td>
<td>180</td>
</tr>
</tbody>
</table>

Substances

1. Met-enkephalin, i.e. Peptid-M (LUPEX®, Biofactor, Germany), $M_r$ 573.
2. Peptide-D (IPPKY, Biofactor, Germany; Lot No. B-0158), >97% purity, $M_r$ 616.8.
3. Thymus Peptide C® (Biofactor, Germany).
4. JAN 50® (Biofactor, Germany).
5. Naloxone hydrochloride (Sigma, USA), >99% purity.
6. Haloperidol (Sigma, USA), >99% purity.
7. 0.9% NaCl
8. $\alpha$-MSH (Commonwealth Biotechnologies, USA; Lot No. 010030399), >97% purity.

NMR Measurements

The $^1$H and $^{13}$C one- and two-dimensional NMR spectra of Met-enkephalin (LUPEX®) and haloperidol were recorded with a Varian Gemini 300 spectrometer, operating at 75.5 MHz for the $^{13}$C nucleus. All samples were measured from DMSO-$d_6$ solution at 20 °C in 5 mm NMR tubes. Chemical shifts, in ppm, are referred to TMS. Digital resolution in $^1$H NMR spectra was 0.20 Hz (Gemini 300), while in $^{13}$C NMR spectra it was 0.63 Hz per point. The following spectra were recorded: broadband proton decoupling, gated proton decoupling, COSY-45, long-range (delayed) COSY-45, NOESY and HETCOR. In all experiments, proton decoupling was performed by
Waltz-16 modulation. Standard pulse sequences were used in two-dimensional experiments. COSY-45 and delayed COSY-45 spectra were measured in the magnitude mode, while NOESY spectra in the phase-sensitive mode. In COSY-45, delayed COSY-45 and NOESY spectra, 1024 points in $F_2$ dimension and 256 increments in $F_1$ dimension, subsequently zero-filled to 1024 points, were used. Each increment was obtained with 16 scans, 3000 Hz spectral width and a relaxation delay of 1 s. Thus, the digital resolution was 5.9 Hz/point and 11.7 Hz/point in $F_2$ and $F_1$ dimensions, respectively. The delayed COSY-45 spectra were measured with delay time, $D_3$, of 0.25 s. The NOESY spectra were measured with several mixing times (0.45–1.2 s). The HETCOR spectra were recorded with 2048 points in $F_2$ dimension and 256 increments in $F_1$ dimension, zero-filled to 512 points. Increments were recorded with 180 scans, relaxation delay of 1 s and spectral width of 20000 Hz in $F_2$ and 4500 Hz in $F_1$ dimensions. Corresponding digital resolution was 19.53 and 17.6 Hz/point in $F_2$ and $F_1$ dimensions, respectively. In broadband proton decoupled spectra of haloperidol and its mixtures with Met-enkephalin, carbon-fluorine spin-spin couplings through one (ca. 251.8 Hz), two (21.7 Hz) and three (9.4 Hz) bonds were observed.

RESULTS

Evaluation of the protective effects of Met-enkephalin, $\alpha$-MSH and their combination on the ethanol induced lesions of the gastric mucosa are presented in Tables I and III. It is evident that Met-enkephalin, $\alpha$-MSH and combination Met-enkephalin + $\alpha$-MSH have a protective effect on gastric mucosa (Table III). The best protective effect was obtained by the combination of Met-enkephalin and $\alpha$-MSH (Table III).

Similarly to the ethanol model, the administration of Met-enkephalin (–30 min) exhibited also statistically significant cytoprotection in the standard cysteamine model, when 400 mg/kg cysteamine (i.e. 80 mg/mL aqua ad injectabilia) was administered to Wistar rats. The area of gastric lesions in Met-enkephalin treated animals was significantly smaller (4.7 ± 1.9 mm$^2$, $p < 0.05$) than in control animals (10.5 ± 2.3 mm$^2$, $\bar{X} \pm$ SEM).

Two immunoregulating thymus peptides (Thymus Peptide C® and JAN 50®) exhibited significant cytoprotective effects in the model of ethanol induced gastric lesions (Table III). Statistically significant synergistic cytoprotective action with Met-enkephalin was more pronounced for Thymus Peptide C®, while parallel administration of JAN 50® and Met-enkephalin had less influence on the amount of cytoprotection already achieved by JAN 50® (Table III).

Haloperidol abolished Met-enkephalin induced gastric cytoprotection (Table IV).

Effects of Met-enkephalin, haloperidol, naloxone and Peptide-D on the Straub tail reaction are presented in Table VI. Met-enkephalin has a statis-
tically significant Straub tail inducing effect versus control. Haloperidol, naloxone and Peptide-D antagonised its effect.

DISCUSSION

Ethanol ingestion results in gastritis characterised by mucosal edema, subepithelial haemorrhage, cellular exfoliation, and an inflammatory cell infiltrate. The initial event in the pathogenesis of mucosal ulcerations is microvascular dysfunction. Exposure of the mucosa to ethanol (50–100%)

### TABLE I

<table>
<thead>
<tr>
<th>Substance</th>
<th>Schedule</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>-1 h</td>
</tr>
<tr>
<td>0.9% NaCl (control)</td>
<td>application</td>
</tr>
<tr>
<td>Met-enkephalin (10 mg/kg ip)</td>
<td>application</td>
</tr>
<tr>
<td>α-MSH (3.3 mg/kg ip)</td>
<td>application</td>
</tr>
<tr>
<td>Thymus Peptide C® (50 mg/kg ip)</td>
<td>application</td>
</tr>
<tr>
<td>JAN 50® (100 mg/kg ip)</td>
<td>application</td>
</tr>
<tr>
<td>Met-enkephalin + α-MSH</td>
<td>application</td>
</tr>
<tr>
<td>Met-enkephalin + Thymus Peptide C®</td>
<td>application</td>
</tr>
<tr>
<td>Met-enkephalin + JAN 50®</td>
<td>application</td>
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</table>

### TABLE II

<table>
<thead>
<tr>
<th>Substance</th>
<th>Schedule</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-1 h</td>
</tr>
<tr>
<td>0.9% NaCl (control)</td>
<td>application</td>
</tr>
<tr>
<td>haloperidol (10 mg/kg ip)</td>
<td>application</td>
</tr>
<tr>
<td>Met-enkephalin (10 mg/kg ip)</td>
<td>application</td>
</tr>
<tr>
<td>Met-enkephalin + haloperidol</td>
<td>application</td>
</tr>
</tbody>
</table>
results in arteriolar vasodilatation, vascular congestion or stasis, haemoconcentration, albumin leakage, and capillary damage.\textsuperscript{10} This is associated with significant reductions in mucosal blood flow,\textsuperscript{10,12} and the extent of mucosal injury is correlated with the magnitude of the blood flow reduction.\textsuperscript{12} Microvascular dysfunction is reminiscent of the vascular phase of acute inflammation.\textsuperscript{10–12} Also, neutrophils have been implicated in the mucosal injury associated with ethanol-induced gastritis.\textsuperscript{13,14} In this context, the lesions resemble some human disease lesions, \textit{e.g.} in inflammatory bowel disease, gastritis, \textit{etc.}

Effects of Met-enkephalin and \textit{\alpha}-MSH are beneficial in the cytoprotective model investigated, especially their combination, which exhibits strong

\begin{table}
\centering
\caption{Results for substances from Table I}
\begin{tabular}{lll}
\hline
Substance & Results & Statistical significance vs. control \\
\hline
\textit{\alpha}-MSH & 18 & 0 & 24 & \textit{p} < 0.001 \\
Met-enkephalin & 225 & 172 & 298 & \textit{p} < 0.05 \\
Thymus Peptide C\textsuperscript{\textregistered} & 25 & 5 & 29 & \textit{p} < 0.01 \\
JAN 50\textsuperscript{\textregistered} & 28 & 8 & 32 & \textit{p} < 0.01 \\
Met-enkephalin + \textit{\alpha}-MSH & 0 & 0 & 4 & \textit{p} < 0.001 \\
Met-enkephalin + Thymus Peptide C\textsuperscript{\textregistered} & 17 & 0 & 22 & \textit{p} < 0.01 \\
Met-enkephalin + JAN 50\textsuperscript{\textregistered} & 31 & 0 & 46 & \textit{p} < 0.01 \\
\hline
\end{tabular}
\end{table}

\begin{table}
\centering
\caption{Results for substances from Table II}
\begin{tabular}{lll}
\hline
Substance & Results & Statistical significance vs. control \\
\hline
\textit{\alpha}-MSH & 18 & 0 & 24 & \textit{p} < 0.001 \\
Met-enkephalin & 225 & 172 & 298 & \textit{p} < 0.05 \\
Thymus Peptide C\textsuperscript{\textregistered} & 25 & 5 & 29 & \textit{p} < 0.01 \\
JAN 50\textsuperscript{\textregistered} & 28 & 8 & 32 & \textit{p} < 0.01 \\
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Met-enkephalin + JAN 50\textsuperscript{\textregistered} & 31 & 0 & 46 & \textit{p} < 0.01 \\
\hline
\end{tabular}
\end{table}
synergistic effects and almost completely protects the gastrointestinal mu-
cosa. This result has potential clinical application because Met-enkephalin
and α-MSH are products of the same precursor molecules.\textsuperscript{15}

Considering our results and the common pathways of peptide action, it
is reasonable to assume that both peptides should be applied together to
achieve optimal clinical and pharmacologic efficacy. The application of Met-
enkephalin + α-MSH combination in severe autoimmune disease, which in

\textbf{TABLE V}

<table>
<thead>
<tr>
<th>Substance</th>
<th>Schedule</th>
<th>observation – every 5 minutes for 50 minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9% NaCl (control)</td>
<td>0.9% NaCl</td>
<td>0.9% NaCl</td>
</tr>
<tr>
<td>Met-enkephalin (10 mg/kg ip)</td>
<td>0.9% NaCl</td>
<td>Met-enkephalin</td>
</tr>
<tr>
<td>haloperidol (10 mg/kg ip)</td>
<td>haloperidol</td>
<td>0.9% NaCl</td>
</tr>
<tr>
<td>naloxone (10 mg/kg ip)</td>
<td>naloxone</td>
<td>0.9% NaCl</td>
</tr>
<tr>
<td>Peptide-D (5 mg/kg ip)</td>
<td>Peptide-D</td>
<td>0.9% NaCl</td>
</tr>
<tr>
<td>Met-enkephalin + haloperidol</td>
<td>haloperidol</td>
<td>Met-enkephalin</td>
</tr>
<tr>
<td>Met-enkephalin + naloxone</td>
<td>naloxone</td>
<td>Met-enkephalin</td>
</tr>
<tr>
<td>Met-enkephalin + Peptide-D</td>
<td>Peptide-D</td>
<td>Met-enkephalin</td>
</tr>
</tbody>
</table>

\textbf{TABLE VI}

<table>
<thead>
<tr>
<th>Substance</th>
<th>Straub tail reaction (group mean ± SEM)</th>
<th>Statistical signifiance vs. control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9% NaCl (control)</td>
<td>0.33 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>Met-enkephalin</td>
<td>0.57 ± 0.07</td>
<td>(p &lt; 0.05)</td>
</tr>
<tr>
<td>haloperidol</td>
<td>0.28 ± 0.04</td>
<td>n.s.</td>
</tr>
<tr>
<td>naloxone</td>
<td>0.35 ± 0.05</td>
<td>n.s.</td>
</tr>
<tr>
<td>Peptide-D</td>
<td>0.35 ± 0.09</td>
<td>n.s.</td>
</tr>
<tr>
<td>Met-enkephalin + haloperidol</td>
<td>0.25 ± 0.04</td>
<td>n.s.</td>
</tr>
<tr>
<td>Met-enkephalin + naloxone</td>
<td>0.33 ± 0.08</td>
<td>n.s.</td>
</tr>
<tr>
<td>Met-enkephalin + Peptide-D</td>
<td>0.34 ± 0.06</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

n.s. = not significant (\(p > 0.05\)).
addition to Met-enkephalin requires administration of a low/moderate corticosteroid dose, may enable tapping of the corticosteroids. In the context of experimentally defined 5 : 1 ratio of Met-enkephalin : α-MSH, the starting dose for humans could be estimated at 5–10 mg Met-enkephalin + 1–2 mg α-MSH daily (5 times weekly for 4 weeks, then gradually reduced every four weeks to once weekly). This is analogous to the extrapolation of animal (rat) Met-enkephalin dose of other cytoprotective experimental models<sup>5</sup> to effective human doses in clinical practice.<sup>4,18</sup>

Met-enkephalin and α-MSH protect gastric mucosa in a different way than non-steroidal antiinflammatory drugs (NSAID). NSAID have a negative effect on gastric mucosal integrity<sup>1–3,16,17</sup> because of non-selective inhibition of cyclooxigenase I. Even selective cyclooxigenase II inhibitors are not side-effect free.<sup>3,16,17</sup> Our preliminary investigation indicates that this is not the case of the Met-enkephalin and α-MSH combination. Effects of both peptides include analgesia, antipiretic and antioxidant action,<sup>7,18</sup> which opens a possibility to achieve pharmacologic effects of both steroidal and non-steroidal antiinflammatory drugs, without most of their side-effects.

Met-enkephalin also exhibits synergistic effects on immunomodulating thymus peptide fractions present in thymus glands of young calves, since parallel administration of Thymus Peptide C® in ethanol induced gastric

![Figure 1. Neutralisation of Met-enkephalin (Peptid-M, LUPEX<sup>®</sup>) induced Straub tail reaction by Peptide-D is comparable to naloxone effects.](image-url)
lesions model (Table III) showed significant enhancement of the cytoprotection. This effect was somewhat less pronounced for JAN 50\(^{®}\) immunoregulating and correcting thymus peptide fraction of very young calves. Since Met-enkephalin is also found in larger quantities in fetal thymus, the synergistic action of both peptides with respect to the well known Met-enkephalin immunomodulatory actions\(^4–6,18\) has a potential therapeutic relevance.

According to our experimental results in the rat model, the estimated human daily dose for Met-enkephalin + Thymus Peptide C\(^{®}\) combination would be 5–10 mg of Met-enkephalin + 25–50 mg Thymus Peptide C\(^{®}\). For Met-enkephalin + JAN 50\(^{®}\), the starting daily dose would be 5–10 mg of Met-enkephalin + 50–100 mg JAN 50\(^{®}\). Our experimental result is in agreement with recent clinical pilot trials in which Thymus Peptide C\(^{®}\) or JAN 50\(^{®}\) were combined to Met-enkephalin and administered according to the standard Met-enkephalin protocols (5 times weekly for 4 weeks, then gradually reduced every four weeks to once weekly; M. Ć. Pešić, personal communication, Štambuk et al.\(^{18}\)).

\(^1\)H and \(^13\)C NMR study of haloperidol, Met-enkaphalin and their mixtures showed, through changes of chemical shifts and amid protons and carbonyl carbons, molecular interaction of both substances, which seems to be responsible for the antagonisation of cytoprotective Met-enkephalin effects. In this context, our data suggest that parallel administration of Met-enkephalin and haloperidol in human studies may be contraindicated, i.e. it should be avoided prior to further comparative studies in mammals.

Interaction of Met-enkephalin with the dopamine system may be influenced by molecular reactions of the peptide with dopamine antagonists (e.g. haloperidol). Dopamine 2 receptor fragments (D2DR_HUMAN D, aa 298–300, 403–405) correspond to the first 3 amino acids of the Met-enkephalin complementary antagonist denoted Peptide-D.\(^6\) Peptide-D residues IPP bind Met-enkaphalin residues YGG, and Peptide-D was shown to neutralise Met-enkephalin effects in several models,\(^6\) including the Straub tail reaction presented in Figure 1 and Table VI. Those effects were shown to be independent of the opioid system. Consequently, the neutralisation of Met-enkephalin induced Straub tail response by means of Peptide-D indicates the presence of new non-opioid (naloxone independent) receptor system in the region containing Peptide-D sequence (calpastatin 201–205 aa).

The results of our study indicate that the Met-enkephalin molecule, in addition to different synergistic effects with ACTH system and thymus peptides on cytoprotection during inflammation, also exhibits complex relationships with several receptor classes at the molecular level. The pharmacological importance of these relationships remains to be determined.
Acknowledgement. – The support by the Ministry of Science and Technology of the Republic of Croatia is highly appreciated (research grants No. 00981108 and No. 00980802).

REFERENCES

Zaštitni učinci Met-enkefalina pri oštećenjima želučane sluznice izazvanima alkoholom

Paško Konjevoda, Nikola Štambuk, Dražen Vikić-Točić, Alenka Boban-Blagaić, Smiljka Vikić-Točić, Vladimir Mrljak, Josip Pavan, Pero Ramadan i Zdenko Bidin