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Protective Effects of Met-enkephalin on Alcohol Induced Gastric Lesions

Paško Konjevoda,[a] *Nikola Štambuk,*[b,*] *Dražen Vikić-Topić,*[b] *Alenka Boban-Blagaić,*[c] *Smiljka Vikić-Topić,*[d,†] *Vladimir Mrljak,*[e] *Josip Pavan,*[f] *Pero Ramadan,*[e] *and Zdenko Biđin*[e]

[a] *Department of Pharmacology, Zagreb University School of Medicine, Šalata 2, 10000 Zagreb, Croatia*

[b] *Rudjer Bošković Institute, Bijenička 54, 10001 Zagreb, Croatia*

[c] *MOLO dd, Maksimirska 21, 10000 Zagreb, Croatia*

[d,†] *Pliva Research Institute, Baruna Filipovića 25, 10000 Zagreb, Croatia*

[e] *Faculty of Veterinary Medicine, University of Zagreb, Heinzelova 55, 10000 Zagreb, Croatia*

[f] *Clinical Hospital Dubrava, Avenija Gojka Šuška bb, 10000 Zagreb, Croatia*

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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-

* Author to whom correspondence should be addressed. (E-mail: stambuk@rudjer.irb.hr)

† M. Sc. Vikić-Topić coauthored this paper in her private capacity.

nificant and dose dependent Straub tail response *versus* control in mice. Haloperidol, naloxone and Peptide-D antagonised its effects. NMR spectrospic 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[1] because of insufficient efficacy and many side effects of antiinflammatory drugs.[1] The most frequent side effects of antiinflammatory drugs are gastric lesions.[2,3]

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.[4–8]

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.[6]

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.[9]

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 mm^2, 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.[9] The Straub tail reaction is believed to be produced by the contraction of the sacrococcygeus dorsalis muscle. It is the prime model for opiod effects assessment *in vivo*. The Straub tail reaction is recorded every 5 minutes for 50 minutes after 0.9% NaCl or Met-enkephalin application.[9] 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[9] as follows:

Score	Degrees of erection
0.5	< 45
1	45
1.5	45–90
2	90
2.5	90–180
3	180

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. Haloperodol (Sigma, USA), > 99% purity.
7. 0.9% NaCl
8. α-MSH (Commonwealth Biotechnologies, USA; Lot No. 010030399), >97% purity.

NMR Measurements

The ^1H 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 ^1H 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, D3, 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, α-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, α-MSH and combination Met-enkephalin + α-MSH have a protective effect on gastric mucosa (Table III). The best protective effect was obtained by the combination of Met-enkephalin and α-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 iniectabilia) was administered to Wistar rats.[6] 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, $X \pm$ 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-

TABLE I

Influence of Met-enkephalin (Peptid-M, LUPEX®), α-MSH and two thymus peptides (Thymus Peptide C® and JAN 50®) on 96% ethanol induced gastricmucosal lesions – experimental design

Substance	Schedule		
	–1 h	0 h	+ 1 h
0.9% NaCl (control)	application	+ ethanol 1mL/rat	sacrifice
Met-enkephalin (10 mg/kg ip)	application	+ ethanol 1mL/rat	sacrifice
α-MSH (3.3 mg/kg ip)	application	+ ethanol 1mL/rat	sacrifice
Thymus Peptide C® (50 mg/kg ip)	application	+ ethanol 1mL/rat	sacrifice
JAN 50® (100 mg/kg ip)	application	+ ethanol 1mL/rat	sacrifice
Met-enkephalin + α-MSH	application	+ ethanol 1mL/rat	sacrifice
Met-enkephalin + Thymus Peptide C®	application	+ ethanol 1mL/rat	sacrifice
Met-enkephalin + JAN 50®	application	+ ethanol 1mL/rat	sacrifice

TABLE II

Influence of Met-enkephalin (Peptid-M, LUPEX®) and haloperidol on 96% ethanol induced gastric mucosal lesions – experimental design

Substance	Schedule		
	–1 h	0 h	+1 h
0.9% NaCl (control)	application	+ ethanol 1mL/rat	sacrifice
haloperidol (10 mg/kg ip)	application	+ ethanol 1mL/rat	sacrifice
Met-enkephalin (10 mg/kg ip)	application	+ ethanol 1mL/rat	sacrifice
Met-enkephalin + haloperidol	application	+ ethanol 1mL/rat	sacrifice

tically significant Straub tail inducing effect *versus* control. Haloperidol, naloxone and Peptide-D antagonised its effect.

DISCUSSION

Ethanol ingestion results in gastritis characterised[10] by mucosal edema, subepithelial haemorrhage, cellular exfoliation, and an inflammatory cell infiltrate. The initial event in the pathogenesis of mucosal ulcerations is microvascular dysfunction.[10,11] Exposure of the mucosa to ethanol (50–100%)

TABLE III

Results for substances from Table I

Substance	Results			
	Area of lesions (mm^2)			Statistial signifiance
	median	min	max	*vs.* conrol
0.9% NaCl (control)	360	294	473	
Met-enkephalin	225	172	298	$p < 0.05$
α-MSH	18	0	24	$p < 0.001$
Thymus Peptide C®	25	5	29	$p < 0.01$
JAN 50®	28	8	32	$p < 0.01$
Met-enkephalin + α-MSH	0	0	4	$p < 0.001$
Met-enkephalin + Thymus Peptide C®	17	0	22	$p < 0.01$
Met-enkephalin + JAN 50®	31	0	46	$p < 0.01$

TABLE IV

Results for substances from Table II

Substance	Results			
	Area of lesions (mm^2)			Statistical significance
	median	min	max	*vs.* control
0.9% NaCl (control)	385	373	432	
Met-enkephalin	242	191	305	$p < 0.05$
haloperidol	430	342	460	$p < 0.05$
Met-enkephalin + haloperidol	449	420	497	$p < 0.05$

results in arteriolar vasodilatation, vascular congestion or stasis, haemoconcentration, albumin leakage, and capillary damage.[10] This is associated with significant reductions in mucosal blood flow,[10,12] and the extent of mucosal injury is correlated with the magnitude of the blood flow reduction.[12] Microvascular dysfunction is reminiscent of the vascular phase of acute inflammation.[10–12] Also, neutrophils have been implicated in the mucosal injury associated with ethanol-induced gastritis.[13,14] In this context, the lesions resemble some human disease lesions, *e.g.* in inflammatory bowel disease, gastritis, *etc.*

Effects of Met-enkephalin and α-MSH are beneficial in the cytoprotective model investigated, especially their combination, which exhibits strong

TABLE V

Straub tail reaction – substances and experimental design

Substance	Schedule		
	–15 min	0 h	observation – every 5 minutes for 50 minutes
0.9% NaCl (control)	0.9% NaCl	0.9% NaCl	observation
Met-enkephalin (10 mg/kg ip)	0.9% NaCl	Met-enkephalin	observation
haloperidol (10 mg/kg ip)	haloperidol	0.9% NaCl	observation
naloxone (10 mg/kg ip)	naloxone	0.9% NaCl	observation
Peptide-D (5 mg/kg ip)	Peptide-D	0.9% NaCl	observation
Met-enkephalin + haloperidol	haloperidol	Met-enkephalin	observation
Met-enkephalin + naloxone	naloxone	Met-enkephalin	observation
Met-enkephalin + Peptide-D	Peptide-D	Met-enkephalin	observation

TABLE VI

Results for substances from Table V

Substance	Results	
	Straub tail reaction (group mean ± SEM)	Statistical signifiance *vs.* control
0.9% NaCl (control)	0.33 ± 0.05	
Met-enkephalin	0.57 ± 0.07	$p < 0.05$
haloperidol	0.28 ± 0.04	n.s.
naloxone	0.35 ± 0.05	n.s.
Peptide-D	0.35 ± 0.09	n.s.
Met-enkephalin + haloperidol	0.25 ± 0.04	n.s.
Met-enkephalin + naloxone	0.33 ± 0.08	n.s.
Met-enkephalin + Peptide-D	0.34 ± 0.06	n.s.

n.s. = not significant ($p > 0.05$).

synergistic effects and almost completely protects the gastrointestinal mucosa. This result has potential clinical application because Met-enkephalin and α-MSH are products of the same precursor molecules.[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

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[5] to effective human doses in clinical practice.[4,18]

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[1–3,16,17] because of non-selective inhibition of cyclooxigenase I. Even selective cyclooxigenase II inhibitors are not side-effect free.[3,16,17] 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,[7,18] which opens a possibility to achieve pharmacologic effects od 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

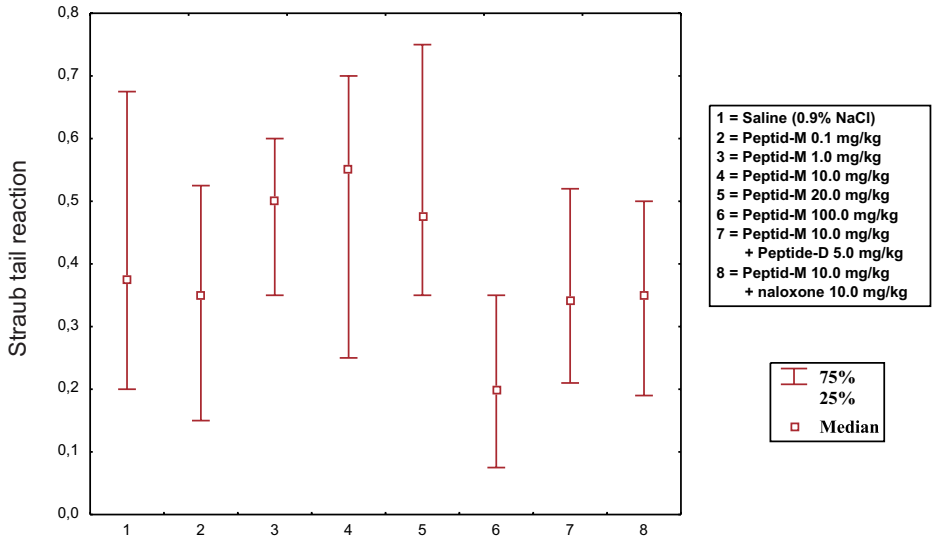

Figure 1. Neutralisation of Met-enkephalin (Peptid-M, LUPEX®) induced Straub tail reaction by Peptide-D is comparable to naloxone effects.

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. Č. 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 additon 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.

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SAŽETAK

Zaštitni učinci Met-enkefalina pri oštećenjima želučane sluznice izazvanima alkoholom

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

Etanolom izazvana oštećenja želučane sluznice koristan su eksperimentalni model za procjenu citoprotektivnih efekata različitih tvari. Spomenuti je model korišten za procjenu citoprotektivnog efekta dva neuropeptida, Met-enkefalina (Peptid-M, LUPEX®) i melanocitnog stimulirajućeg hormona α (α-MSH). Oba neuropeptida izazvala su znatne i sinergističke citoprotektivne efekte. Najbolja terapijska efikasnost postignuta je pri omjeru doza Met-enkefalina i α-MSH 3–5:1. Značajna citoprotekcija u etanolskom modelu opažena je i za dva timusna imunomodulirajuća peptida, Timusni Peptid C® i JAN 50®. Timusni Peptid C® pokazao je izrazitiji sinergistički citoprotektivni efekt s Met-enkefalinom nego JAN 50®. Uz citoprotekciju na etanolskom modelu štakora, Met-enkefalin je u miša izazvao pri visokim dozama statistički značajan Straubov refleks repa. Haloperidol, naloxon i Peptid-D antagonizirali su spomenuti refleks. NMR spektri Met-enkefalina, haloperidola i njihovih smjesa ukazali su na molekulsku interakciju hormona i lijeka, a gotovo identični nalazi neutralizacije Straubova refleksa naloxonom i Peptidom-D potvrđuju da uz opioidne (naloxon-ovisne) receptore postoji i interakcija Met-enkefalina s receptorskim sistemom koji sadrži sekvenciju Peptid-D (kalpastatin 201–205 aa).