Acta Veterinaria (Beograd), Vol. 52. No. 2-3, 69-78, 2002.

UDK 619:612.017.1

THE EFFECT OF OPIOID PEPTIDES ON SUPEROXIDE ANION PRODUCTION IN RAT PERITONEAL MACROPHAGES STIMULATED WITH ZYMOSAN: INVOLVEMENT OF μ, δ AND κ OPIOID RECEPTORS

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(Received 12. March 2002)

It is well documented that endogenous opioid peptides modulate the activity of immune and inflammatory cells. Opioid peptides exert biological effects through at least three opioid receptor types, known as μ, δ and κ. In the present study we have investigated which types of opioid receptors are involved in the opioid peptide-induced modulation of superoxide anion release from macrophages. Rat peritoneal macrophages were stimulated with zymosan, and treated in vitro with opioid peptides and/or antagonists specific for different opioid receptor types. The results showed that the μ opioid receptor agonist, beta-endorphin, and the κ opioid receptor agonist, dynorphin A, decreased superoxide anion production. Conversely, methionine-enkephalin (Met-Enk) and leucine-enkephalin, that have a preference for δ opioid receptors, did not affect superoxide anion release. Furthermore, we have tested the effect of Met-Enk on superoxide anion release from macrophages under blockade of all receptors with specific antagonists except for one receptor subtype. Accordingly, it was found that the Met-Enk induced increase and decrease of superoxide anion production were mediated through δ1,2 and μκ opioid receptors, respectively. It can be concluded that opposing activity of μ and κ receptors vs. δ receptors together with the affinity for a certain receptor type determines the effect of a particular endogenous opioid peptide on superoxide anion release from rat peritoneal macrophages.

Key words: macrophages, methionine-enkephalin, opioid receptors μ, δ and κ, superoxide anion

INTRODUCTION

Endogenous opioid peptides comprise three peptide families: enkephalins, endorphins and dynorphins. Together with their acknowledged effects on the central nervous system opioid peptides modulate immune (Radulović and

Janković, 1994; Radulović *et al.*, 1995a; Radulović *et al.*, 1996) and inflammatory reactions (Janković and Marić, 1994; Vujić-Redžić *et al.*, 2000). Macrophages constitute the first line of immunological defense of the organism against pathogenic agents and participate in both non-specific and specific immunity. Furthermore, macrophages are involved in the development and resolution of inflammatory reactions. Opioid peptides were shown to modulate several activities of macrophages, such as antibody-mediated cytotoxicity (Foris *et al.*, 1984), IL-1 (Yung and Li, 1989) and IL-6 secretion (House *et al.*, 1996) and NO production (Kowalski, 1998). These effects of opioid peptides were naloxone reversible, suggesting that opioid receptors were involved in the regulation of macrophage functions.

Phagocytosis is one of the early events in the macrophage effector function that comprises a cascade of activation steps resulting in the respiratory burst and release of reactive oxygen intermediates. It has been reported that opioid pentapeptide methionine-enkephalin (Met-Enk) dose-dependently modulated phagocytosis and superoxide anion production *in vitro* (Foris *et al.*, 1984; Fischer and Falke, 1987; Casselas *et al.*, 1991). Likewise, we have previously demonstrated that Met-Enk induced a dose - and strain dependant increase or decrease of hydrogen peroxide release from rat peritoneal macrophages stimulated with phorbol myristate acetate (Radulović *et al.*, 1995b). In addition, both effects were antagonized with naloxone and a highly selective δ opioid receptor antagonist (Radulović *et al.*, 1995b), underlying the role of specific opioid receptor type expressed on the macrophages.

Recent advances in the molecular biology of opioid receptors led to the identification of three different receptor types δ, μ and κ, thereby supporting the results of earlier pharmacological studies which postulated their existence. However, the molecular biology data generated so far did not confirm the presence of various subtypes of the three receptor types that were recognized pharmacologically (i.e. $\delta_{1,2}$, $\mu_{1,2}$, $\kappa_{1,2,3}$). Moreover, all three types of opioid receptors were detected on macrophages (Carr *et al.*, 1989; Sedqui, 1995), and it was shown that opioid peptides might interact with more than one type of opioid receptor (Corbett *et al.*, 1991; Radulović et al., 1996; Vujić-Redžić *et al.*, 2000). Numerous experiments *in vitro* utilizing direct stimulation of cells with opioid peptides, opioids and opioid receptor agonists reveal that μ and κ opioid receptors mainly mediate suppressive effects (Taub *et al.*, 1991; Bian *et al.*, 1995; Radulović *et al.*, 1995a) on immune responses while δ receptors mediate potentiation of immune function (House *et al.*, 1996; Sharp *et al.*, 1996).

The objective of the present study was to investigate the involvement of the specific opioid receptor type/subtype in the opioid-induced changes in phagocytic function of macrophages. For that purpose, rat peritoneal macrophages stimulated with zymosan, were tested for nitro blue tetrazolium reduction, as a measure of superoxide anion release, after *in vitro* treatment with different opioid peptides. The following opioid peptides were used: Met-Enk and leucine-enkephalin (Leu-Enk, δ opioid receptor agonists), beta-endorphin (beta-End, a mainly μ opioid receptor agonist) and dynorphin A (Dyn A, a κ opioid receptor agonist). In the antagonistic study, specific antagonists of different opioid receptor types/subtypes were employed: naltrindole (δ), benzylidenenaltrexone (δ_1), naltriben (δ_2), β-funaltrexamine (μ) and

nor-binaltorphimine (κ). Since several studies demonstrated that opioid receptor antagonists per se might influence the functions of immune and inflammatory cells (Radulović *et al.*, 1995b; Radulović *et al.*, 1996), their effects on superoxide anion release from macrophages, with or without Met-Enk, were also tested.

MATERIAL AND METHODS

Animals

Male Wistar rats (60 ± 3 days of age; 200-210 g body weight) were obtained from the Breeding Colony of the Medical Military Academy, Belgrade. Animals were housed in Plexiglas cages with free access to food pellets and tap water.

Drugs

The opioid pentapeptides Met-Enk (Tyr-Gly-Gly-Phe-Met) and Leu-Enk (Tyr-Gly-Gly-Phe-Leu) were obtained from Serva (Heidelberg, Germany). Beta-endorphin (beta-End) and Dynorphin A 1-13 (Dyn-A) were purchased from Sigma (St. Louis, USA). The following opioid receptor antagonists were obtained from Tocris (United Kingdom): δ receptor antagonist Naltrindole hydrochloride (NTI); δ_1 receptor antagonist 7-Benzylidenenaltrexone maleate (BNTX); δ_2 receptor antagonist Naltriben mesylate (NTB); μ receptor antagonist β-Funaltrexamine hydrochloride (beta-FNA); κ_1 receptor antagonist nor-Binaltorphimine dihydrochloride (nor-BNI). All drugs were solubilized at a concentration of 10^{-2} M, aliquoted and stored at –20°C.

Macrophage harvest

Rats received an intraperitoneal injection of Thioglycolate medium (15 ml). Macrophages were obtained one week later by washing the peritoneal cavity with 10 ml of minimal essential medium (MEM) without phenol red. Individual cell suspensions (n = 8-12) were washed 3 times, adjusted to 2.5×10^6 cells/ml and plated 100 μl/well in 96-well flat-bottomed tissue culture plates (Nunc). Plates were incubated for 2h at 37 °C in 95% air-5% CO_2. Nonadherent cells were removed by washing the plates twice with warm phenol red-free MEM.

Superoxide anion production

Superoxide anion release was determined as previously described (Pick *et al.*, 1981). Macrophages were stimulated with zymosan (125 μg/ml) in phenol red-free MEM with or without opioid peptides and/or antagonists of opioid receptors (10^{-12} - 10^{-6} M) in the presence of nitro blue tetrazolium (NBT, 0.5 mg/ml phenol red-free MEM) for 30 min at 37 °C in 95% air-5% CO_2. The supernatants were discarded and cells were fixed with methanol. Optical densities (OD) were determined at 540 nm using cells treated with iodacetamide 5 mM/phenol red-free MEM and NBT (0.5 mg/ml phenol red-free MEM) as a blank. Results are expressed as stimulatory index ($OD_{zymosan}$ / OD_{MEM}).

Statistical analysis

Data were analyzed by one-factor ANOVA for repeated measurements (factor: concentration or treatment). Differences are regarded as statistically significant if $p < 0.05$. The statistical was analysis performed using the StatView II package.

RESULTS AND DISCUSSION

Met-Enk and Leu-Enk did not influence superoxide anion production in rat peritoneal macrophages stimulated with zymosan, since there were no differences between the stimulatory index of cells treated with each peptide and control cells (Fig. 1). In contrast, both beta-End and Dyn-A decreased the stimulatory index of zymosan-stimulated rat peritoneal macrophages at concentrations of 10^{-6} M (Fig. 2). Since beta-End and Dyn-A exert biological effects through μ and κ opioid receptors, respectively, it is most likely that these two receptor types mediated the suppressive effect of opioid peptides on superoxide anion production in rat macrophages. The release of superoxide anion from zymosan-stimulated macrophages is a consequence of phagocytosis of zymosan particles and subsequent activation of a respiratory burst in the cells. Our results are in accordance with the reported inhibitory effects of both μ and κ opioid receptor agonists on phagocytosis of Candida albicans (Szabo *et al.*, 1993) and sheep red blood cells (Casellas *et al.*, 1991) by peritoneal macrophages in mice. With respect to δ opioid receptor agonist Met-Enk, both a decrease (Foris *et al.*, 1984; Foris *et al.*, 1986; Casellas *et al.*, 1991) and an increase (Foris *et al.*, 1986; Petty and Berg, 1988) of phagosytosis were reported. However, the most consistent finding in studies with δ opioids was potentiation of various macrophage functions, such as hydrogen peroxide release (Radulović *et al.*, 1995b), IL-6 (House *et al.*, 1996) and IL-1 (Yung and Li, 1989) secretion and NO production (Kowalski, 1998).

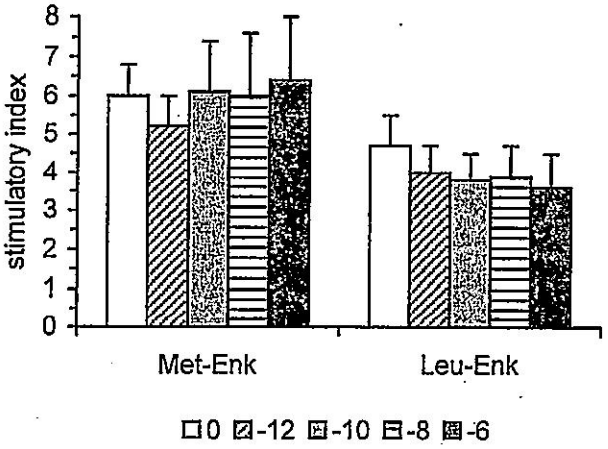

Figure 1. No effect of δ opioid receptor agonists Met-Enk and Leu-Enk (concentrations 10^{-12} M -10^{-6} M) on superoxide anion production in rat peritoneal macrophages stimulated with zymosan.

We have reported previously that central effects of δ opioid-preferring Met-Enk and Leu-Enk on immune system functions could be also mediated through μ and κ opioid receptors (Radulović *et al.*, 1996; Dimitrijević *et al.*, 2000). Accordingly, we have investigated the effect of Met-Enk on superoxide anion release from macrophages under selective blockade of different opioid receptor

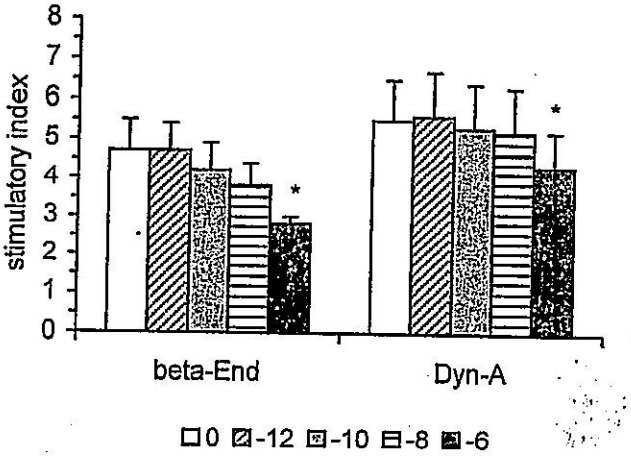

Figure 2. The effect of μ (beta-End) and κ (Dyn-A) opioid receptor agonists (concentrations 10^{-12} M -10^{-6} M) on superoxide anion production in rat peritoneal macrophages stimulated with zymosan. Statistically significant differences: * $p<0.01$ vs. control.

types, enabling interacion of Met-Enk with a single receptor type/subtype. The possible influence of specific opioid receptor antagonists on superoxide anion release was also tested.

The effect of δ opioid receptor antagonists on superoxide anion production in rat peritoneal macrophages stimulated with zymosan is shown on Fig. 3. NTI, an antagonist of both δ_1 and δ_2 opioid receptors, tended to increase the stimulatory index of peritoneal macrophages, but this change was not statistically significant. BNTX, an antagonist of δ_1 opioid receptors, had no effect, while NTB, an antagonist of δ_2 opioid receptors, decreased the stimulatory index at

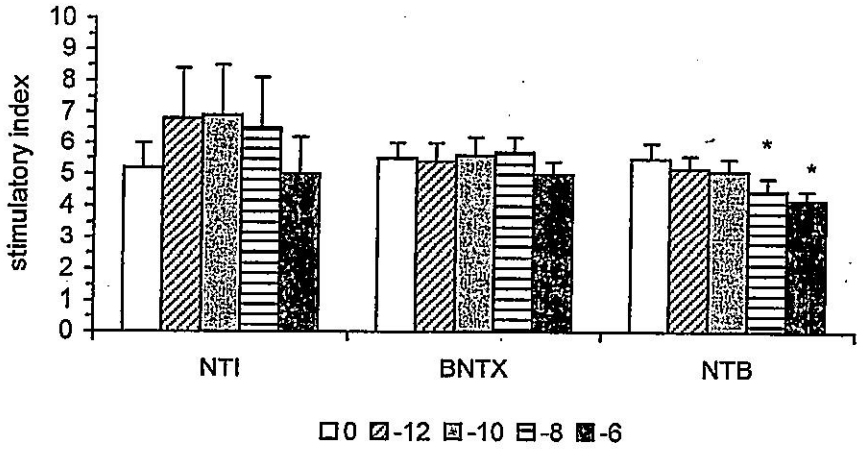

Figure 3. The effect of antagonists of δ (NTI), δ_1 (BNTX), and δ_2 (NTB) opioid receptors (concentrations 10^{-12} M -10^{-6} M) on superoxide anion production in rat peritoneal macrophages stimulated with zymosan. Statistically significant differences: * $p< 0.01$, vs. control.

concentrations 10^{-8} M and 10^{-6} M. Beta-FNA (an antagonist of μ opioid receptors) did not affect superoxide anion production (Fig. 4). However, nor-BNI (antagonist of κ opioid receptors) significantly decreased the stimulatory index of peritoneal macrophages stimulated with zymosan in all concentrations tested (10^{-12} M - 10^{-6} M, Fig. 4).

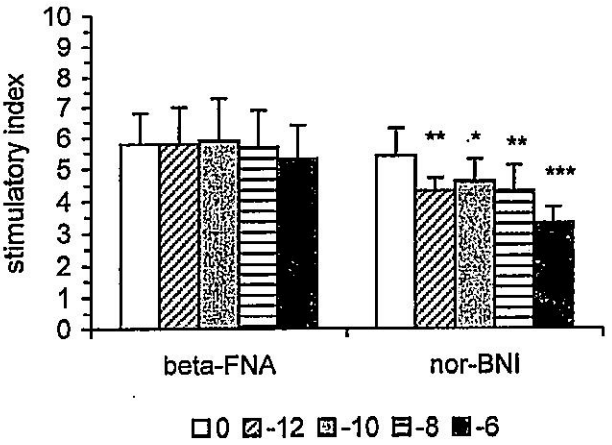

Figure 4. The effect of antagonists of μ (beta-FNA) and κ (nor-BNI) opioid receptors (concentrations 10^{-12} M -10^{-6} M) on superoxide anion production in rat peritoneal macrophages stimulated with zymosan. Statistically significant differences: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.0001$ vs. control.

When rat peritoneal macrophages were simultaneously treated with 10^{-12} M of Met-Enk and a combination of antagonists of δ_2, μ and κ opioid receptors (NTB, beta-FNA, nor-BNI), or 10^{-12} M of Met-Enk and antagonists of δ_1, μ and κ opioid receptors (BNTX, beta-FNA, nor-BNI), an increase in stimulatory index was observed (Fig. 5). These results suggested that Met-Enk could potentiate superoxide anion release through activation of δ_1 or δ_2 opioid receptors. However, concomitant treatment with Met-Enk (10^{-8} M) and a combination of antagonists of d and k opioid receptors (NTI and nor-BNI), or Met-Enk (10^{-10} M - 10^{-8} M) and a combination of antagonists of δ and μ opioid receptors (NTI and beta-FNA), significantly decreased the stimulatory index in zymosan-stimulated rat peritoneal macrophages (Fig. 6). These findings confirmed that activation of μ or κ opioid receptors result in suppression of superoxide anion release from macrophages.

Higher doses of NTB, a δ opioid receptor antagonist, displayed inverse-agonist activity when applied alone, but behaved as a true opioid antagonist in the presence of the agonist Met-Enk. It is suggested that the presence of the agonist induced conformational changes in the receptor molecule, making the allosteric site available for reaction with the antagonist. When the allosteric site interacts with an antagonist, it prevents the agonist from inducing an effect (Archer and Michne, 1976). Apart from that, the κ opioid receptor antagonist, nor-BNI, suppressed superoxide anion release, acting as a partial opioid agonist alone, which corroborated with our previous results

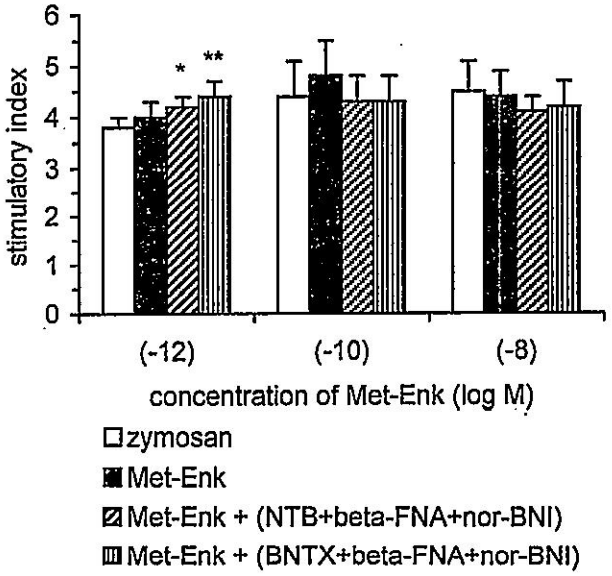

Figure 5. The effect of Met-Enk (concentrations 10^{-12} M -10^{-6} M) applied simultaneously with a combination of antagonists of δ_2, μ and κ opioid receptors (NTB, beta-FNA and nor-BNI, concentration 10^{-8} M), or antagonists of δ_1, μ and κ opioid receptors (BNTX, beta-FNA and nor-BNI, concentration 10^{-8} M) on superoxide anion production in rat peritoneal macrophages stimulated with zymosan. Statistically significant differences: * $p < 0.05$, and ** $p < 0.01$ vs. control.

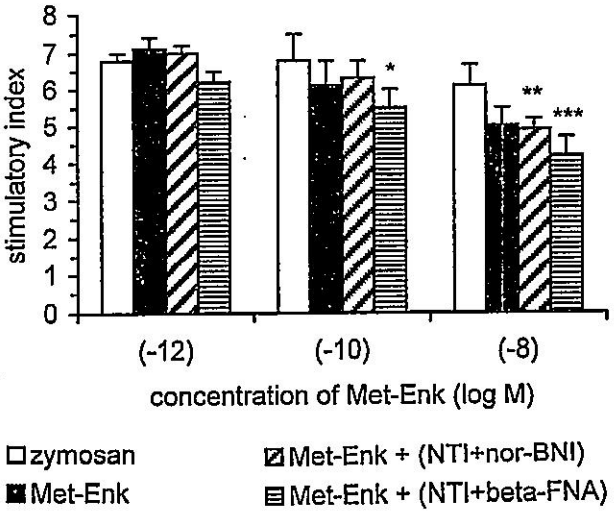

Figure 6. The effect of Met-Enk (concentrations 10^{-12} M -10^{-6} M) applied simultaneously with a combination of antagonists of δ and κ opioid receptors (NTI and nor-BNI, concentration 10^{-8} M), or a combination of antagonists of d and m opioid receptors (NTI and beta-FNA, concentration 10^{-8} M) on superoxide anion production in rat peritoneal macrophages stimulated with zymosan. Statistically significant differences: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.0001$ vs. control.

considering the central effect of nor-BNI on the humoral immune response in the rat (Dimitrijević *et al.*, 2000).

CONCLUSION

Our results showed that opioid peptides could modulate superoxide anion release from zymosan-stimulated peritoneal macrophages in the rat. Potentiation and suppression of superoxide anion release were mediated through $\delta_1\delta_2$ and $\mu\kappa$ opioid receptors, respectively. Since endogenous opioid peptides originating from peripheral nerves and/or immune cells were shown at the site of inflammation, it was suggested that they could affect inflammatory reactions by modulating superoxide anion release from the macrophages. It was concluded that endogenous opioid peptides, depending on their affinity for certain opioid receptor type/subtypes and activation of μ and κ receptors vs. d receptors, contribute to the complex regulation of inflammatory reactions.

Acknowledgments

This work was supported by the Serbian Ministry of Science, Technology and Development, Belgrade, Yugoslavia, Grant No 1239. We thank Zuzana Tomaš for technical assistance.

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UTICAJ OPIOIDNIH PEPTIDA NA PRODUKCIJU SUPEROKSID ANJONA U PERITONEALNIM MAKROFAGAMA PACOVA STIMULISANIM ZIMOZANOM: ULOGA μ, δ I κ OPIOIDNIH RECEPTORA

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SADRŽAJ

Poznato je da endogeni opioidni peptidi modulišu aktivnost imunskih i inflamatornih ćelija. Opioidni peptidi ostvaruju biološke efekte preko tri tipa receptora, μ, δ i κ. U ovom radu ispitivali smo koji tipovi opioidnih receptora učestvuju u opioidnoj modulaciji oslobađanja superoksid anjona iz peritonealnih makrofaga. Peritonealne makrofage pacova su stimulisane zimozanom, i tretirane in vitro sa opioidnim peptidima i / ili specifičnim antagonistima različitih tipova opioidnih receptora. Rezultati su pokazali da beta-endorphin, agonist μ opiodnih

receptora, i dynorphin A, agonist κ opiodnih receptora, smanjuju produkciju superoksid anjona. S druge strane, metionin-enkefalin (Met-Enk) i leucin-enkefalin, koji se prevashodno vezuju za δ opioidne receptore, nisu uticali na oslobađanje superoksid anjona. Pored toga, ispitivali smo uticaj Met-Enk na oslobađanje superoksid anjona iz makrofaga u uslovima blokade svih receptora sa specifičnim antagonistima izuzev jednog tipa/podtipa opioidnog receptora. Shodno tome pokazali smo da Met-Enk povećava oslobađanje superoksid anjona preko $\delta_{1,2}$ a smanjuje oslobađanje superoksid anjona preko μκ opioidnih receptora. Može se zaključiti da je uticaj endogenih opioidnih peptida na oslobađanje superoksid anjona iz peritonealnih makrofaga pacova određen suprotnom aktivnošću μ i κ receptora u odnosu na δ receptore, kao i različitim afinitetom peptida za pojedine tipove opioidnih receptora.