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ORIGINAL ARTICLE

Oxidative and nitrative modifications of enkephalins by human neutrophils: effect of nitroenkephalin on leukocyte functional responses

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Abstract Neutrophils play a major role in acute inflammation by generating reactive oxygen/nitrogen species. Opioid peptides, including enkephalins, are present at inflammation sites. Neutrophils contribute to protect against inflammatory pain by releasing opioid peptides. In this investigation, the ability of human polymorphonuclear cells to induce oxidative and nitrative modifications of Leu-enkephalin has been investigated in vitro. Activated human neutrophils mediate the oxidation of Leu-enkephalin resulting in the production of dienkephalin. In the presence of nitrite at concentrations observed during inflammatory and infectious process (10–50 μM), nitroenkephalin, a nitrated derivative of Leu-enkephalin, is additionally formed. The yield of nitroenkephalin increases with nitrite concentration and is significantly inhibited by the addition of catalase or 4-aminobenzoic acid hydrazide (ABAH), a specific inhibitor of peroxidases. These results suggest that neutrophils induce nitration of Leu-enkephalin by a mechanism that is dependent on myeloperoxidase activity and hydrogen peroxide. Oxidative/nitrative modifications of Leu-enkephalin have been also evidenced when cells were treated with the NO-donor molecule, DEANO. The nitrated enkephalin has been examined for its effect on leukocyte functional responses. The data reveal that nitroenkephalin at micromolar concentrations inhibits superoxide anion generation and degranulation of azurophilic granules of human polymorphonuclear cells. Moreover, nitroenkephalin inhibits spontaneous apoptosis of neutrophils, as evaluated by measuring caspase-3 activity. Collectively, our data indicate that the nitrated enkephalin attenuates neutrophil activation and promotes the short-term survival of these cells, suggesting a possible role of the nitrocompound in the efficiency and resolution of inflammatory processes.

Keywords Enkephalin · Nitroenkephalin · Neutrophils · Protein nitration · Inflammation · Apoptosis

Abbreviations

ABAH	4-Aminobenzoic acid hydrazide
Ac-DEVD-AMC	Acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (caspase-3 substrate)
DEANO	Diethylamine NONOate
fMLP	*N*-Formyl-methionyl-leucyl-phenylalanine
HRP	Horseradish peroxidase
Leu-enkephalin	Leucine enkephalin
MeO-Suc-Ala-Ala-Pro-Val-AMC	*N*-Methoxysuccinyl-Ala-Ala-Pro-Val-7-amido-4-methylcoumarin (elastase substrate)
MPO	Myeloperoxidase
ODQ	1*H*-[1,2,4]Oxadiazole[4,3-a]quinoxalin-1-one
PMA	Phorbol 12-myristate 13-acetate
PMNs	Polymorphonuclear cells
SOD	Superoxide dismutase

Introduction

Enkephalins belong to a class of bioactive peptides known for their endogenous opiate agonist activity (Frederickson

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1977; Hughes et al. 1975). Mainly enkephalins, endorphins and dynorphins represent the endogenous opioid peptides. Endorphins and enkephalins share a common amino-terminal sequence and exhibit a tyrosine residue at the amino terminus, which is essential for their biological activity (Terenius et al. 1976). The aromatic amino acid residues are especially susceptible to oxidation by various forms of reactive oxygen species (ROS). In particular, enkephalins and opioid peptides react easily with ROS giving rise to oxidized products (Fontana et al. 2001; Nagy et al. 2009; Rosei 2001).

Opioid peptides, including enkephalins, are present at inflammation sites (Padrós et al. 1989; Sibinga and Goldstein 1988; Vindrola et al. 1990). During inflammatory process, the migration of opioid-containing leukocytes to inflamed tissues is induced. The secretion of opioid peptides by polymorphonuclear cells (PMNs), monocytes and lymphocytes can relieve inflammatory pain by interacting with peripheral opioid receptors (Cabot 2001; Przewlocki et al. 1992; Rittner et al. 2001; Stein et al.1990). Therefore, inflammatory response can be associated with the release of high levels of opioid peptides. It has been reported that opioid peptides, such as methionine-enkephalin and beta-endorphin, are involved in neutrophil priming and that beta-endorphin also exerts immunosuppressive potency (McCain et al. 1982; Pasnik et al. 1999). Opioid peptides have been reported to bind to specific receptors on human phagocytic leukocytes and to modulate the activation of neutrophils (Diamant et al. 1989; Lopker et al. 1980; Menzebach et al. 2003).

Activated neutrophils produce a variety of powerful inflammatory mediators such as reactive oxygen species, enzymes and cytokines that play a key role in the host defenses against invading microorganisms, but can also be responsible for tissue damage. The reactive oxygen species are produced by neutrophils as a result of NADPH oxidase activation (reactions named "respiratory burst") (Hampton et al. 1998; Witko-Sarsat et al. 2000). NADPH oxidase can be activated by various stimuli such as chemotactic peptide, *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) or phorbol 12-myristate 13-acetate (PMA). Neutrophil function in vivo can be also regulated through NO-dependent mechanisms (Belenky et al. 1993; Fialkow et al. 2007; Moilanen et al. 1993). Nitric oxide (NO) modulates neutrophil pro-inflammatory responses and plays a protective role against priming and activation of these cells (Armstrong 2001; Clancy et al. 1992; Dal Secco et al. 2003; Grisham et al. 1999). However, when generated at elevated levels during inflammation by inducible NO synthase, NO is readily transformed into potent nitrating and oxidizing species, including peroxynitrite ($ONOO^-$), nitrogen dioxide radical ($^{\cdot}NO_2$), and nitrous acid (HONO) (Pryor and Squadrito 1995; Radi et al. 2001), which can modify both the structure and the function of numerous biomolecules (Eiserich et al. 1998a; Lim et al. 2002; Vadseth et al. 2004; Quijano et al. 2005; Souza et al. 2008). It has been observed that various reactive nitrogen species react in vitro with Leu-enkephalin. As a consequence of these reactions, the tyrosine amino-terminal residue of enkephalin is converted either to 3-nitrotyrosine producing nitroenkephalin or dityrosine with the production of an enkephalin dimer, the dienkephalin (Fontana et al. 2006). Up to now, no study has been published on nitrative biochemistry of enkephalins with neutrophil-generated reactive oxidants.

In the present investigation, we explored the ability of human PMNs to induce oxidative/nitrative modifications of Leu-enkephalin. Moreover, the nitrated enkephalin has been examined for its effect on leukocyte functional response. The data reveal that nitroenkephalin inhibits the respiratory burst and degranulation of azurophilic granules of human PMNs and their spontaneous apoptosis in vitro. These findings are of potential interest, inasmuch nitroenkephalin may act as modulator of tissue inflammatory response.

Materials and methods

Chemicals

Leucine-enkephalin (Leu-enkephalin), horseradish peroxidase (HRP), diethylamine NONOate sodium salt (DEANO), phorbol 12-myristate 13-acetate (PMA), *N*-formyl-Met-Leu-Phe (fMLP), *N*-methoxysuccinyl-Ala-Ala-Pro-Val-7-amido-4-methylcoumarin (MeO-Suc-Ala-Ala-Pro-Val-AMC, elastase substrate), acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC, caspase-3 substrate), porcine pancreas elastase, human recombinant caspase-3, naloxone and 4-aminobenzoic acid hydrazide (ABAH) were obtained from Sigma-Aldrich, Inc (St. Louis, MO, USA). 3-Nitrotyrosine, potassium nitrite, hydrogen peroxide, cytochrome *c* from horse heart and cytochalasin B were from Fluka Chemie GmbH (Buchs, CH). 1*H*-[1,2,4]oxadiazole [4,3-a]quinoxalin-1-one (ODQ, guanylate cyclase inhibitor) was from Alexis Co (Lausen, CH). All other chemicals were of analytical grade.

Synthesis and purification of nitroenkephalin

Nitroenkephalin was enzymatically synthesized via nitration of Leu-enkephalin by peroxidase-catalyzed oxidation of nitrite. Briefly, Leu-enkephalin (2 mM) was incubated with 1 μM horseradish peroxidase (HRP) and 10 mM potassium nitrite in 20 mM K-phosphate buffer at pH 7.4.

The reaction was started by the addition of 1 mM H_2O_2 and allowed to proceed at 37°C. The reaction was stopped after 1 h by the addition of 10 nM catalase. Aliquots of the reaction mixture were chromatographed on HPLC to accomplish the purification of nitroenkephalin. Elution was performed on a Prep Nova-Pak HR C_{18} column (7.8 × 300 mm; 6 µm particle size). The mobile phases were—A: 0.2% aqueous trifluoroacetic acid containing 10% acetonitrile; B: acetonitrile:water (50:50, v/v); linear gradient: from A to 100% B over 30 min; and flow rate: 1.5 mL/min. The eluent was monitored at 274 and 360 nm and the fractions eluting between 28 and 30 min, corresponding to nitroenkephalin, were pooled and lyophilized (freeze/dried). The product was analyzed by HPLC as already described (Fontana et al. 2006) and nitroenkephalin was quantified from peak area using 3-nitrotyrosine as reference standard. Nitroenkephalin was stored in K-phosphate buffer, pH 7.4, at −20°C until use.

Isolation of neutrophils

Leukocytes were purified from heparinized human blood freshly drawn from healthy donors. Leukocyte preparations containing 90–98% neutrophils and apparently free of contaminating erythrocytes were obtained by a one-step procedure involving centrifugation of blood samples layered on Ficoll-Hypaque medium (Polymorphprep, Axis-Shield, Oslo, Norway) (Ferrante and Thong 1980). The cells were suspended in isotonic phosphate-buffered saline, pH 7.4, with 5 mM glucose and stored on ice. Each preparation produced cells with a viability higher than 90% up to 6 h after purification. The viability of the cells was measured by trypan blue exclusion test.

Oxidative/nitrative modifications of Leu-enkephalin by neutrophils

Human PMNs (2×10^6 cells) suspended in phosphate-buffered saline containing 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 5 mM glucose were incubated with 1 mM Leu-enkephalin at 37°C in the absence or in the presence of different concentrations of potassium nitrite. After 5 min of incubation, PMNs were stimulated by the addition of 1 µg/mL PMA or 0.1 µM fMLP. Cytochalasin B (1 µg/mL) was added to f-MLP-stimulated PMNs. Reactions were terminated by placing tubes on ice and immediately adding SOD (90 U/mL) and catalase (500 U/mL) to scavenge residual oxidants. PMNs were pelleted by centrifugation (1,200*g*, 5 min, 4°C) and the products in the supernatant determined by HPLC as already described (Fontana et al. 2006). When used, ABAH (100 µM final concentration) was added to the incubation mixture 5 min before neutrophil activation.

Respiratory burst of neutrophils

Superoxide production by NADPH oxidase was estimated by measuring the rate of superoxide dismutase-inhibitable reduction of cytochrome *c* at 550 nm ($\varepsilon = 21{,}100$ M^{-1} cm^{-1} for ferrocytochrome *c*) by a modification of the method described by Lehmeyer et al. (1979). The incubation mixture contained 2×10^6 cells/mL, 80 µM cytochrome *c* in phosphate-buffered saline containing 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 5 mM glucose. After 3 min of preincubation at 37°C, the reaction was started by adding 1 µg/mL PMA or 0.1 µM fMLP. Cytochalasin B (1 µg/mL) was added to fMLP-stimulated PMNs. The controls contained, in addition, 20 µg/mL superoxide dismutase. Steady-state velocity of superoxide production was estimated from the linear part of the reaction curve. Where used, the soluble guanylate cyclase inhibitor 1*H*-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (ODQ, 4 µM) was preincubated 20 min with cells before the assay.

Determination of neutrophils degranulation by elastase release

Degranulation of azurophilic granules was determined by elastase release (Sklar et al. 1982). Elastase release was measured by hydrolysis of the elastase substrate (MeO-Suc-Ala-Ala-Pro-Val-AMC). Briefly, isolated neutrophils (2×10^6 cells/mL) were resuspended in phosphate-buffered saline containing 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose and 1 µg/mL cytochalasin B at 37°C. The elastase substrate (MeO-Suc-Ala-Ala-Pro-Val-AMC) was added at a final concentration of 40 µM. After 3 min of preincubation at 37°C, the reaction was started by adding 1 µM fMLP, and elastase activity was monitored fluorometrically (excitation wavelength 380 nm, emission wavelength 460 nm).

Detection of neutrophil apoptosis by caspase-3 activity assay

Caspase-3 activity was tested in neutrophil lysates by measuring the release of the fluorescent 7-amino-4-methylcoumarin (AMC) moiety from the synthetic substrate acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC). Neutrophils (5×10^6 cells), preincubated at 37°C for 3.5 h in phosphate-buffered saline containing 0.5 mM $MgCl_2$, 1 mM $CaCl_2$ and 5 mM glucose were collected by centrifugation and lysed in 0.5 mL volumes of 50 mM HEPES, 5 mM 3-[3-(cholamidopropyl)dimethylammonio]-1-proanesulfonate (CHAPS), 5 mM dithiothreitol (DTT), pH 7.4, 10 µM 4-amidinophenylmethanesulfonyl fluoride (APMSF), 10 µg/mL pepstatin and 10 µg/mL aprotinin. The reaction was started by adding 100 µl aliquots

of the lysates in 2 mL solutions containing 16 μM Ac-DEVD-AMC, 20 mM HEPES, 0.1% CHAPS, 5 mM DTT- and 2 mM EDTA, pH 7.4. The assay mixture was incubated at 20°C in the dark for 1 h. The fluorescence (excitation wavelength 360 nm, emission wavelength 460 nm) increase was compared with an appropriate blank control containing 10 μM acetyl-Asp-Glu-Val-Asp-al, a specific caspase-3 inhibitor (Nicholson et al. 1995) or a standard preparations of recombinant caspase-3 (Sigma). A calibration curve obtained with standard AMC solutions was employed for quantitative analysis.

Statistical analysis

Results are expressed as mean ± SD for at least three separate experiments. Graphics and data analysis were performed using GraphPAD prism 4 software. Statistical analyses were performed using the ANOVA and Bonferroni post hoc test. $p \leq 0.05$ was deemed significant.

Results

Oxidative/nitrative modifications of Leu-enkephalin by neutrophils

Human neutrophils utilize myeloperoxidase (MPO)/H_2O_2/NO_2^- system to generate reactive nitrogen species (Brennan et al. 2002; Eiserich et al. 1998b). Recently, we reported that oxidants formed by this enzymatic system are able to convert the tyrosine amino-terminal residue of Leu-enkephalin either to 3-nitrotyrosine producing nitroenkephalin or to dityrosine with the production of an enkephalin dimer, the dienkephalin (Fontana et al. 2006). Figure 1 shows that 30 min incubation of 1 mM Leu-enkephalin with human PMNs activated with PMA yielded a detectable level (0.82 ± 0.10 μM) of dienkephalin. Addition of NO_2^- to this reaction system produced nitroenkephalin as an additional product. The extent of the conversion of Leu-enkephalin to the nitroderivative was found to increase with the concentration of nitrite added, while dienkephalin production leveled off at approximately 25 μM NO_2^-. At higher nitrite concentrations (>200 μM), the yield of nitroenkephalin exceeded that of dienkephalin (data not shown). Unstimulated PMNs did not induce any modification of Leu-enkephalin.

To elucidate the mechanism by which NO_2^- participates in PMN-mediated nitration reaction, the system was investigated in the presence of SOD and catalase. As seen in Fig. 1 (inset), SOD exhibited only a marginal effect on the production of nitroenkephalin, whereas catalase strongly reduced the yield of the nitropeptide. In addition, 4-aminobenzoic acid hydrazide (ABAH), an inhibitor of

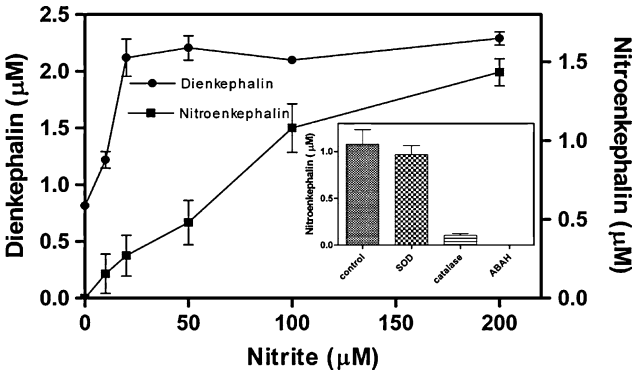

Fig. 1 Oxidative/nitrative modification of Leu-enkephalin by PMA-stimulated neutrophils. Leu-enkephalin (1 mM) was incubated with 2×10^6 neutrophils at 37°C and potassium nitrite (0–200 μM). After 5 min, neutrophils were stimulated by the addition of 1 μg/mL PMA. After 30 min, the reaction was stopped by the addition of SOD (90 U/mL) and catalase (500 U/mL). Neutrophils were pelleted by centrifugation and the reaction products in the supernatant were determined by HPLC. *Inset*, effect of SOD, catalase and ABAH on Leu-enkephalin nitration by PMA-stimulated neutrophils. Leu-enkephalin (1 mM) was incubated with 2×10^6 neutrophils at 37°C and 100 μM potassium nitrite in the presence of SOD (90 U/mL), catalase (500 U/mL) or ABAH (100 μM). The reaction was stopped after 30 min as above and the reaction products were determined by HPLC

MPO (Kettle et al. 1995), completely abolished nitroenkephalin formation. Collectively, these data support a mechanism for PMN-mediated nitration of enkephalin that is dependent on active MPO and hydrogen peroxide.

The oxidative/nitrative modifications of Leu-enkephalin by human neutrophils have been also investigated in the presence of an NO-producing system. PMNs activated with PMA or fMLP were exposed to a continuous flux of NO, using the NO-donor molecule DEANO. The yields of dienkephalin and nitroenkephalin produced after 30 min of incubation with 1 mM Leu-enkephalin are reported in Fig. 2. It can be seen that PMNs activated with PMA are capable of nitrating and oxidizing enkephalin with higher efficiency compared with fMLP-activated PMNs.

Effect of nitroenkephalin on superoxide generation by human PMNs

To investigate if enkephalin, undergoing oxidative/nitrative modification, could influence neutrophil functions, the nitrated peptide was explored for its effect on respiratory burst. Addition of 100 μM Leu-enkephalin or nitroenkephalin to human PMN in suspension did not stimulate superoxide generation. However, nitroenkephalin added to neutrophils 2 min before activation by 1 μg/mL PMA or 0.1 μM fMLP led to a concentration-dependent inhibition of superoxide generation (Fig. 3b). Nitroenkephalin half maximal inhibition of superoxide anion generation was 48.4 ± 1.3 and 66.9 ± 1.3 μM for PMA- and fMLP-

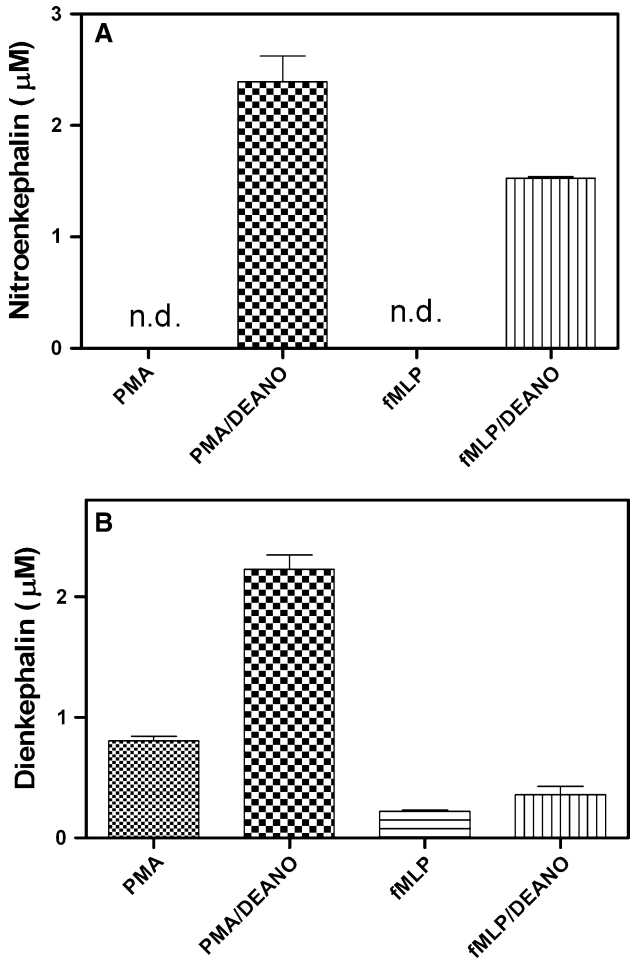

Fig. 2 Oxidative/nitrative modification of Leu-enkephalin by PMA- or fMLP-stimulated neutrophils in the presence of the nitric oxide donor, DEANO. Leu-enkephalin (1 mM) was incubated with 2×10^6 neutrophils at 37°C and 1 mM DEANO. After 5 min, neutrophils were stimulated by the addition of 1 μg/mL PMA or 0.1 μM fMLP. Cytochalasin B (1 μg/mL) was added to f-MLP-stimulated PMNs. After 30 min, the reaction was stopped by the addition of SOD (90 U/mL) and catalase (500 U/mL). Neutrophils were pelleted by centrifugation and the reaction products in the supernatant were determined by HPLC; *n.d.* not detected. Control values are without DEANO. **a** Nitroenkephalin; **b** Dienkephalin

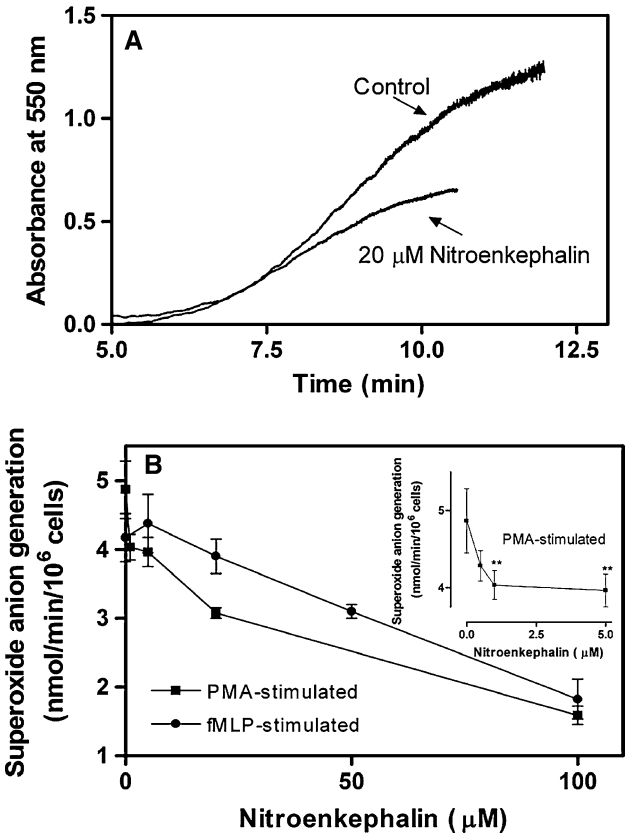

Fig. 3 Inhibition of neutrophil superoxide anion generation in response to either PMA or fMLP by nitroenkephalin. Superoxide anion generation was estimated by measuring cytochrome *c* reduction as described in "Materials and methods". Neutrophils (2×10^6 cells) in 1 mL phosphate-buffered saline containing 80 μM cytochrome *c* at 37°C were activated by 1 μg/mL PMA or 0.1 μM fMLP and the absorbance at 550 nm was monitored. Nitroenkephalin was added 2 min before activation. Steady-state velocity of superoxide production was estimated from the linear part of the reaction curve. **a** Time course of superoxide anion generation by PMA-activated neutrophils with/without 20 μM nitroenkephalin. **b** Dose-dependent inhibition of PMA- and fMLP-stimulated neutrophil superoxide anion generation by nitroenkephalin. **b** *Inset*, dose-dependent inhibition of PMA-stimulated neutrophil superoxide anion generation by ≤5 μM nitroenkephalin. $^{**}p < 0.05$, compared with the control value (PMA-stimulated neutrophil superoxide anion generation in the absence of nitroenkephalin)

stimulated human PMNs, respectively. Control experiments, using the enzymatic system xanthine/xanthine oxidase to generate superoxide anion, indicated that nitroenkephalin did not act as a scavenger of superoxide anion (not shown). In Fig. 4a, it is also shown that Leu-enkephalin and the free nitrated amino acid, 3-nitrotyrosine, did not affect the oxidative burst of PMA-stimulated neutrophils.

Previous investigations indicated that NO or NO-releasing compounds increased cyclic GMP (cGMP) in human neutrophils with concomitant inhibition of PMN functions including superoxide anion generation (Moilanen et al. 1993). To evaluate the possible involvement of the NO/cGMP system in the mechanism of inhibition exerted by nitroenkephalin, fMLP-stimulated human PMNs were incubated with nitroenkephalin in the presence of the soluble guanylate cyclase inhibitor, ODQ. It can be observed that the inhibitory effect of nitroenkephalin was not reversed by the presence of ODQ indicating that the inhibition of superoxide anion generation is not cGMP mediated (Fig. 4b).

Enkephalins are known to bind to specific opioid receptors on human neutrophils (Menzebach et al. 2003). To evaluate whether the inhibitory activity of

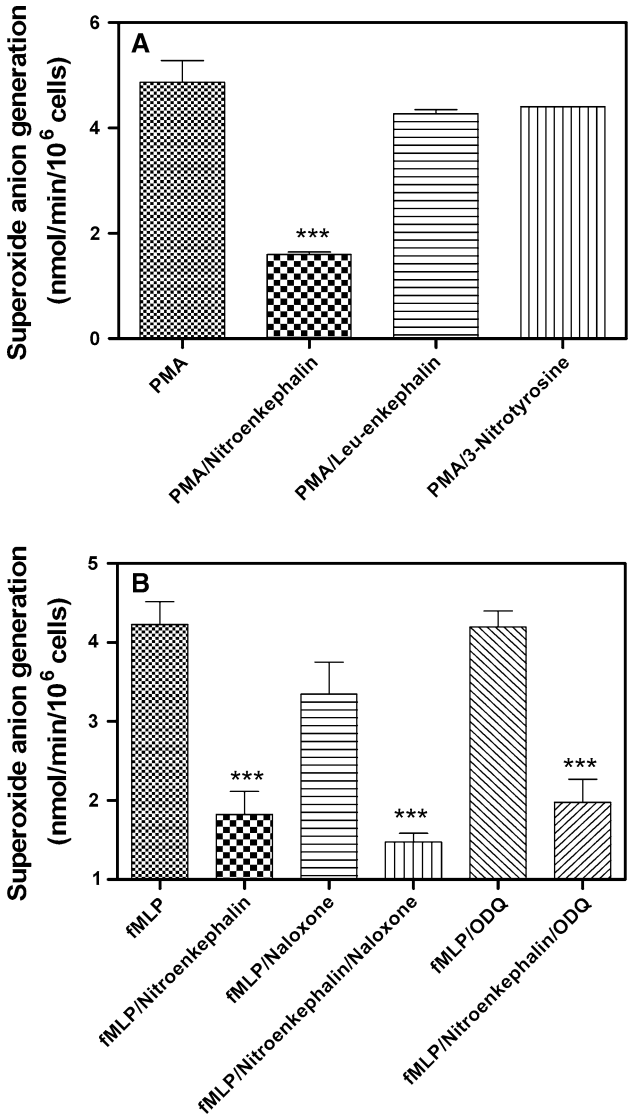

Fig. 4 Comparison of the effect of nitroenkephalin with various bioactive molecules on PMA- or fMLP-stimulated neutrophil superoxide anion generation. Superoxide anion generation was estimated by measuring cytochrome *c* reduction as described in "Materials and methods". Neutrophils (2×10^6 cells) in 1 mL phosphate-buffered saline containing 80 μM cytochrome *c* at 37°C were activated by 1 μg/mL PMA or 0.1 μM fMLP, and the absorbance at 550 nm was monitored. Nitroenkephalin and other test compounds were added 2 min before activation. ODQ was preincubated 20 min with cells before the assay. Steady-state velocity of superoxide production was estimated from the linear part of the reaction curve. **a** Comparison of the effect of 100 μM nitroenkephalin, 100 μM Leu-enkephalin and 100 μM 3-nitrotyrosine on PMA-stimulated neutrophil superoxide anion generation. **b** Comparison of the effect with/without nitroenkephalin of 100 μM naloxone and 4 μM ODQ on fMLP-stimulated neutrophil superoxide anion generation. $***p < 0.001$, compared with the corresponding control (the first column of each panel)

nitroenkephalin is mediated by its binding to opioid receptors, the effect of nitroenkephalin on respiratory burst was examined in the presence of naloxone, a competitive opioid receptor antagonist. Figure 4b shows that the addition of 100 μM naloxone did not modify the inhibitory effect of nitroenkephalin on superoxide anion release, suggesting that the mechanism of inhibition does not involve opioid receptors.

Nitroenkephalin attenuation of fMLP-induced degranulation of human PMNs

Pretreatment of neutrophils with 100 μM nitroenkephalin strongly reduced fMLP-induced azurophilic degranulation as evaluated by measuring the activity of released elastase (Fig. 5a). Figure 5b shows that the inhibitory effect of nitroenkephalin was concentration-dependent with half

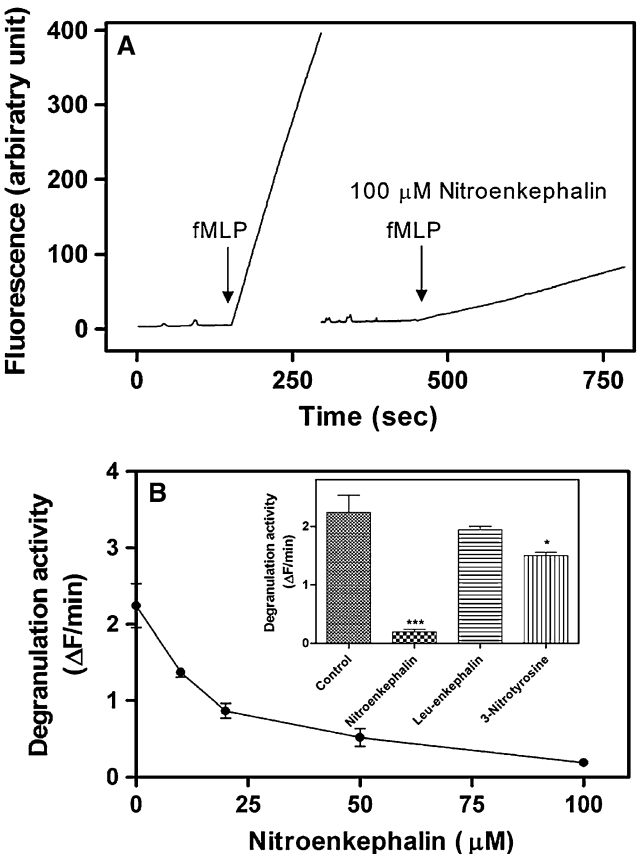

Fig. 5 Inhibition of fMLP-stimulated neutrophil degranulation by nitroenkephalin. Degranulation acitivity was determined by elastase release. Elastase activity was measured by hydrolysis of the elastase substrate (MeO-Suc-Ala-Ala-Pro-Val-AMC) as described in "Materials and methods". Neutrophils (2×10^6 cells) were incubated with 1 μg/mL cytochalasin B and 40 μM MeO-Suc-Ala-Ala-Pro-Val-AMC at 37°C for 3 min before activation with 1 μM fMLP, as indicated by *arrows*. Nitroenkephalin was added 2.5 min before activation. **a** Time course of elastase release by fMLP-activated neutrophils with/without 100 μM nitroenkephalin. **b** Dose-dependent inhibition of fMLP-stimulated neutrophil elastase release by nitroenkephalin. **b** *Inset*, comparison of elastase release by fMLP-stimulated neutrophils in the absence (control) and presence of 100 μM nitroenkephalin, 100 μM Leu-enkephalin and 100 μM 3-nitrotyrosine. $*p < 0.05$ and $***p < 0.001$, compared with the control (the first column of the panel)

maximal inhibition of 13.8 ± 1.5 μM. Leu-enkephalin (100 μM) was ineffective and 3-nitrotyrosine (100 μM) showed a 37% inhibition of elastase release (Fig. 5b, inset). Addition of nitroenkephalin to unstimulated PMNs had no effect on their degranulation activity. Control experiments using commercial elastase from porcine pancreas indicate that nitroenkephalin (100 μM) did not affect the elastase activity (not shown).

Influence of nitroenkephalin and Leu-enkephalin on neutrophil spontaneous apoptosis

Neutrophil spontaneous apoptosis was evaluated by measuring caspase-3 activity in lysates of neutrophils (5×10^6 cells/mL) that were preincubated at 37°C for 3.5 h. When the preincubation step was performed in the presence of Leu-enkephalin (5–100 μM), no considerable effect on the caspase-3 activity was observed (Fig. 6). Conversely, the addition of nitroenkephalin induced a decrease of caspase-3 activity that was concentration dependent. With 50 μM nitroenkephalin, the reduction of caspase-3 activity was close to 40% ($p < 0.05$). Control experiments indicated that neither nitroenkephalin nor the parent peptide Leu-enkephalin affected the activity of recombinant caspase-3 (not shown).

To rule out toxic effects exerted by nitroenkephalin, cell viability was measured by the trypan blue exclusion test. Neutrophil viability was evaluated after incubation of the cells at 37°C for 3.5 h. Nitroenkephalin showed no effect in the range 10–100 μM (Fig. 6).

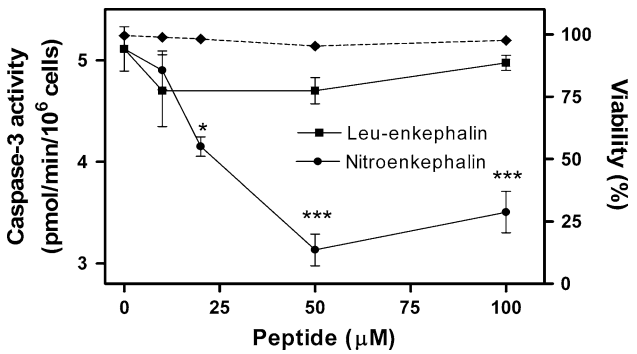

Fig. 6 Effect of Leu-enkephalin and nitroenkephalin on neutrophil caspase-3 activity. Neutrophils (2×10^6 cells) were incubated in the absence (control) and in the presence of various concentrations of Leu-enkephalin or nitroenkephalin at 37°C for 3.5 h. Intracellular caspase-3 activity was determined as described in "Materials and methods". Cell viability (*filled diamonds*) was estimated by the trypan blue exclusion test and is expressed as percentage of untreated cells. Values are given as mean ± SD ($n = 4$). *$p < 0.05$, and ***$p < 0.001$, compared with the control value

Discussion

In a recent paper, we showed that reactive nitrogen species, such as peroxynitrite or nitrite/singlet oxygen or peroxidase-catalyzed oxidation of nitrite, are capable of nitrating and oxidizing the bioactive peptide Leu-enkephalin to give nitroenkephalin and dienkephalin (Fontana et al. 2006). The results presented in this paper demonstrate that activated human neutrophils, in the presence of nitrite, mediate nitration and dimerization of Leu-enkephalin leading to nitroenkephalin and dienkephalin formation. It should be noted that addition of nitrite at concentrations observed during inflammatory and infectious process (10–50 μM) (Farrel et al. 1992; Torre et al. 1996) induced both nitration and dimerization of Leu-enkephalin. The relative yields of nitroenkephalin and dienkephalin produced by human PMNs depend on nitrite concentration. At low concentrations of nitrite, dimerization of enkephalin is the predominant pathway, whereas at higher concentration of nitrite (>200 μM), nitroenkephalin is the main product. These findings suggest that enkephalin dimerization competes with nitroenkephalin formation, which becomes more significant with increasing levels of nitrite. As already reported, PMN-mediated nitration reactions involve reactive oxygen/nitrogen species and myeloperoxidase activity (Brennan et al. 2002; Eiserich et al. 1998b). Accordingly, our data show a critical role of hydrogen peroxide as revealed by inhibition of the nitroenkephalin formation in the presence of catalase. Furthermore, inhibition of myeloperoxidase by ABAH completely abolishes the conversion of enkephalin to nitroenkephalin. Collectively, these results support a mechanism for PMN-mediated modification of Leu-enkephalin that is dependent on myeloperoxidase activity and hydrogen peroxide production. Nitrative/oxidative modifications of tyrosine by MPO in the presence of hydrogen peroxide and nitrite has been attributed to direct action of MPO Compound I and II on both nitrite and tyrosine producing nitrogen dioxide radical ($^{\cdot}NO_2$) and tyrosyl radical (Tyr$^{\cdot}$), respectively (Burner et al. 2000). Then, Tyr$^{\cdot}$ can either react with $^{\cdot}NO_2$ ($k = 1.3 \times 10^9$ M^{-1} s^{-1}) to form nitrotyrosine or dimerize ($k = 2.25 \times 10^8$ M^{-1} s^{-1}) to give dityrosine (Hunter et al. 1989; Prutz et al. 1985). With enkephalin as a target, the tyrosine amino-terminal residue of the peptide can be converted either to 3-nitrotyrosine producing nitroenkephalin or to dityrosine, with the production of an enkephalin dimer, the dienkephalin.

Recently, it has been reported that activated neutrophils use MPO to oxidize Leu-enkephalin to their corresponding tyrosyl free radical, which preferentially reacts with respiratory burst-derived superoxide anion ($k = 1.5 \times 10^9$ M^{-1} s^{-1}) (Jin et al. 1993) to form a hydroperoxide derivative (Nagy et al. 2010). Here, we demonstrate that stimulated neutrophils, in the presence of nitrite, promote as a further

modification of enkephalins nitration of amino-terminal tyrosine residue to give nitroenkephalin. As the rate constant for the reaction of tyrosyl radical with superoxide anion is similar to that with $^{\cdot}NO_2$ ($k = 4.5 \times 10^9\ M^{-1}\ s^{-1}$) (Goldstein et al. 1998), it is possible (but remain to be proved) that Leu-enkephalin may be partly oxidized via superoxide anion-mediated mechanism also under our experimental conditions.

Nitrite is the major end product of NO metabolism, and inducible NO synthase is contained in neutrophil primary granules (Evans et al. 1996). To simulate a physiological mechanism of nitrite formation, activated neutrophils were exposed to a continuous flux of NO, using the NO-donor molecule, DEANO. These conditions also promoted nitration and dimerization of Leu-enkephalin. Although NO readily reacts with superoxide anion to form the potent oxidative/nitrative agent, peroxynitrite, it has been previously reported that NO fluxes induce tyrosine nitration predominantly via MPO/H_2O_2-dependent oxidation of nitrite to $^{\cdot}NO_2$. In line with this, we found that with fMLP-activated PMNs, the yield of nitroenkephalin was 1.6-fold less, a consequence possibly related to decreased MPO release (Eiserich et al. 1998b).

In our studies on the PMNs-mediated nitrative reactions, we used enkephalin concentrations much higher than those present in plasma. Circulating enkephalins are normally in the nanomolar range; however, their amount can significantly increase under some pathological conditions (Clement-Jones et al. 1980; Pierzchala and Van Loon 1990; Semmoum et al. 2001). Moreover, locally synthesized enkephalins in inflamed tissues could produce interstitial enkephalin concentrations much higher than those observed in plasma. The concept of local enkephalin is supported by the finding that inflammatory cells contain enkephalins and the mRNA for their precursor, proenkephalin (Padrós et al. 1989; Przewlocki et al. 1992; Rittner et al. 2001). Furthermore, enkephalins undergo rapidly hydrolysis, underestimating the actual concentration.

It has been shown that opioid peptides may prime or modulate the activity of leukocytes (McCain et al. 1982; Menzebach et al. 2003; Pasnik et al. 1999; Rabgaoui et al. 1993; Sulowska et al. 2002). To characterize a possible bioactivity of the nitrated enkephalin, its influence on human neutrophil functional responses has been examined. Nitroenkephalin at micromolar concentrations inhibits neutrophil activation in response to PMA or fMLP, as indicated by the inhibition of superoxide anion generation and of degranulation of azurophilic granules. Under the same experimental conditions, the parent peptide, Leu-enkephalin, showed no inhibitory activity on these human neutrophil functions.

Neutrophil apoptosis is an important process because it provides a signal for PMN removal (Savill and Fadok 2000) and it results in the loss of functional neutrophil responsiveness (Lee et al. 1993; Savill et al. 2002; Simon 2003). Thus, modulation of apoptosis may have a major effect on the inflammatory process. As several studies suggested a critical role of caspase-3 in both spontaneous and Fas receptor-mediated apoptosis in neutrophils (Daigle and Simon 2001; Khwaja and Tatton 1999; Ottonello et al. 2002; Pongracz et al. 1999; Weinmann et al. 1999), we studied the effect of nitroenkephalin and the parent peptide on spontaneous apoptosis by measuring the caspase-3 activity in cell lysates (Fig. 6). Our results indicate that the nitroenkephalin influences the life span of human PMNs by inhibition of spontaneous apoptosis.

It is recognized that the production of reactive oxygen species by activated cells accelerate the apoptosis and that superoxide release is required for spontaneous apoptosis (Kettritz et al. 1997; Ottonello et al. 2002; Scheel-Toellner et al. 2004). In agreement, blood neutrophils from patients with a genetic defect of NADPH oxidase, thus not generating reactive oxygen species, demonstrate delayed spontaneous apoptosis (Kasahara et al. 1997; Hampton et al. 2002; Savill et al. 1989). Thus, it is possible that the nitrated enkephalin decreases spontaneous apoptosis of human neutrophils by inhibiting superoxide anion production. Accordingly, the parent Leu-enkephalin, which does not inhibit superoxide anion generation, does not decrease spontaneous apoptosis.

The cellular mechanism by which nitroenkephalin exerts an inhibitory effect on neutrophil functional responses requires further study. At the moment, the observation that naloxone, a competitive opioid receptor antagonist, failed to reverse the inhibition of superoxide anion generation by nitroenkephalin, indicates that the opioid receptor is not involved in the mechanism of inhibition. Furthermore, cGMP has been observed to have an inhibitory effect on fMLP-induced responses (Ervens et al. 1991). Under our experimental conditions, the soluble guanylate cyclase inhibitor, ODQ, did not interfere with the inhibitory effect of nitroenkephalin providing evidence that the inhibition on fMLP-induced respiratory burst occurs through a cGMP-independent pathway.

In conclusion, the results of our in vitro studies indicate that Leu-enkephalin, present at inflammation sites, might exert regulatory effects on the efficiency of inflammatory processes, after its conversion to nitroenkephalin, induced by activated human neutrophils. Although the bioactivity of nitroenkephalin has been observed at concentrations higher than those eventually formed in vivo, our findings may lead to further understanding of biochemical

inflammatory mechanisms and open the route to the development of new therapeutic strategies for inflammation.

Conflict of interest The authors declare that they have no conflict of interest.

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