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Oxidative and nitrative modifications of enkephalins by reactive nitrogen species

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Abstract
The interaction of Leucine-enkephalin (Leu-enkephalin) with reactive nitrogen species has been investigated. Reactive nitrogen species are capable of nitrating and oxidizing Leu-enkephalin. HPLC analysis shows the formation of two major enkephalin derivatives by peroxynitrite. The tyrosine amino-terminal residue of Leu-enkephalin is converted either to 3-nitrotyrosine thus producing nitroenkephalin and to dityrosine by dimerization with the production of an enkephalin dimer. The evidence of the formation of the nitroenkephalin and of the enkephalin dimer—dienkephalin—was achieved by electrospray ionisation mass spectrometry. In addition to peroxynitrite, the methylene blue photosensitized oxidation of enkephalin in the presence of nitrite leads to the formation of the nitrated peptide. Moreover, the nitropeptide can be also obtained by peroxidase-generated nitrogen reactive species.

Keywords: *Enkephalin, peroxynitrite, singlet oxygen, dityrosine, nitroenkephalin, photooxidation*

Abbreviations: *ABTS, 2, 2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid); DTPA, diethylenetriaminepentaacetic acid; DHR, dihydrorhodamine 123; HRP, horseradish peroxidase; LPO, lactoperoxidase; Leu-enkephalin, leucine enkephalin; LC-MS, liquid chromatography-mass spectrometry; MPO, myeloperoxidase; SIN-1, 3-Morpholinosydnonimine*

Introduction

Enkephalins are known as neurotransmitters and neuromodulators whose opiate agonist activity [1,2] is removed by the action of specific peptidases [3,4]. Enkephalins belong to a class of bioactive peptides that exhibit as a common feature the presence of a tyrosine residue at the amino terminus, which is essential for their biological activity [5]. Proteolytic cleavage of the pentapeptides was regarded as the unique metabolic pathway and no investigation to alternative pathways had been pursued. In the last years, we have demonstrated that, besides peptidases, also oxidative enzymes can act upon enkephalins [6,7]. In particular, enkephalins can be converted into enkephalin dimers—dienkephalins—by the peroxidase/H_2O_2 system and into dopaenkephalins by tyrosinase [6–8]. The latter enzyme as well as reactive oxygen species, like hydroxyl radical ($^{\cdot}OH$), can transform enkephalins and opioid peptides into melanin pigments retaining the peptide moiety [9–11], named enkmelanins and opiomelanins, respectively. Furthermore, as a consequence of the reaction of enkephalins with tyrosinase in the presence of cysteine, cysteinyldopaenkephalins can be obtained [12]. These enkephalin derivatives by means of the peroxidase/H_2O_2 system and by reactive oxygen species, are converted into another class of melanin pigments: the pheoopiomelanins [12,13]. Enkephalins, their cysteinyl derivatives and all these newly synthesized melanins exhibit antioxidant

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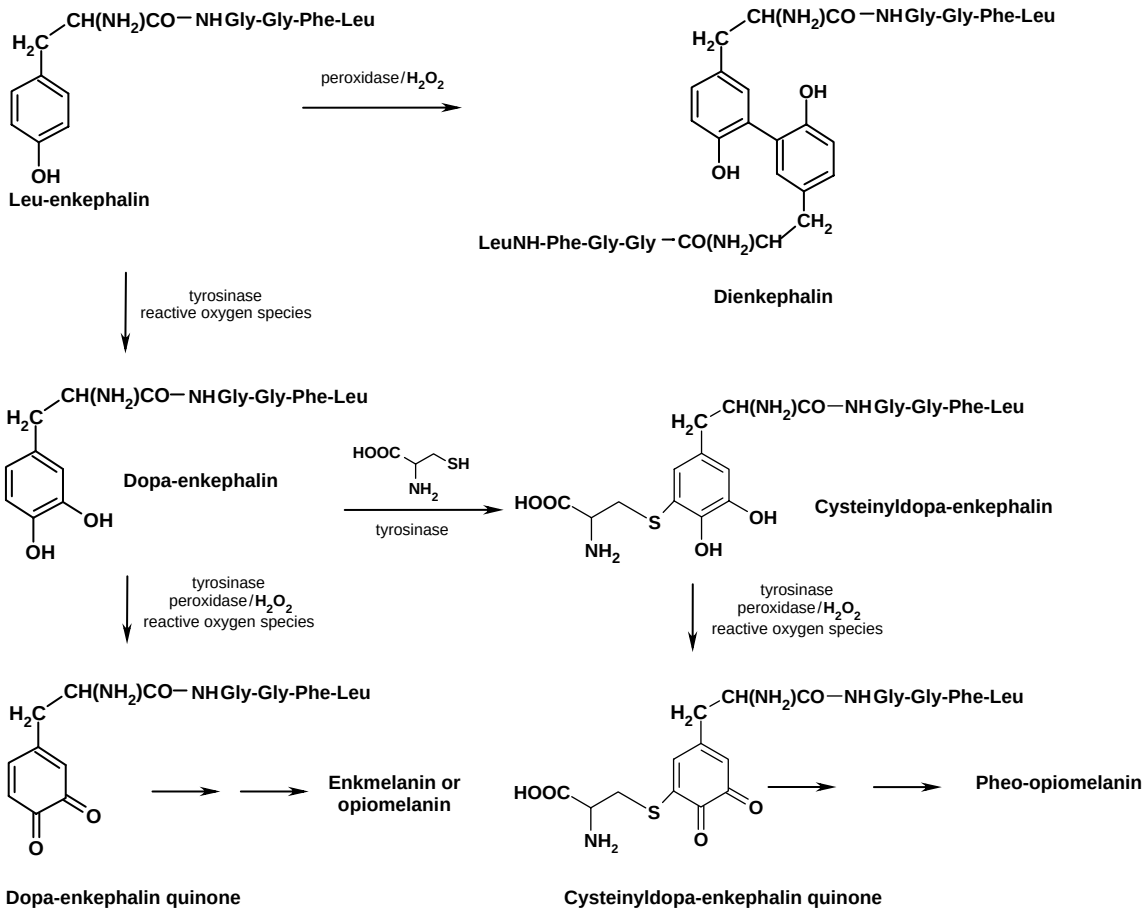

Scheme 1. Oxidative transformations of Leu-enkephalin.

properties, scavenging activity and the capacity to inhibit lipid peroxidation [11,13,14]. The overall studies about enkephalin oxidative routes have been recently reviewed [15] (Scheme 1).

On this trail, enkephalin reactions involving nitrogen reactive species have been taken into consideration. Peroxynitrite represents a highly oxidizing and nitrating agent which can be produced by the reaction of nitric oxide with superoxide anion [16–18]; peroxynitrite is quite stable but upon protonation to peroxynitrous acid ($pK_a = 6.8$), it rapidly decays to nitrate generating highly oxidizing and nitrating reactive species like $^{\cdot}OH$ and nitrogen dioxide ($^{\cdot}NO_2$) [19]. Under physiological conditions, the nitration ability of peroxynitrite is enhanced by its reaction with carbon dioxide (CO_2) to the short-lived adduct peroxynitrite-CO_2 which decomposes to nitrate thereby producing carbonate radical anion ($CO_3^{\cdot-}$) and $^{\cdot}NO_2$ [20–23]. Though the role of peroxynitrite as a central species in biological nitration has been more recently questioned [24,25] and the relative contribution of alternative routes *in vivo* is still a matter of dispute [26], peroxynitrite is actually able to convert free tyrosine and tyrosine protein residue into the corresponding nitrated molecules. Free tyrosine and tyrosine protein residues nitration can also be achieved through mechanisms involving peroxidase-dependent oxidation of nitrite [27–29] or by singlet oxygen in the presence of nitrite [30].

Nitration of tyrosine residues in proteins may alter protein function, which may have both physiological and pathological significance [26,31]. The occurrence of 3-nitrotyrosine is considered the molecular footprint left by reaction of nitrogen reactive species with biomolecules [32,33] and nitrated tyrosine residues are actually considered as biomarkers in a variety of pathophysiological conditions such as chronic inflammation, cancer and neurodegenerative disease [34–36].

In the present investigation, the oxidation and nitration of the bioactive peptide, Leu-enkephalin by peroxynitrite, by singlet oxygen in the presence of nitrite or by peroxidase-generated reactive nitrogen species has been studied.

Materials and methods

Chemicals

Leu-enkephalin, 3-morpholinosydnonimine (SIN-1), dihydrorhodamine 123 (DHR), 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), horseradish

peroxidase (HRP), bovine lactoperoxidase (LPO), methylene blue and diethylenetriaminepentaacetic acid (DTPA) were obtained from Sigma-Aldrich, Inc (St Louis, MO, USA). L-Tyrosine, 3-nitrotyrosine, human myeloperoxidase (MPO), potassium nitrite and hydrogen peroxide (H_2O_2) were from Fluka Chemie GmbH (Buchs, CH). Dityrosine was synthesized according to the enzymatic preparation described by Malencik et al. [37]. All other chemicals were analytical grade. DTPA was included in all reaction mixtures to avoid interfering reactions with contaminating metal ions.

Peroxynitrite was synthesized from potassium nitrite and H_2O_2 under acidic conditions as previously described [38], and excess H_2O_2 was removed by treatment with granular manganese dioxide. Typical peroxynitrite concentration after freeze fractionation was 600–700 mM as determined at 302 nm using a molar absorption coefficient (ε_{302}) of 1670 M^{-1} cm^{-1}. Stock solutions were diluted with 0.1 M NaOH immediately before use to achieve the desired concentration.

Leu-enkephalin nitration by peroxynitrite and HPLC analysis

Leu-enkephalin (1 mM) was reacted with 1 mM peroxynitrite in 0.5 M K-phosphate buffer, pH 7.4, the concentration of Na-bicarbonate, when present was 25 mM. After 5 min incubation at 25°C, the reaction products were analysed by HPLC. Elution was performed on a C_{18} (3.9 × 150 mm), 4 μm, with a linear gradient for 30 min from 50 mM K-posphate/H_3PO_4, pH 3.0, containing 10% acetonitrile to acetonitrile–water (50:50, v/v) at a flow rate of 1.0 ml/min. Eluates were monitored by absorbance at 274 and 360 nm using a Waters 996 photodiode array, or by fluoroscence detection, using a Perkin-Elmer LS-1LC with a 260 nm excitation filter and a 410 nm emission wavelength. Peaks were quantified using Millenium 32 software (Waters, Milford, MA, USA).

For preparative HPLC, the column was a 7.8 × 300 mm Prep Nova-Pak HR C_{18}, 6 μm. 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 fractions corresponding to the main reaction products were collected. The eluted fractions of 5 HPLC runs were pooled and lyophilised (freeze/dried) before submitting to mass spectrometry.

Leucine enkephalin (0.5 mM) was also treated with SIN-1 (0.5 mM), which at neutral pH decomposes to release nitric oxide and superoxide anion [39]. Small aliquots were added to the reaction mixture, which was then incubated at 37°C for 1 h. The reaction products were analysed by HPLC as described above.

Peroxynitrite formed from NO/superoxide anion was estimated by use of the DHR oxidation assay [40]. The rate of DHR oxidation by 0.5 mM SIN-1 was 0.20 ± 0.02 μM min^{-1}. The amount of oxidized DHR was calculated using molar absorption coefficient (ε_{500}) of 78,780 M^{-1} cm^{-1}.

Mass spectrometry analysis

HPLC fractions were applied on RP-HPLC and mass spectra analyses performed on-line with their elution (liquid chromatography-mass spectrometry, LC-MS). Lyophilised HPLC fractions were suspended in 0.2 ml 0.2% aqueous trifluoroacetic acid, loaded on a Vydac (Hesperia, CA, USA) C_{18} column (150 × 2.1 mm; 5 μm particle size) and eluted using a LabService Analytical (Bologna, Italy) HPLC system equipped with a UV detector and connected to a mass spectrometer by a T splitter. Elution was obtained by a 60 min linear gradient from 0.2% aqueous trifluoroacetic acid to 0.2% trifluoroacetic acid in acetonitrile–water 90/30. After elution differently splitted aliquots of the column effluent were simultaneously monitored for the absorbance at 220 nm and mass spectrometric analysis using an electrospray ion trap mass spectrometer (LCQ, Finnigan, San Jose, CA). MS spray voltage was 5.15 kV, the heated capillary was maintained at 260°C and sheath gas flow was 80 (arbitrary units). Mass spectra were collected operating in positive mode in mass spectrum range of m/z 500–2000.

Photosensitized oxidation of Leu-enkephalin in the presence of nitrite

A reaction mixture containing 1 mM leu-enkephalin, 10 μM methylene blue and potassium nitrite (0–1 mM) in 20 mM K-phosphate buffer, pH 5.8 was illuminated with a 200-W tungsten halogen lamp at a distance of about 10 cm from the solution and allowed to proceed at 25°C for 30 min. Reaction mixtures were analyzed by HPLC as described above.

Leu-enkephalin nitration by horseradish peroxidase (HRP) and nitrite

Leu-enkephalin (1 mM) was incubated with 1 μM HRP and various concentration of potassium nitrite in 20 mM K-phosphate buffer at pH 7.4. The reaction was started by addition of 1 mM H_2O_2 and allowed to proceed at 37°C. Reaction was stopped after 10 min by addition of 10 nM catalase. As controls, parallel experiments without enzyme or with heat-inactivated enzyme were carried out. Reaction mixtures were analysed by HPLC as described above.

The nitration rate of enkephalins (Leu-enkephalin and Met-enkephalin), of tyrosine aminoterminal peptides and of free tyrosine by HRP was followed

spectrophotometrically at 430 nm using ε_{430} nitrotyrosine = 4400 M^{-1} cm^{-1} at alkaline pH [41,42]. The reaction was carried out at 37°C in 1 ml of 20 mM K-phosphate buffer, pH 7.4 containing 1 μM HRP and 1 mM potassium nitrite. The reaction was started by the addition of 1 mM H_2O_2. The nitration rate of 1 mM Leu-enkephalin or free tyrosine by mammalian peroxidases was carried out at 37°C in 1 ml 20 mM K-phosphate buffer, pH 7.4, containing 1 μM MPO or LPO and 10 mM potassium nitrite. The reaction was started by the addition of 1 mM H_2O_2. In order to compare the catalytic activity of various peroxidase systems, we have normalized experimental data to one enzymatic unit defined as follows: One unit is the amount of enzyme that catalyzes the oxidation of 1 μmol of ABTS (ε_{412} of oxidized ABTS = 32,400 M^{-1} cm^{-1}) per min in an incubation mixture containing 2 mM ABTS and 0.05 mM H_2O_2 in 1 ml of 100 mM K-phosphate buffer, pH 5.5, at 25°C.

Acid or enzymatic hydrolysis

Acid or enzymatic hydrolysis was carried out by incubating aliquots of pooled fractions obtained by HPLC, respectively, in sealed tubes in 6 N HCl at 110°C for 20 h or in the presence of microsomal leucine aminopeptidase (0.5 U) in 100 mM K-phosphate buffer, pH 7.4, at 37°C for 1 h. At the end of incubation time, samples were appropriately diluted and analyzed by HPLC using the analysis conditions already described for nitrotyrosine and dityrosine by Pecci et al. [30].

Statistical analysis

Results are expressed as means ± SEM for at least three separate experiments. Graphics and data analysis were performed using GraphPAD prism 4 software.

Results

Leu-enkephalin nitration by peroxynitrite: HPLC analysis

Leu-enkephalin and the products of the reaction with peroxynitrite were separated successfully from each other using HPLC (Figure 1). HPLC analysis of the reaction mixture, monitored at 274 and 360 nm, showed, in addition to the unreacted Leu-enkephalin, the presence of reaction products named *a*, *b* and *c*, respectively. The more consistent one (product *a*) exhibited a retention time of 19.3 min (Figure 1). The absorption spectrum—taken at the top—exhibited the same absorption features of 3-nitrotyrosine (Figure 1, inset) suggesting that this product could represent the nitrated derivative of Leu-enkephalin.

Product *b*—not presenting absorbance at 360 nm—showed a retention time of 22.3 min. HPLC analysis performed by recording the effluent with the fluorometric detector operating at λ_{ex} 260 and 410 nm showed an unique peak with the same retention time (22.3 min). This behaviour recognized as typical of dityrosine [37], suggested the presence in the product *b* of a dityrosine moiety.

In order to inequivocally determine the production of nitroenkephalin and enkephalin dimer, the eluates corresponding to product *a* and *b* were separately collected, subjected to enzymatic (product *a*) or acid (product *b*) hydrolysis and analysed by HPLC for the release of 3-nitrotyrosine in sample *a* and dityrosine in sample *b* (not shown). This was indeed the case, thus permitting to identify product *a* as the nitroenkephalin derivative and product *b* as the enkephalin dimer (dienkephalin), as demonstrated by the presence of peaks coeluting with authentic standard of 3-nitrotyrosine in enzymatic hydrolysed product *a* and with authentic standard of dityrosine in acid hydrolysed product *b*.

The peak corresponding to product *c*—a very small one indeed—with retention time of 14.3 min was identified as dopaenkephalin, i.e. the hydroxylated Leu-enkephalin, using an authentic sample of dopaenkephalin synthetized as described by Larsimont et al. [8].

Leu-enkephalin nitration by peroxynitrite: Mass spectrometry

In order to confirm the formation of nitroenkephalin and dienkephalin after reaction of Leu-enkephalin with peroxynitrite, the eluates corresponding to products *a* and *b*, respectively, were collected separately using preparative HPLC. The lyophilised pooled fractions were dissolved in an acid aqueous solution and loaded on a reverse-phase chromatographic column. Elution was analyzed by ESI mass spectrometry. In Figure 2, the mass spectra of the two HPLC fractions are shown. Full scan ion spectrum, acquired in single MS positive ion mode, shows (Figure 2, top) the predominant ions at $m/z = 601$, for the product *a* and the predominant ion at $m/z = 1109$ for the collected product *b* (Figure 2, bottom). These values are in full agreement with the expected $[MH]^+$ ions for nitroenkephalin and dienkephalin, respectively.

Leu-enkephalin nitration by peroxynitrite: Effect of bicarbonate

Exposure of Leu-enkephalin (1 mM) to increasing concentration of peroxynitrite (0–1 mM) resulted in an increase of the nitroderivative production and a subsequent decrease in the level of Leu-enkephalin (not shown). Nitration was observed with as little as 1 μM peroxynitrite added to 1 mM Leu-enkephalin. In the presence of 25 mM Na-bicarbonate, the

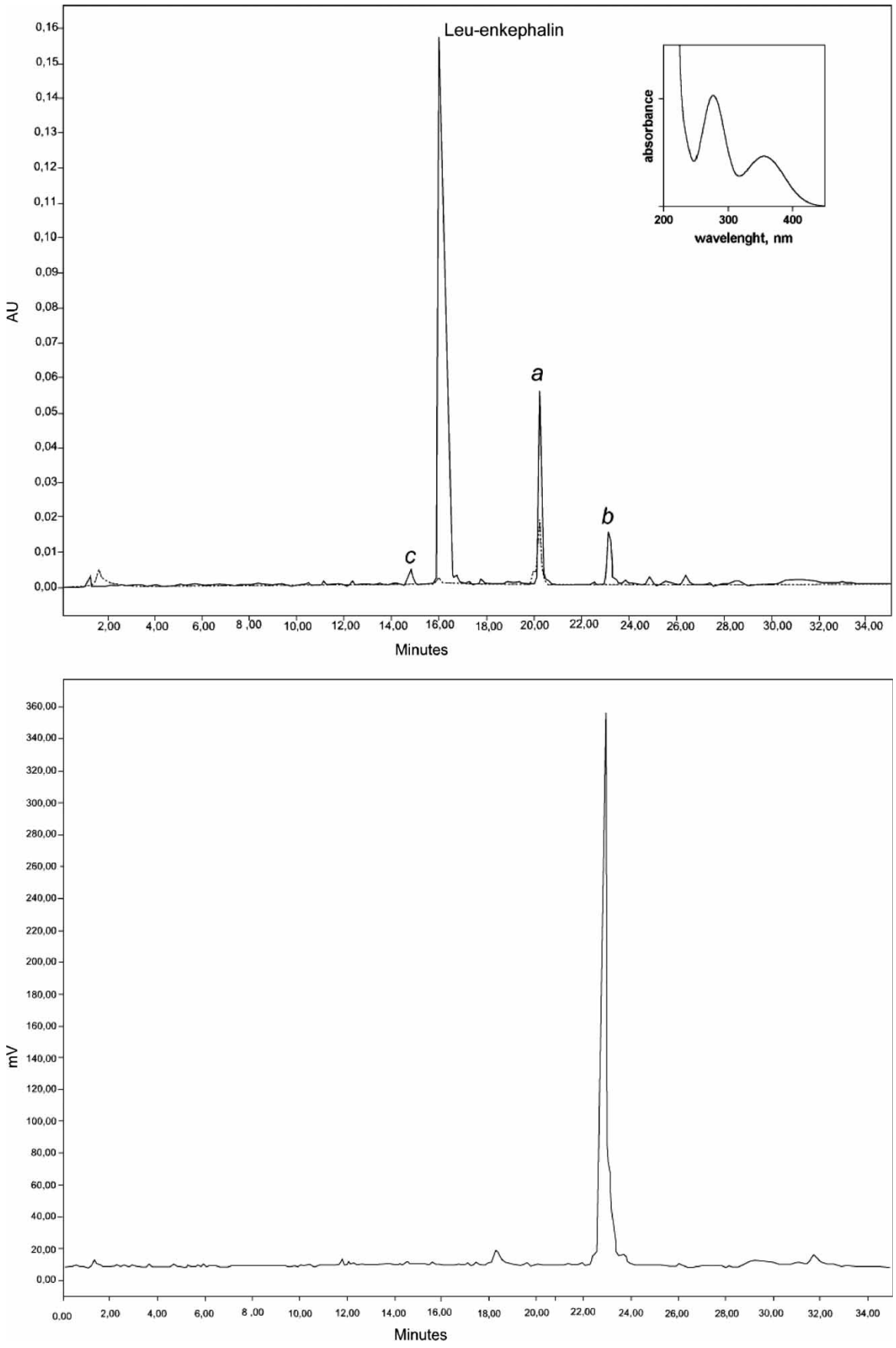

Figure 1. HPLC elution profile of the products formed by the reaction of Leu-enkephalin with peroxynitrite. Leu-enkephalin (1 mM) was reacted with 1 mM peroxynitrite in 0.5 M K-phosphate buffer, pH 7.4. The reaction mixture was analysed by HPLC as described in "Materials and Methods" section. Top: UV detection at 274 nm (—) and 360 nm (–). Inset: UV spectrum of product *a*. Bottom: spectrofluorometric detection (λ_{ex} 260 and 410 nm).

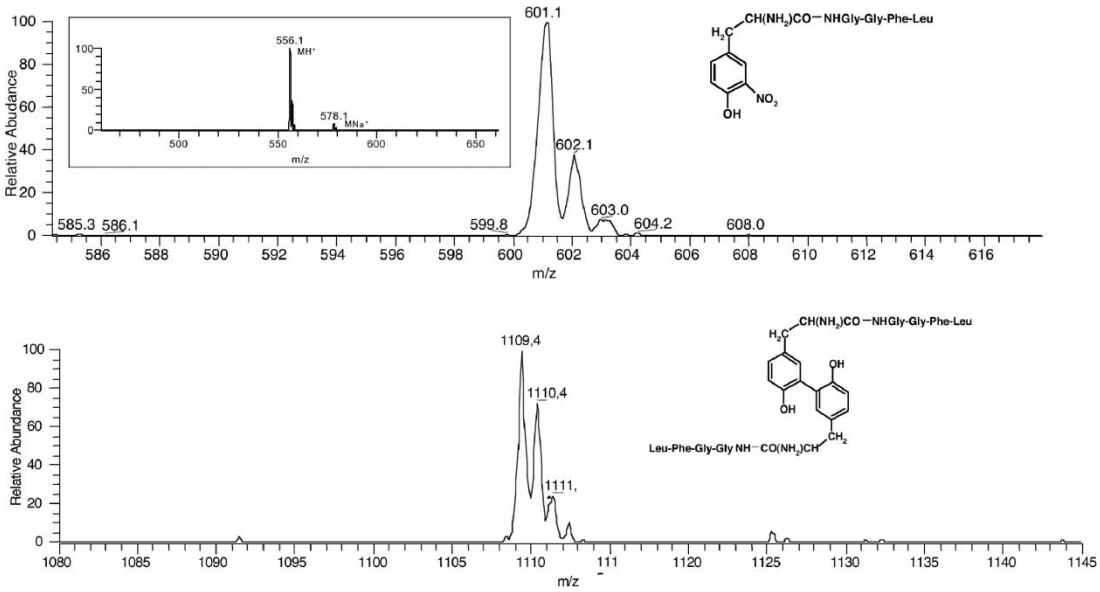

Figure 2. ESI mass spectrometry of products *a* and *b* obtained by reaction of Leu-enkephalin with peroxynitrite and purified by RP-HPLC. Top: Mass spectrum of product *a*, identified as nitroenkephalin; Bottom: Mass spectrum of product *b*, identified as dienkephalin. Inset: Mass spectrum of the parent compound, Leu-enkephalin, and its Na^+-adduct.

nitration reaction was enhanced with a two-fold increase in the yield of nitroenkephalin, while the production of dienkephalin slightly decreased. The yield of nitroenkephalin and dienkephalin in the presence and in the absence of 25 mM bicarbonate is reported in Figure 3. The content of nitroenkephalin and dienkephalin was determined from peak area using 3-nitrotyrosine and dityrosine as reference standards, respectively.

Modification of Leu-enkephalin was also studied using sydnonimine SIN-1, a compound which at neutral pH decomposes to form nitric oxide and superoxide anion simultaneously, at similar rates, and thus forming peroxynitrite [43]. The reaction of Leu-enkephalin (0.5 mM) with equimolar SIN-1 at pH 7.4 for 60 min at 37°C results in formation of 1.40 ± 0.11 μM nitroenkephalin and 1.81 ± 0.22 μM dienkephalin.

Photosensitized oxidation of Leu-enkephalin in the presence of nitrite

It has been reported that the interaction of tyrosine with photochemically generated singlet oxygen leads, through a tyrosyl radical intermediate, to the oxidative modification of aromatic ring and dityrosine has been detected as one of the products [30]. Using methylene blue as sensitizer for the production of singlet oxygen, the photooxidation of Leu-enkephalin was performed in the presence of 1 mM nitrite. In these experimental conditions at mild acidic pH (pH 5.8), nitroenkephalin has been found by HPLC analysis of the reaction mixture. After 30 min illumination of 1 mM Leu-enkephalin in the presence of 10 μM methylene blue and 1 mM nitrite, the yield of nitroenkephalin was 15.5 ± 2.7 μM. No detectable nitroenkephalin was found in illuminated controls lacking methylene blue. In dark controls, 2.3 μM nitroenkephalin was produced. The exposure to light for 30 min of 1 mM Leu-enkephalin in the presence of methylene blue at pH 5.8 resulted in the formation of 34.8 ± 4.4 μM dienkephalin. The yield of dienkephalin decreased to 2.2 ± 0.3 μM when 1 mM nitrite was present. As shown in Figure 4, the formation of nitroenkephalin by the photochemical system at pH 5.8 was found to increase with the concentration of nitrite added. At pH > 7, the amount of nitroenkephalin fells sharply and no nitroenkephalin is detectable above neutrality.

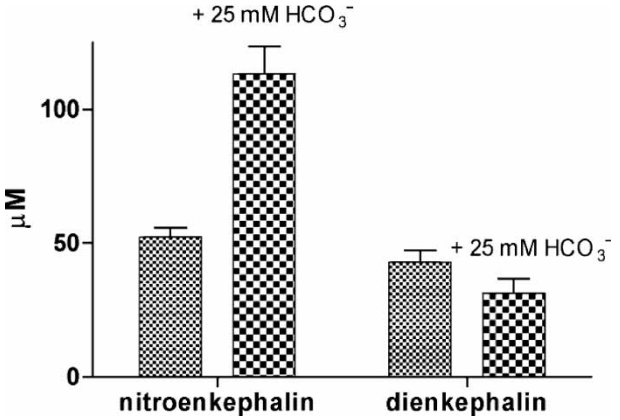

Figure 3. Yield of the oxidative/nitrative products formed by the reaction of Leu-enkephalin with peroxynitrite. Effect of added bicarbonate. Leu-enkephalin (1 mM) was reacted with 1 mM peroxynitrite in 0.5 M K-phosphate buffer, pH 7.4, the concentration of Na-bicarbonate, when present, was 25 mM. The reaction products were analysed by HPLC as described in "Materials and methods" section.

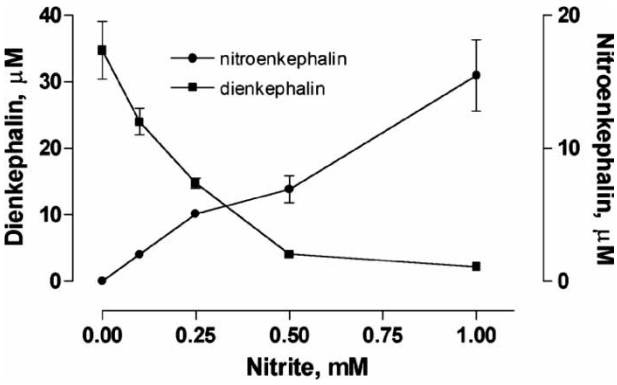

Figure 4. Photosensitized oxidation of Leu-enkephalin in the presence of nitrite. A reaction mixture containing 1 mM Leu-enkephalin, 10 μM methylene blue and potassium nitrite (0–1 mM) in 20 mM K-phosphate buffer, pH 5.8 was illuminated with a 200-W tungsten halogen lamp at a distance of about 10 cm from the solution and allowed to proceed at 25°C for 30 min. Reaction mixtures were analysed by HPLC as described in "Materials and methods" section.

Leu-enkephalin nitration by peroxidase-catalyzed oxidation of nitrite

In the presence of H_2O_2, peroxidases such as HRP or MPO are known to catalyze oxidation of the tyrosine aminoterminal residue of enkephalin to form tyrosyl radicals, as indicated by the production of a dimerization product containing a dityrosine moiety [7]. When the former reaction is allowed to proceed in the presence of nitrite, nitroenkephalin is formed as an additional product as shown after HPLC analysis of the reaction mixture. The identity of nitroenkephalin was confirmed by spectral analysis and by the presence of 3-nitrotyrosine after enzymatic hydrolysis of the collected HPLC peak. The formation of nitroenkephalin was found to increase parallelly with the concentration of nitrite, while the dimer of enkephalin—dienkephalin—decreased,

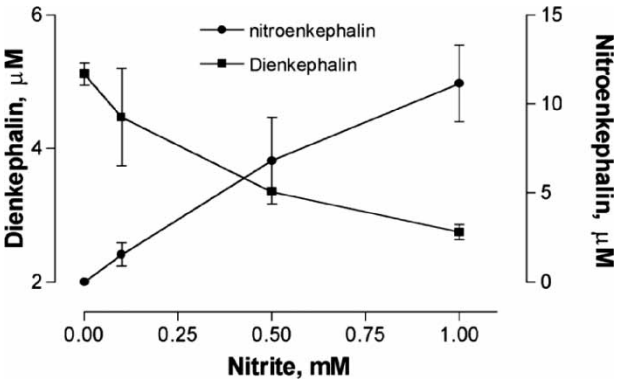

Figure 5. Leu-enkephalin nitration by peroxidase-catalyzed oxidation of nitrite. Leu-enkephalin (1 mM) was incubated with 1 μM HRP and various concentration of potassium nitrite in 20 mM K-phosphate buffer at pH 7.4. The reaction was started by addition of 1 mM H_2O_2 and allowed to proceed at 37°C. Reaction was stopped after 10 min by addition of 10 nM catalase. The reaction products were analysed by HPLC as described in "Materials and methods" section.

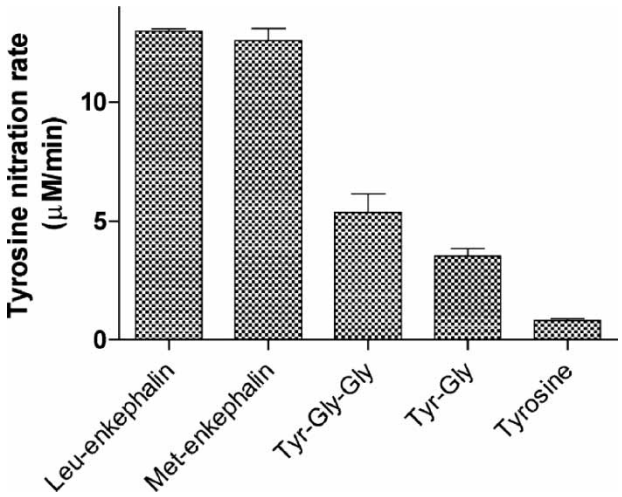

Figure 6. Nitration rate of enkephalins and tyrosine aminoterminal peptides by peroxidase-catalyzed oxidation of nitrite. The nitration rate was followed at 430 nm. The reaction was carried out in 1 ml of 20 mM K-phosphate buffer, pH 7.4, containing 1 mM test compound, 1 μM HRP and 1 mM potassium nitrite. The reaction was started by the addition of 1 mM H_2O_2.

suggesting that nitrite competes with Leu-enkephalin for oxidation by HRP/H_2O_2 (Figure 5). No detectable Leu-enkephalin nitration was observed in the absence of HRP or nitrite.

The nitration rate of Leu-enkephalin was measured spectrophotometrically at 430 nm. Under the reaction conditions, nitroenkephalin was produced at a linear rate. In addition, the nitration rate of enkephalins (Leu-enkephalin and Met-enkephalin) and tyrosine aminoterminal peptides (Tyr-Gly-Gly and Tyr-Gly) by HRP/nitrite/H_2O_2 is compared in the Figure 6 with free tyrosine. In order to establish if Leu-enkephalin could be converted to nitroenkephalin also by mammalian peroxidases, the nitration rate of Leu-enkephalin by HRP was compared with human MPO and LPO in the presence of 10 mM nitrite. In Table I a comparison of the activity of various peroxidase systems on Leu-enkephalin and free tyrosine is reported.

Discussion

In previous experiments, we demonstrated that enkephalins and opioid peptides react easily with oxyradicals giving rise to oxidative products ultimately leading to enkmelanins and opiomelanins. The results presented in this paper provide evidence that peroxynitrite or nitrite/singlet oxygen or peroxidase-catalyzed oxidation of nitrite, are capable of nitrating and oxidizing Leu-enkephalin producing nitroenkephalin and dienkephalin. HPLC analysis shows the formation of two major enkephalin derivatives by peroxynitrite: (a) spectrophotometric analysis of one peak exhibits an absorption spectrum with the spectroscopic properties of 3-nitrotyrosine, indicating the conversion of tyrosine into the

Table I. Nitration rate of Leu-enkephalin and free tyrosine by reaction with various peroxidase/H_2O_2/NO_2^- systems.

Enzyme	Nitroenkephalin (μM/min)	3-Nitrotyrosine (μM/min)
HRP	29.4 ± 6.6	4.1 ± 0.7
LPO	813 ± 5	623 ± 12
MPO	132 ± 36	432 ± 53

The nitration rate was followed at 430 nm. The reaction was carried out in 1 ml of 20 mM K-phosphate buffer, pH 7.4, containing 1 mM Leu-enkephalin or free tyrosine, 1 μM HRP, LPO or MPO and 10 mM potassium nitrite. The reaction was started by the addition of 1 mM H_2O_2. The reported values correspond to the rate of formation of the nitrocompound (μM/min) normalized to enzyme unit.

nitrocompound at the amino terminus of enkephalin; (b) the second peak shows the fluorescence properties of a derivative bearing a dityrosine moiety. The evidence of the formation of the nitroenkephalin and of the enkephalin dimer, dienkephalin, was achieved unequivocally by ESI mass spectrometry. Leu-enkephalin nitration by 1 mM peroxynitrite yields about 5.2 ± 0.5% nitroenkephalin; in the presence of bicarbonate, the nitration reaction, as already known for free tyrosine nitration, was enhanced with a two-fold increase in the nitropeptide yield.

The methylene blue photosensitized oxidation of Leu-enkephalin in the presence of nitrite leads also to the formation of the nitrated peptide. In these experimental conditions, it has been demonstrated for free tyrosine that the oxidation is mediated by singlet oxygen with generation of intermediate tyrosyl radicals which can dimerize to form dityrosine or react with a nitrite-derived species (NO_x) with production of 3-nitrotyrosine [30].

Moreover, nitropeptides can be also obtained by the enzymatic system involving HRP-catalyzed oxidation of nitrite. Though all the peptides tested are converted into nitropeptides, the rate of nitration proceeds differently for the various peptides. Leu-enkephalin and Met-enkephalin appear to be more easily nitrated than tyr-gly and free tyrosine by the HRP/H_2O_2/nitrite system. In contrast, a slight difference in the nitration rate of Leu-enkephalin compared to free tyrosine for either LPO- or MPO-dependent reaction has been noted. However, biochemical experiments involving enkephalin interaction with oxidative enzyme [7,9] have always demonstrated that the rate of oxidation of peptidic substrates is faster than that of free tyrosine. In the same manner, in our experimental conditions, the formation of the nitrated compound is directly correlated with peptide length so that the peptidic bond confirms to be a favourable factor for the oxidative attack. This has been explained by the fact that the carboamidic linkage—permitting a resonance system—can adjuvate the formation of the tyrosyl radical which represents the trigger point for the successive steps in the synthesis of both dimeric linkage and nitration of the phenolic ring [10].

Furthermore, it is noteworthy the relative yields of nitroenkephalin and dienkephalin produced by photochemical system and by peroxidase-generated reactive nitrogen species with different concentrations of nitrite. The dienkephalin formation decreased at higher nitrite concentrations and was inversely related to enkephalin nitration. These results suggest that nitroenkephalin formation competes with enkephalin dimerization, formation of dienkephalin being more significant at low nitrite levels. This competition would imply that both products are formed by a related mechanism, via intermediate formation of tyrosyl radical [24,44]. The tyrosyl radical could either react with NO_x to produce nitroenkephalin or dimerize to give dienkephalin. Combination of tyrosyl radical and $\dot{N}O_2$ radical has been reported to occur with second order rate constant of $3 \times 10^9\,M^{-1}\,s^{-1}$, and a rate constant of $2.25 \times 10^8\,M^{-1}\,s^{-1}$ was reported for the combination of two tyrosyl radicals [45,46]. Such reaction kinetics is in agreement with our results with enkephalin. At low nitrite concentrations, dimerization of enkephalin is the predominant pathway, whereas nitration becomes the predominant reaction at high nitrite (and thus NO_x) concentrations.

Various analogues of enkephalins with modification of tyrosine aminoterminal residue have been described in order to find a modification able to modulate the biological effects of these peptides [47]. It has been found that the enkephalin analogues—dopaenkephalin and cysteinyldopaenkephalin—are still able to bind to δ and μ opioid receptor sites but with a decreased receptor affinity [8,12], while salsolinolenkephalin, a tetrahydroisoquinoline derivative of Leu-enkephalin, shows a total falling in the affinity towards the opioid binding sites [48].

The results of our experiments allows us to hypothesize a further new oxidative/nitrative modification of enkephalins. The data, here reported, clearly demonstrate that nitroenkephalin and dienkephalin can be easily formed, at least *in vitro*, starting from native enkephalin by simple reactions of reactive nitrogen species. Recently, it has been reported that some secretory peptide hormones such as LHRH, angiotensin, vasopressin, oxytocin and enkephalins have a second function as biochemical antioxidants [49] and significant effects *in vitro* can be observed at nanomolar concentrations, which make these peptides comparable in potency with classic antioxidants having low molecular mass. Hence, bioactive peptides, such as enkephalins, may represent also target structures for free radical species [11,49]. Whether enkephalins, undergoing such oxidative/nitrative modifications by reactive species, can display novel and unique biological roles needs to be determined.

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