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Interaction of enkephalins with oxyradicals

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Abstract

The interaction of enkephalins (leu-enkephalin and met-enkephalin) and other tyrosine amino-terminal peptides with reactive oxygen species has been investigated. All the peptides tested exhibited hydroxyl radical and superoxide anion scavenging ability and the capacity to reduce the rate of lipid peroxidation induced by 2,2′-azobis(2-amidinopropane). The scavenging activity was observed in the 0.1–1 mM concentration range. It has been observed that enkephalins underwent an oxidative modification by Fenton systems. The tyrosine amino-terminal residue was attacked by hydroxyl radical, being converted to dopa. The overall transformation produced opiomelanin pigments. This oxidative process provides evidence of a possible route for opiomelanin synthesis without any enzyme intervention.

Keywords: Enkephalins; Antioxidants; Scavengers; Oxyradicals; Opiomelanins

1. Introduction

ROS and other free radicals are broadly recognized as main contributors to aging, neurodegenerative diseases and atherosclerosis [1]. Major sources of ROS are encompassed by aerobic metabolism, specialized physiological functions and xenobiotic metabolism [2]. Under oxidative insult, cellular components, including DNA, proteins and phospholipids are gradually damaged. Protein molecules are subjected to substantial modifications through oxidative reactions. The aromatic amino acid residues are especially susceptible to oxidation by various form of ROS, and in particular tyrosine can be converted to dopa that can undergo redox cycling further triggering the production of additional ROS [3,4].

Opioid peptides belong to a class of bioactive compounds of great interest because of their opiate-like activity [5,6]. In the last years, we have published a series of papers regarding the enzymatic transformation of opioid peptides, documenting that besides peptidases also oxidative enzymes can act upon enkephalins [7]. In particular, enkephalins and also the tyrosine amino-terminal peptides, Tyr-Gly and Tyr-Gly-Gly, produced *in vivo* by the action of peptidases [8] function as substrates for tyrosinase giving rise to peptide-linked pigments [9,10]. All these melanin-like pigments belong to a new class of melanins, collectively named opiomelanins [10].

Melanins are efficient photoprotective agents against solar radiation and they have also the capacity to scavenge oxyradicals [11,12]. Melanins are found in the skin, in sensory organs and in some specific regions of the mammalian brain such as *substantia nigra* and *locus coeruleus* (as neuromelanin) [13]. Tyrosinase is considered responsible for melanin synthesis in the skin, whereas this enzyme has not been found in brain [14], hence, other mechanisms have to be involved to explain neuromelanin synthesis.

Phenylalanine and tyrosine hydroxylation by the Fenton reaction has been documented and the conversion of the hydroxylated products to melanin was observed at physiological pH values [15]. Furthermore, in the presence of H_2O_2 various enzymatic systems can perform the oxidation of catecholamines yielding melanin pigments [16–18].

In this paper, the interaction of enkephalins (leu-enkephalin, met-enkephalin) and tyrosine amino-terminal peptides (Tyr-Gly-Gly, Tyr-Gly) with free radicals (hydroxyl radical, superoxide anion, peroxyl radical) was investigated in comparison with the behavior of free tyrosine. We report

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Abbreviations: ABAP, 2,2′-azobis(2-amidinopropane); dopa, dihydroxyphenyl-alanine; H_2O_2, hydrogen peroxide; leu-enk, leu-enkephalin; met-enk, met-enkephalin; LOOH, linoleic acid 13-hydroperoxide; NBT, nitro blue tetrazolium; PMS, phenazine methosulfate; ROS, reactive oxygen species; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances

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evidence that the above mentioned peptides can scavenge oxyradicals and that the hydroxyl radical, generated by a Fenton system, causes the oxidation of enkephalins into the corresponding melanin pigments.

2. Materials and methods

2.1. Materials

Leu-enk, met-enk, Tyr-Gly-Gly, Tyr-Gly, NBT, and linoleic acid were from Sigma Chemical Co. ABAP was purchased by Polysciences Inc. L-Tyrosine, PMS, NADH, deoxyribose, thiobarbituric acid, butylated hydroxytoluene and all other reagents were from Fluka. All the solutions were prepared using deionized water of very high purity (resistance > 18 MΩ/cm^2) and treated with a Chelex 100 resin, Na^+ form (Sigma Chemical Co.). LOOH was synthesized enzymatically by soybean lipoxidase according to Schilstra et al. [19]. LOOH concentration was checked prior to the experiment using a molar extinction coefficient of 27000 $M^{-1}cm^{-1}$ at 234 nm.

2.2. Activity against hydroxyl radical

Hydroxyl radical was generated by the Fenton reaction in the presence of deoxyribose [20]. Incubation mixture contained: 1 mM H_2O_2, 0.4 mM $FeCl_2$, 0.4 mM EDTA, 2 mM deoxyribose in 50 mM K-phosphate saline buffer, pH 7.4. The reaction mixture was incubated for 1 hr at 37°. Deoxyribose degradation by hydroxyl radical at various peptide concentrations was measured at 532 nm as TBARS. At the end of incubation, 0.5 mL of 1% (w/v) thiobarbituric acid in 0.05 M NaOH was added to each tube with 0.5 mL of 2.8% (w/v) trichloroacetic acid. The tubes were heated for 10 min at 100° in the presence of 0.02% butylated hydroxytoluene. The tubes were cooled on ice and centrifuged; the resulting absorbance was read at 532 nm against appropriate blanks.

2.3. Superoxide anion generation

The effect of enkephalins and tyrosine amino-terminal peptides as superoxide anion scavengers was assayed by the inhibition of NBT reduction by NADH in the presence of PMS [21]. Incubation mixture contained: 73 μM NADH, 15 μM phenazine methosulfate, 50 μM NBT in 20 mM K-phosphate buffer, pH 7.4 and the test compounds at various concentrations. Absorbance variations were determined at 560 nm, measuring the initial rate of superoxide-induced NBT reduction. The inhibition of the NBT reduction rate by 20 μg SOD was taken as 100%.

2.4. Inhibition of lipid peroxidation

Linoleic acid peroxidation was induced by a water-soluble initiator, ABAP. Lipid peroxidation was detected by conjugated dienes formation. The inhibition of lipid peroxidation by test compounds was measured according to the method of Pryor et al. [22]. Linoleic acid (2.6 mM) was dispersed in micelles of 0.1 M SDS in 50 mM Na-phosphate buffer, pH 7.4 as described by Longoni et al. [23]. In a typical assay, 2.4 mL of linoleic acid suspension was added to the sample cuvette and thermostated at 37°. In the reference cuvette was added the same solution without linoleic acid. The conjugated dienes formation was monitored at 236 nm. Then, 5 μL of 0.5 M ABAP was added both in the sample and in the reference cuvette and incubation was carried out until the autoxidation rate became constant. Finally, the test compound was added to both cuvettes and the decrease in the rate of linoleic acid peroxidation produced by enkephalins and tyrosine amino-terminal peptides was measured.

2.5. Melanin detection

Leu-enk and Tyr-Gly-Gly oxidation into the corresponding melanin pigments was carried out in the presence of iron(II)-EDTA and H_2O_2. 1 mM peptide was incubated at room temperature in the presence of 0.2 mM ammonium iron(II) sulfate, 0.2 mM EDTA and 0.5 mM H_2O_2 in 50 mM K-phosphate buffer saline solution, pH 7.4. Blanks without iron(II)-EDTA or H_2O_2 were simultaneously run. After 24 hours, the reaction was stopped by addition of 2 N HCl to reach pH 2. The incubation mixture was centrifuged at 12000 *g*; then, the pellet was collected and dissolved in 0.1 M K-phosphate buffer, pH 7.4. Absorbance was measured within the range 220–600 nm to verify the melanin generation.

2.6. Amino acid analysis

Aliquots (500 μL) of the above reaction mixture at the indicated times were hydrolyzed in 0.6 N HCl under vacuum at 110° overnight to assess the amino acid residues modification. The amino acid content of the sample was determined by HPLC analysis [24] after precolumn derivatization with *o*-phthaldialdehyde according to Jarret et al. [25]. HPLC was a Waters-Millipore apparatus with a reverse phase column (150 mm $\times$ 4 mm Hypersil ODS, 5 μ). Eluates were monitored by a Perkin-Elmer Model LS-1 LC fluorescence detector using a 340 nm filter for excitation with an emission wavelength of 450 nm. The amino acid amount was calculated by computer-assisted peak area integration from calibration curves obtained with standard solutions of tyrosine, dopa, glycine, phenylalanine and leucine.

2.7. Statistical treatment of data

All the experiments were repeated at least three times with similar results. The figures show either single representative results or means ($\pm$ SE).

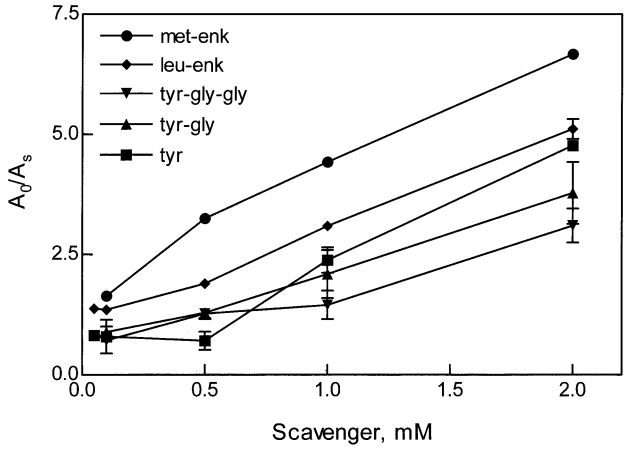

Fig. 1. Effect of enkephalins and tyrosine amino-terminal peptides on deoxyribose degradation induced by the Fenton reagent. The mixture contained 2 mM deoxyribose, 0.4 mM Fe(II)/EDTA, 1 mM H_2O_2 in 50 mM K-phosphate saline buffer, pH 7.4 and was incubated for 1 hr at 37° in the presence of the indicated amount of test compound. A_0/A_s indicates the relative extent of deoxyribose degradation in the absence (A_0) and in the presence (A_s) of the test compound as determined by TBARS assay. Data are means ± SE.

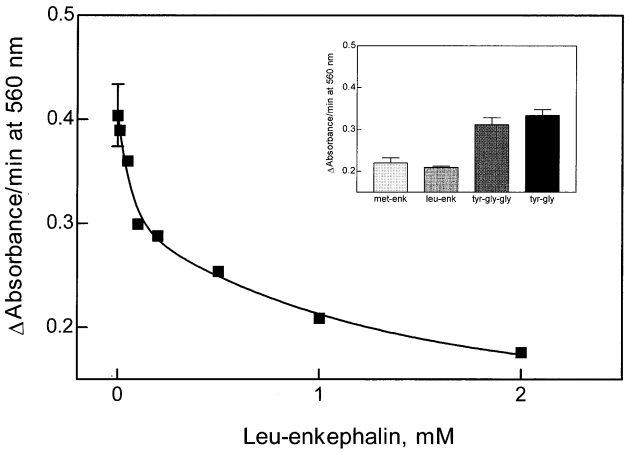

Fig. 2. Superoxide scavenging activity of leu-enk as a function of peptide concentration. Incubation mixture contained 73 μM NADH, 15 μM PMS, 50 μM NBT, and the indicated amount of leu-enk in 1 mL 20 mM K-phosphate buffer, pH 7.4. Inset: Superoxide scavenging activity exerted by 1 mM enkephalins and tyrosine amino-terminal peptides. Reaction rate has been measured as ΔAbsorbance/min at 560 nm. Data are means ± SE.

3. Results

The aim of this article was to study the free radical scavenging ability of opioid peptides and their inhibition of lipid peroxidation *in vitro*. Furtherly, the oxidative modifications of these peptides throughout the Fenton reaction have been determined.

Experiments have been carried out in order to test the scavenging activity of opioid peptides both on hydroxyl radicals and on superoxide anion. The effect of varying concentrations of enkephalins, Tyr-Gly-Gly, Tyr-Gly and tyrosine on the hydroxyl radical-induced degradation of deoxyribose is shown in Fig. 1. All the peptides show a dose-dependent inhibitory effect. Leu-enk and met-enk appear to be more active than tyrosine and tyrosine amino-terminal peptides as determined by the TBARS assay. The superoxide anion scavenging ability of leu-enk, as a function of the peptide concentration is exhibited in Fig. 2. The comparison of enkephalins with tyrosine amino-terminal peptides at 1 mM concentration is reported in the inset of Fig. 2. All the peptides exhibit a dose-dependent activity: the percentage inhibition of the NBT reduction rate by 1 mM leu-enk and met-enk is 48.9% and 46.3%, respectively, whereas the other peptides are less effective inhibitors. A very similar dose-dependent inhibitory effect was exhibited by enkephalins, Tyr-Gly-Gly, Tyr-Gly and tyrosine on lipid peroxidation. The increase in the absorption at 236 nm, due to the formation of conjugated dienes hydroperoxide of linoleic acid induced by ABAP, was measured, to reveal that all the tyrosine amino-terminal peptides reduce the rate of linoleic acid peroxidation. The response curve is shown in Fig. 3: at 100 μM enkephalin, the reduction of the rate of lipid peroxidation is 52.7% and 50.5% for leu-enk and met-enk, respectively.

In order to determine the products obtained by reaction of the peptides with hydroxyl radical, Tyr-Gly-Gly and leu-enk oxidation in the presence of iron(II)-EDTA and H_2O_2 were investigated spectrophotometrically. In both cases an increase in the absorbance around 310 nm, indicative of opiochrome formation, is observed (Fig. 4) [26]. After prolonged incubation (24 hr) and upon acidification, opiomelanins from these bioactive peptides are easily recovered by centrifugation. The UV-vis spectrum of oxidized Tyr-Gly-Gly by the Fenton system is compared with the spectrum obtained by the tyrosinase action on Tyr-Gly-

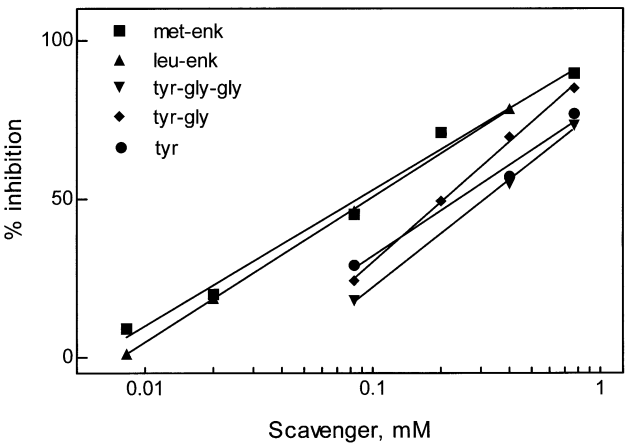

Fig. 3. Inhibition of lipid peroxidation by enkephalins and tyrosine amino-terminal peptides. Incubation mixture contained 2.4 mL of 2.6 mM linoleic acid dispersed in micelles of 0.1 M SDS in 50 mM Na-phosphate buffer, pH 7.4. The rate of conjugated diene formation was measured at 236 nm. The inhibition was calculated according to the equation: % inhibition = 100 − [(rate with inhibitor/rate without inhibitor) × 100]. Data are means ± SE.

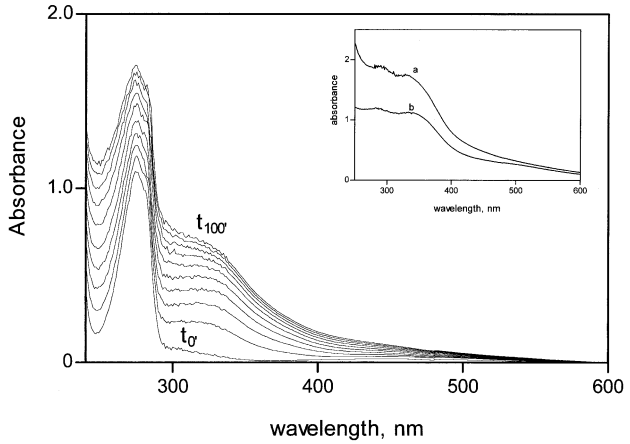

Fig. 4. Tyr-Gly-Gly oxidation by Fenton reagent. 1 mM Tyr-Gly-Gly was incubated in the presence of 0.2 mM EDTA/Fe(II) and 0.5 mM H_2O_2 in 50 mM K-phosphate buffer saline solution, pH 7.4 at room temperature. The spectra were recorded every 10 min ($t_{0'}$–$t_{100'}$). Inset: The spectra of Tyr-Gly-Gly melanin (a) recovered after 24-hr incubation in the same system compared to that obtained by tyrosinase (b).

Gly in the inset of Fig. 4; the typical spectra of opiomelanins can be observed in both conditions [10,27]. Hence, hydroxyl radical and tyrosinase give rise to an identical pigment.

In order to assess the modification of amino acid residues during oxidation by iron(II) chelate and H_2O_2, leu-enk hydrolysis at various times was carried out and the amino acid content analyzed by HPLC. The results demonstrate that the amino-terminal tyrosine residue is the main target of the hydroxyl radical attack (Fig. 5). The analysis provides evidence of the immediate production of dopa, whose amount reaches a maximum within 15 min, disappearing after 60 min, because of further oxidation and then conversion into the melanin pigment. Comparing enkephalin oxidation performed by iron(II) chelate and H_2O_2 with that obtained by iron(II)-EDTA/LOOH system, although both reactions exhibit identical spectral changes around 310 nm, a difference can be found in the yield of dopa, much smaller in the latter system.

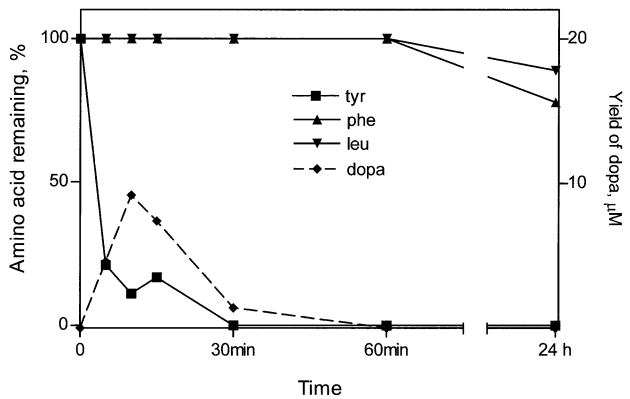

Fig. 5. Leu-enk amino acid residues decay and modification by Fenton system as a function of time. 1 mM leu-enk was incubated in the presence of 0.2 mM EDTA/Fe(II), 2 mM H_2O_2 in 50 mM K-phosphate buffer saline solution, pH 7.4. At the indicated times, aliquots were hydrolyzed in 6.0 N HCl under vacuum at 110° overnight. Amino acids were determined by HPLC as reported in experimental procedures.

4. Discussion

The results presented in this paper provide evidence that opioid peptides can interact with oxyradicals. Furthermore, the data support the hypothesis of hydroxyl radical mediated synthesis of opiomelanins from enkephalins.

All the opioid peptides tested have hydroxyl radical and superoxide anion scavenging properties, and the capacity to reduce the rate of lipid peroxidation. The scavenging activity towards hydroxyl radical and the protection of lipid peroxidation is higher than superoxide anion scavenging ability at the effective concentrations of the various compounds examined. In our experimental conditions the effect is dose-dependent and the peptides exhibit the same rank order of activity. Regarding the differences among the various tyrosine amino-terminal peptides studied, leu-enk and met-enk are more effective as scavengers than the other peptides and free tyrosine, these latter compounds showing a three-fold lower antioxidant activity than enkephalins. Met-enk is slightly more effective than leu-enk as a radical scavenger probably because of the known antioxidant role of the thioether moiety [28]. After the reaction with oxyradicals, enkephalins undergo an oxidative modification. The main target of oxyradicals is the tyrosine amino-terminal residue which is attacked by hydroxyl radical and converted into dopa. The oxidation further proceeds producing a brownish pigment that is easily collected by acidification and centrifugation. The UV-vis spectrum of the pigment shows an absorption pattern indicative of opiomelanin formation [10].

Previous works demonstrated that ROS attack on proteins converts intramolecular tyrosine residues into dopa which can be involved in redox reactions [3,4], whereas free tyrosine could scavenge oxyradicals forming tyrosyl radicals which can dimerize to dityrosine [29]. We demonstrate the occurrence of further oxidative modifications of dopa residues in peptides which are converted into melanin pigments when the oxidized tyrosine is amino-terminal.

Our results indicate that enkephalins, bearing an amino-terminal tyrosine, effectively counteract free radical species and that this action is higher than that exerted by free tyrosine. Enkephalins oxidation by hydroxyl radical generates melanin pigments that are known to actively counteract ROS and thus further contribute to the antioxidant effect [11]. Enkephalins can reach high levels at synaptic vesicles and at inflammation sites, where the concentration of these bioactive peptides may be close to that found in our experiments.

Up to now, opiomelanins have been synthesized by tyrosinase oxidation of opioid peptides [10]. Melanin pigments formation from enkephalins by a Fenton-like system is indicative of a possible role of hydroxyl radicals in key transformation of enkephalins, which, under special conditions, can lead to opiomelanins without any enzyme intervention.

The mammalian brain seems to be lacking in tyrosinase, hence the eventual biosynthesis of neuromelanin from dopamine as well as that of opiomelanins from enkephalins remains an open question [14]. As a matter of fact, monoaminergic synaptic terminals, like nigrostriatal dopaminergic pathways, are important districts of free radical sources, thereby significantly exposed to oxidative stress [30]. Some authors suggested a possible role for free radicals in dopamine oxidation in the brain leading to neuromelanin synthesis [31,32]. Indeed, it has been established that melanization in these regions occurs as a consequence of cell oxidative stress actually envisaged as responsible for the onset of Parkinson's disease and of related nigrostriatal disorders [33]. In this scenario, considering the co-localization of dopamine and enkephalins at the synaptic terminal in the same neural cell [34], enkephalins exposure *in vivo* to ROS cannot be ruled out. Our results are in keeping with previously published papers on ROS involvement in neuromelanin generation and provide insight into the possible generation of melanin pigments from enkephalins by the action of oxyradicals.

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