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NEUTROPHIL-CATALYSED DIMERISATION OF TYROSYL PEPTIDES

SAÏDA SALMAN-TABCHEH, NAJAT RABGAOUI and JEAN TORREILLES*

INSERM unité 58, 60 rue de Navacelles, 34090 Montpellier - France

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Evidence is given that tyrosyl-peptides are dimerised by polymorphonuclear leukocytes leading to a new family of compounds. The products formed are homo- and hetero-dimeric peptides with linkage between the tyrosyl residues. This corresponds to a dityrosine structure as determined by analytic and spectroscopic data.

KEY WORDS: Peroxidase, tyrosyl-peptide, neutrophil.

Abbreviation: PMN, polymorphonuclear leukocyte; PMA, phorbol myristate acetate; HRP, horseadish peroxidase; MPO, myeloperoxidase; SOD, superoxide dismutase; Leu-enkephalin, tyrosyl-glycyl-glycyl-phenylalanyl-leucine; Kyotorphin, tyrosyl-arginine

INTRODUCTION

When they ingest bacteria or parasites or are involved in the inflammatory response to tissue injury, polymorphonuclear leukocytes (PMN) undergo the so-called "oxidative burst" reaction and release a variety of toxic agents outside of the cell: oxygen-derived reactive species ($O_2^{\cdot}$, H_2O_2, $HO\cdot$ and HOCl), proteinases (elastase and cathepsins); lipids (arachidonic acid and metabolits) peptides, (defensins, enkephalins, ACTH) and a PMN-specific peroxidase: myeloperoxidase (MPO). At the inflammation site, these agents could have cytotoxic functions through a variety of mechanisms. They could also react to each other and produce new biochemical species in cell environments, thus creating a network of positive and negative stimuli for the original cells, there inflammatory cell neighbours and tissue cells in the environment.

Peroxidases are known to catalyse oxidation of a large variety of substrates[1] used as hydrogen donors, including phenols[2,3], aromatic amines[4] and tyrosine[5,6]. The reaction leads to formation of radical intermediates which are deactivated by forming oligomeric products.

Rosei *et al.*[7,8] recently observed that leu-enkephalin or met-enkephalin can act *in vitro* like hydrogen donors for horseradish peroxidase (HRP), myeloperoxidase (MPO) or lactoperoxidase (LPO) by inducing dimer formation with both monomers linked through a dityrosine unit.

MPO is present in human neutrophils in exceptionally high concentrations[9] and several authors have shown that these cells produce and secrete hydrogen peroxide

*All correspondence to J. Torreilles, Unité 58 de l'INSERM, 60, rue de Navacelles, 34090 Montpellier, France; Fax: 67 52 06 77.

and peptide hormones such as endorphins and enkephalins[10,11]. We thus assumed that interactions between opioid peptides and cellular peroxidases could occur in the vicinity of stimulated PMNs present at inflammatory sites, leading to the formation of various "activated" peptides bearing tyrosyl radicals which could dismutate and give rise, as shown in Scheme 1, to stable peptidic homo- and hetero-dimers.

To determine whether dimers from morphinomimetic peptides could be formed *in vivo* through peroxidase-catalysed oxidation, we compared HPLC chromatograms of an equimolar mixture of leu-enkephalin and kyotorphin (previously incubated with human PMA-stimulated PMNs) with HPLC chromatograms of the same peptides incubated with HRP and H_2O_2. The study was extended to non-morphinomimetic peptides with tyrosyl residues in their sequence.

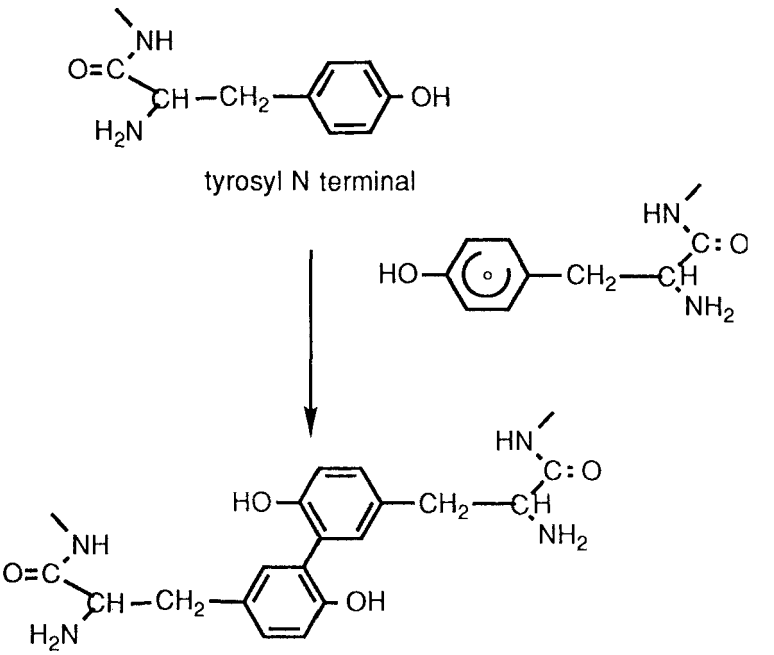
MATERIAL AND METHODS

Reagents

Peptides were from Bachem (Feinchemikalein, AG), HRP was from Boehringer (Mannheim) and H_2O_2 from Merck (Darmstadt).

Cell Preparation

Human peripheral blood obtained from healthy volunteer donors was drawn into heparinized tubes and centrifuged for 10 min at 4000 g. Plasma was withdrawn and PMNs were isolated by differential density centrifugation on Percoll (density 1.13, Pharmacia France S.A.). Contaminating erythrocytes were removed by hypotonic



SCHEME 1 Structure of the dimer.

lysis with Tris [hydroxymethyl] aminomethane buffer (8.3 mM, pH 7.5). The cells were subsequently washed twice in Krebs-bicarbonate solution (pH 7.4) of the following composition (mM): glucose 8.3, KH_2PO_4 1.2, $NaHCO_3$ 2.5, $CaCl_2$ 2.5, KCl 4.7, $MgSO_4$ 7 H_2O 1.2 and NaCl 118.1. The cells were then resuspended to yield 4×10^6 cells/ml.

HPLC Analysis

HPLC analysis was performed with a dual pump LKB 2150 apparatus under the following conditions: samples were applied on a reverse-phase column ODS Si 100 Serva, eluent A was 0.1 M NaH_2PO_4 buffer (pH 2.5) and eluent B was eluent A containing 50% acetonitrile. The gradient went from 0–60% B in 10 min, the flow rate was 1.5 ml/min. The fluorescence emission of the effluents was monitored with a Jasco 820 FP fluorescence detector.

Methods

HRP-catalysed Dimerisation

The assay mixture contained: 2 mM peptide, 4 mM H_2O_2, 3 μM HRP, 0.1 M borate buffer (pH 9.5) in 1 ml final volume. The reaction was incubated for 15 min at 37°.

PMN-catalysed Dimerisation

100 mM kyotorphin or 100 mM leu-enkephalin or an equimolar mixture of kyotorphin and leu-enkephalin (100 mM) were incubated for 7 min with PMNs (4×10^6 cells/ml in Krebs/bicarbonate buffer, pH 7.4) and PMA (100 ng/ml final concentration) was added. After 30 min incubation at 37°, the cell suspensions were centrifuged (35000 rpm for 35 min) and the supernatants recovered for analysis.

Identification of Peptides

For identification of HPLC peaks of dimeric peptides, acid hydrolysis of each peptide was carried out (6N HCl, 110°C for 16 h under vacuum) and the hydrolysate was coinjected on the HPLC apparatus with the authentic standard dityrosine. The amino acid composition of peptides were determined after hydrolysis with a Waters Picotag system (Waters Associates, Milford, MA).

RESULTS

UV and fluorescence spectra obtained using leu-enkephalin or kyotorphin as HRP substrate were very similar to those reported by Rosei *et al.*[7,8] for formation of dimers with dityrosine linkage: two absorbence maxima at 290 nm and 315 nm and a fluorescence emission maximum at 400 nm when excited at 325 nm. We then used these spectroscopic data to monitor dimer formation and record HPLC chromatograms of the reaction mixtures obtained after incubation of various tyrosyl-peptides with HRP and H_2O_2 or with human PNM-stimulated PMNs.

To quantitate the amount of $(kyotorphin)_2$ formed by human PMA stimulated

PMNs, we compared dityrosine production with tyrosine disappearance as function of the incubation time of kyotorphin with the HRP/H_2O_2 system (Figure 1). The increase of dityrosine was monitored by fluorescence emission at 400 nm (excitation at 325 nm). A parallel decrease in tyrosine content was observed by recording fluorescence emission at 300 nm (excitation at 280 nm). The time coursed spectral modifications of kyotorphin incubated with human PMA-stimulated PMNs is shown in Figure 2. A decrease in the emission peak at 300 nm present at zero incubation time parallel to a strong increase in a new emission peak at 400 nm was observed. The similar results obtained in both experiments showed that kyotorphin is substrate for HRP as well as leukocyte MPO.

Since, as reported by Rosei *et al.*[7,8] leu-enkephalin was also used as substrate by both enzymes, producing dimers, we used HPLC to analyse the behaviour of an

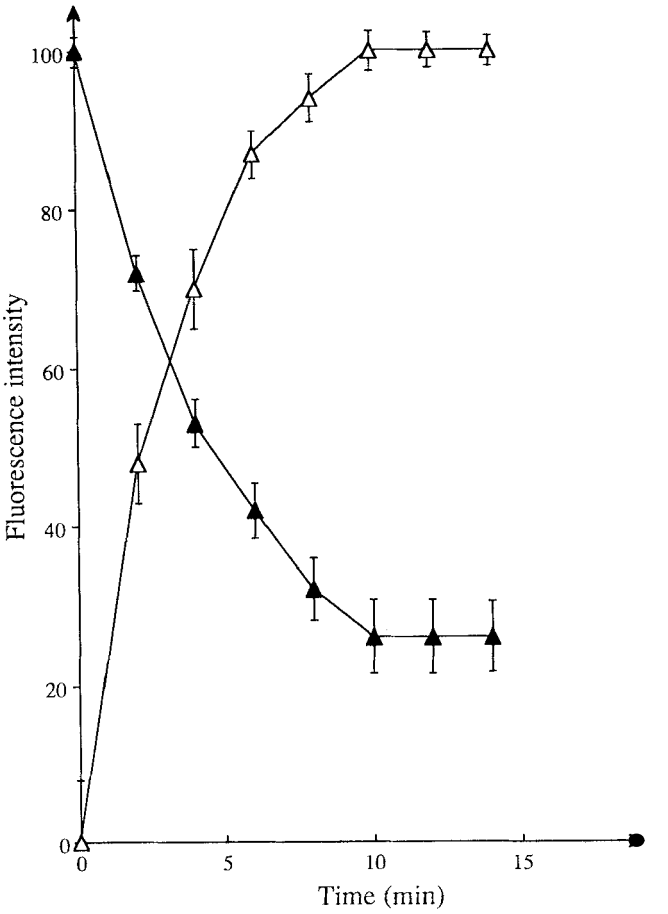


FIGURE 1 Disappearance of kyotorphin and production of $(kyotorphin)_2$ as a function of the incubation time of kyotorphin with HRP/H_2O_2 system. The values were calculated with the initial kyotorphin fluorescence intensity (λ_{em} 300 nm, λ_{ex} 280 nm) or the maximum value of fluorescence intensity of $(kyotorphin)_2$ (λ_{em} 400 nm, λ_{ex} 325 nm) taken as 100. Kyotorphin (▲); $(kyotorphin)_2$ (△). Each value is the average of 3 different experiments.

equimolar mixture of kyotorphin and leu-enkephalin submitted to the action of the HRP/H_2O_2 system or to human PMA-stimulated PMNs.

Figure 3 shows chromatograms obtained when HRP and H_2O_2 were added to the borate buffered solution (pH 9.5) with kyotorphin (Figure 3A) and leu-enkephalin (Figure 3B). Since the chromatograms were recorded with an emission wavelength at 400 nm and an excitation wavelength at 325 nm, the kyotorphin and leu-enkephalin monomers, which have an emission maximum at 300 nm and excitation maximum at 280 nm, appeared as small peaks with retention time (RT) = 3.2 min and RT = 8.8 min respectively (RT of monomers were confirmed by recording HPLC chromatograms at the fluorescence emission maximum of tyrosyl residues).

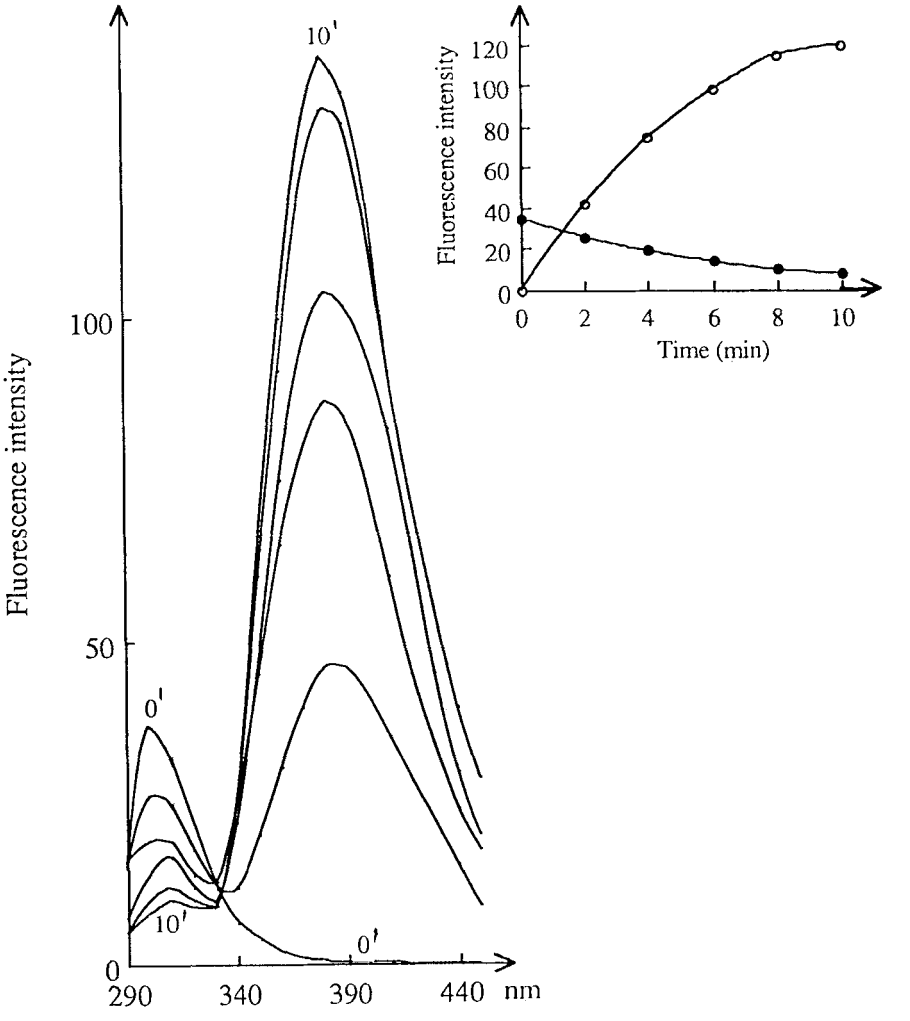


FIGURE 2 Time course of spectral modifications of leu-enkephalin oxidised by action of PMA-stimulated-PMNs in Krebs bicarbonate buffer pH 7.4. Spectra were recorded every 2 min (λ_{ex} 280 nm) from 0′ to 10′. Inset: emission at 300 nm (-●-●-) and 400 nm (-○-○-).

The column eluates corresponding to peaks with RT = 3.5 min in Figure 3A and RT = 11.5 min in Figure 3B were collected and identified by amino acid analysis. They corresponded to homo-dimers (leu-enkephalin)$_2$ and (kyotorphin)$_2$ respectively (Table 1).

Figure 3C shows the chromatogram for an equimolar mixture of leu-enkephalin and kyotorphin incubated for 30 min at 37° with HRP and H_2O_2. Peaks with RT = 3.5 min and RT = 11.5 min corresponded to (kyotorphin)$_2$ and (leu-enkephalin)$_2$ respectively, while the peak with RT = 7.8 min corresponded to a hetero-dimer: (leu-enkephalin-kyotorphin) as confirmed by amino acid analysis.

Since PMNs contain high concentrations of MPO and secrete it extracellularly with H_2O_2 and $O_2^{\cdot}$ during the burst[9], we tested whether similar oxidative dimerisations could be effective in media containing stimulated PMNs by incubating leu-enkephalin or kyotorphin or an equimolar mixture of leu-enkephalin and kyotorphin for 7 min with PMNs suspended in buffer and then stimulated by the addition of PMA.

After 30 min incubation at 37°, cell suspensions were centrifuged and the supernatants tested for fluorescence emission at 400 nm (λ_{ex} 325 nm). An increased fluorescence emission was observed when compared with the cell suspensions incubated in the same conditions but without the addition of PMA. The peptide contents of supernatants were analysed by HPLC and showed very similar chromatograms to those obtained after peptide treatment with the HRP/H_2O_2 system (Figure 3C), thus indicating that stimulated PMNs could promote the formation of homo- and hetero-dimers of tyrosyl-peptides at physiological pH.

As shown in Figure 4, the tyrosyl-peptide dimerisation was dependent on MPO since sodium azide, an MPO inhibitor, inhibited 90% of the fluorescence emission recorded at the maximum wavelength for dityrosine. It was also dependent on H_2O_2 since the addition of catalase, (which converts H_2O_2 into H_2O) inhibited fluorescence emission. Moreover, the addition of SOD enhanced the fluorescence emission by dismutation of the superoxides released by stimulated PMNs, thus increasing the amounts of H_2O_2 in the vicinity of the cells.

These results were extended to non-morphinomimetic peptides by incubating tyrosyl-peptides with specific spectral characteristics: tyrosyl-glycyl-glycyl-phenylalanine (phenylalanine fluorescence at λ_{em} 280 nm and λ_{ex} 260 nm) and tyrosyl-tryptophan (tryptophan fluorescence at λ_{em} 360 nm and λ_{ex} 300 nm), with HRP and H_2O_2 or with stimulated PMNs. HPLC analysis of the obtained peptides were

TABLE 1
Retention times of some homo- (A_2, B_2) and hetero- (AB) dimers prepared from various tyrosyl-peptides (A and B)

Peptides	Monomers		Dimers		
			homo	homo	hetero
Tyr-gly-gly-phe-leu	8.8		11.5		
Tyr-arg		3.2		3.5	7.8
Leu-tyr-leu	8.1		11.3		
β-Ala-tyr		4		5.3	8.2
Gly-tyr-gly	3.3		4.6		
Gly-tyr-gly-gly-phe-met		8.3		10.4	7.9
Tyr-gly-gly-phe	6.7		8.6		
Tyr-gly-gly		3.2		3.7	6.1

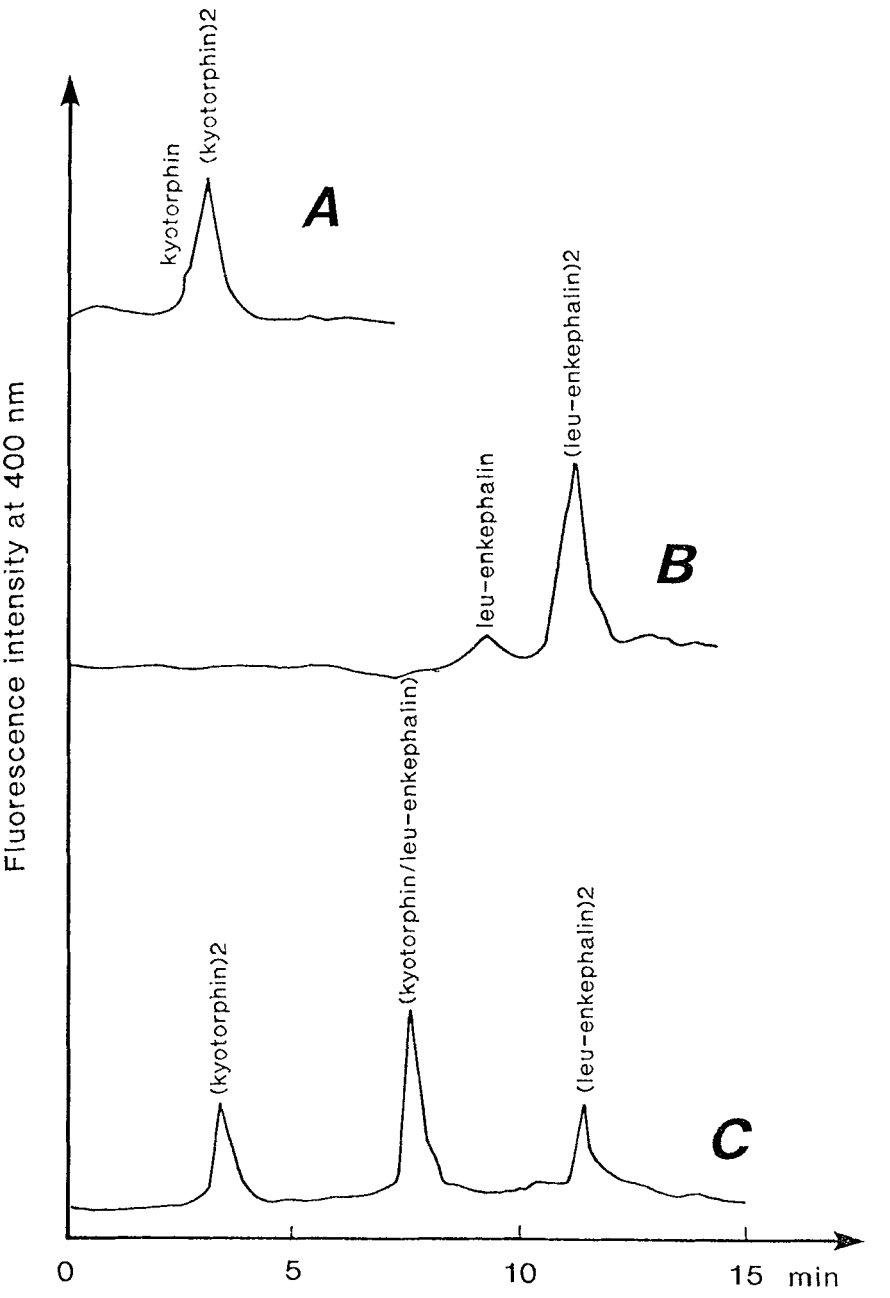


FIGURE 3 HPLC chromatograms of peptides treated with peroxidase/H_2O_2 system in borate buffer pH 9.5. (A) kyotorphin, (B) leu-enkephalin, (C) equimolar mixture of kyotorphin and leu-enkephalin.

performed for identification by recording spectra at the maximum wavelength for dityrosine, phenylalanine and tryptophan (Table 2).

The peptide with RT = 10.5 min which showed absorptions at the maximum

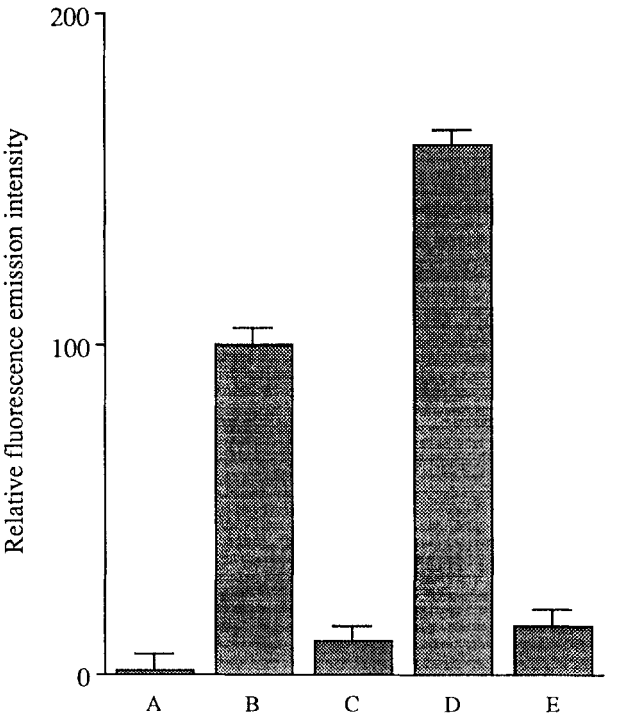


FIGURE 4 Relative fluorescence emission intensities at 400 nm (λ_{ex} 325 nm) of a mixture of 10^{-4} M leu-enkephalin and 10^{-4} M kyotorphin after incubation with 4×10^6 PMNs in Krebs bicarbonate buffer pH 7.4 for 30 min. (A) resting PMNs, (B) PMA-stimulated PMNs, (C) B + 1 mM sodium azide, (D) B + 10 U SOD, and (E) B + 10 U catalase. Each value is the average of 7 different experiments.

TABLE 2
Retention times of peptides produced by incubation of an equimolar mixture of tyrosyl-glycyl-glycyl-phenylalanine and tyrosyl-tryptophan with 4×10^{-6} PMA-stimulated PMNs. For identification, HPLC chromatograms were recorded at the maximum emission wavelength of tyrosine (λ_{em} 300 nm, λ_{ex} 280 nm), dityrosine (λ_{em} 400 nm, λ_{ex} 325 nm, phenylalanine (λ_{em} 280 nm, λ_{ex} 260 nm) or tryptophan (λ_{em} 360 nm, λ_{ex} 300 nm)

Peptides	RT of the peak observed at the emission wavelength of			
	tyrosine	dityrosine	tryptophan	phenylalanine
Tyr-gly-gly-phe	6.7			6.7
Tyr-trp	7.5		7.5	
$(Tyr\text{-}gly\text{-}gly\text{-}phe)_2$		8.6		8.6
(Tyr-gly-gly-phe)-(Tyr-trp)		10.5	10.5	10.5
$(Tyr\text{-}trp)_2$		11.7	11.7	

wavelength of dityrosine, tryptophan and phenylalanine was identified as the hetero-dimer (Tyr-gly-gly-phe)-(Tyr-trp). The peptide with RT = 8.6 min showing absorptions at the maximum wavelength of dityrosine and phenylalanine was identified as the homo-dimer $(Tyr\text{-}gly\text{-}gly\text{-}phe)_2$. The peptide with RT = 8.6 min showing absorptions at the maximum wavelength of dityrosine and tryptophan was identified as the homo-dimer $(Tyr\text{-}trp)_2$.

The formation of dimers from tyrosyl-peptides appears to be caused by radical species generated from reaction of tyrosyl-peptides with HRP/H_2O_2 or with leukocyte MPO released by the PMA-stimulated PMNs. Since tyrosine phenoxy radicals which emit chemiluminescence[12] have been detected in the reaction of tyrosine with HRP/H_2O_2 at pH 9.5, we studied the chemiluminescence emitted from the reaction mixture of leu-enkephalin with HRP/H_2O_2 or with PMA-stimulated PMNs. As shown in Figure 5, chemiluminescence emission was observed, the intensity gradually increased for 35 min suggesting that the peroxidase (HRP or MPO) abstracted hydrogen radicals from tyrosyl residues to give phenoxy radicals which in turn condensed into dimers (Figure 5).

DISCUSSION

The results reported in this paper confirm those of Rosei *et al.*[7,8] showing that *in vitro*, morphinomimetic peptides such as kyotorphin and leu-enkephalin are substrates for HRP and lead to the formation of homo-dimers with two absorbance maxima at 290 and 315 nm and a strong fluorescence emission at 400 nm when excited at 325 nm.

Moreover, our investigations indicate that hetero-dimers are produced when

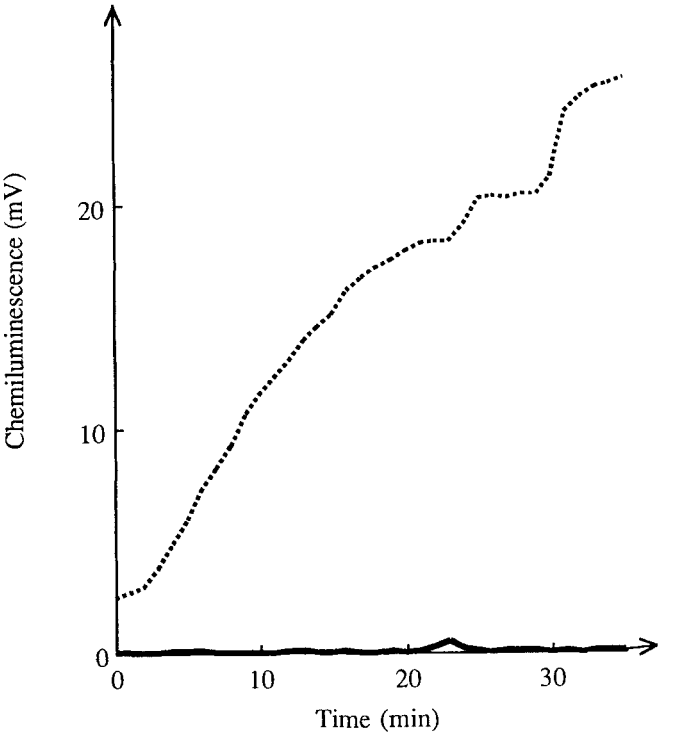


FIGURE 5 Chemiluminescence intensities were measured with an LKB Wallac luminometer connected to an Apple computer. Measurements were carried out in 0.1 M borate buffer (pH 9.5) once per min from 0 to 35 min. (---) 1.35 mM leu-enkephalin, 1 mM H_2O_2 and 10 μg/ml HRP. (—) control without leu-enkephalin.

kyotorphin and leu-enkephalin were used simultaneously as HRP substrates. These reactions could be effective *in vivo*. Indeed, incubation of morphinomimetic peptides with stimulated PMNs, which are known to release MPO and H_2O_2 when degranulated, leads (at physiological pH) to the formation of both homo- and hetero-dimers similar to those obtained by reaction with HRP and H_2O_2 in borate buffer at pH 9.5.

By reaction of non-morphinomimetic tyrosyl-peptides with the peroxidases/H_2O_2 systems, oxidative coupling of tyrosyl residues occurs regardless of the tyrosyl residues in the peptide sequence, giving rise to the formation of both homo- and hetero-dimers.

Fluorescence formation in aged tissues is regarded as an index for aging[13] and there is considerable evidence that lipid peroxidation of tissues and cells can lead to the development of fluorescence by interaction of malonaldehyde with amino acids and proteins[14]. In the present investigation, an alternative mechanism for fluorescence formation emerged. At inflammatory sites, where many PMNs degranulate, tyrosyl-peptides could be widely transformed into various dimers. These fluorescent dimers could account for the formation of lipofuscin[15] whose presence in tissues is generally considered to be proof of intense peroxidative processes.

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