Biochemical Pharmacology, Vol. 43, No. 3, pp. 497–502, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00
Pergamon Press plc

RECIPROCAL EFFECTS BETWEEN OPIOID PEPTIDES AND HUMAN POLYMORPHONUCLEAR LEUKOCYTES—I

CHEMICAL MODIFICATIONS OF LEU-ENKEPHALIN BY PHORBOL MYRISTATE ACETATE-STIMULATED POLYMORPHONUCLEAR LEUKOCYTES

AMAL SLAOUI-HASNAOUI, MARIE-CHRISTINE GUERIN and JEAN TORREILLES*
INSERM U 58, 60 Rue de Navacelles, 34090 Montpellier, France

(*Received* 8 *July* 1991; *accepted* 8 *October* 1991)

Abstract—When L-tyrosyl-glycyl-L-phenylalanyl-L-leucine (Leu-enkephalin) is exposed to the activated oxygen species produced by phorbol myristate acetate (PMA)-stimulated polymorphonuclear leukocytes (PMNs), hydroxylation of the phenylalanyl residue in position 4 of the peptide occurs, producing hydroxy-phenylalanyl derivatives which are identified by HPLC analysis and mass spectrometry. Attack of hydroxyl radicals generated by the Cu (II)/ascorbate system upon Leu-enkephalin also produces isomeric *o*-, *m*- and *p*-hydroxy-phenylalanyl derivatives. When PMNs are incubated with a synthetic peptide, L-tyrosyl-glycyl-glycyl-L-tyrosyl-L-leucine used as a model of hydroxylated Leu-enkephalin, their chemiluminescence response to PMA activation is higher than that of PMNs incubated with Leu-enkephalin.

There has been recent evidence [1, 2] that opiate receptors are present on both human polymorphonuclear leukocytes (PMNs†) and monocytes, and that several cellular functions related to microbicidal and cytotoxic activity, such as phagocytosis, chemotaxis and antibody-dependent cytotoxicity, could be modulated by exposure to opioid peptides.

When stimulated, such phagocytic cells release AOS central to their microbicidal and cytotoxic functions and which have been reported to hydroxylate salicylate [3] and to decarboxylate benzoate [4]. Thus, we sought to determine whether AOS could modify the structure of opioid peptides, by exposure of Leu-enkephalin to stimulated PMNs, and focus our attention on the aromatic amino acid residues, phenylalanyl and tyrosyl, present in the common amino acid sequence of all morphinomimetic peptides: L-tyrosyl-glycyl-glycyl-L-phenylalanyl.

Since hydroxyl radicals seem to be effectively released by activated neutrophils [5], we compared our results with those obtained when Leu-enkephalin reacts with hydroxyl radicals generated by the Cu (II)/ascorbate system [6, 7] which was shown in many reports to hydroxylate aromatic compounds such as benzene, phenol and salicylic acid [8, 9].

The Cu(II)/ascorbate system was chosen for use in this study because neural tissue was found to contain ascorbic acid concentrations that rank among the highest of all mammalian biological tissues [10] and because copper was implicated by Okuyama *et al.* [11] in the activation mechanism of opiate receptors to explain the analgesic activity of copper complexes of non-steroidal anti-inflammatory agents.

* Corresponding author. Tel. (33) 67-54-15-44; FAX (33) 67-52-06-77.

† Abbreviations: AOS, activated oxygen species; PMN, polymorphonuclear leukocyte; PMA, phorbol myristate acetate; CL, chemiluminescence; Leu-enkephalin, L-tyrosyl-glycyl-glycyl-L-phenylalanyl-L-leucine; GGFL, glycyl-glycyl-L-phenylalanyl-L-leucine; YGGYL, L-tyrosyl-glycyl-glycyl-L-tyrosyl-L-leucine; YGG, L-tyrosyl-glycyl-glycine; GF, glycyl-L-phenylalanine; *o*-tyrosine, 2-hydroxy-phenylalanine; *m*-tyrosine, 3-hydroxy-phenylalanine; *p*-tyrosine, 4-hydroxy-phenylalanine.

MATERIALS AND METHODS

Reagents. *o*-Tyrosine, *m*-tyrosine, *p*-tyrosine and ascorbate were of the highest quality available from Fluka AG, cupric sulfate was purchased from Prolabo (France) and PMA from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). PMA was dissolved in dimethyl sulfoxide 1 mg/mL, and stored in aliquots at −20°. Leu-enkephalin and other peptides were from Bachem Feinchemikalein AG, YGGYL was synthesized by Altergen Strasbourg. HPLC grade solvents were from Merck (Darmstadt, Germany). Distilled deionized water was used throughout all experiments.

Cell preparation. Blood from the antecubital veins of healthy human volunteers was drawn into heparinized tubes and centrifuged for 10 min at 400 *g*. The plasma was withdrawn and the PMNs were isolated by differential density centrifugation on Percoll (density 1.13, Pharmacia Fine Chemicals). 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, $MgSO_4$ 7, H_2O 1.2, $CaCl_2$ 2.5, KCl 4.7 and NaCl 118.1, and finally resuspended to yield a concentration of 5×10^6 cells/mL.

Modification of peptides by activated PMNs. PMNs (5×10^6 cells/mL final concentration) in Krebs-bicarbonate solution were added with opiate peptide

Table 1. HPLC retention times of opiate peptides and hydroxylated derivatives

Compounds	Hydroxylating agents	Retention times*			
L-Phenylalanine	None		7.6		
	Cu (II)/ascorbate	5.9 (0.78)	6.2 (0.82)	6.9 (0.92)	7.5
p-Tyrosine	None		5.9		
m-Tyrosine	None		6.2		
o-Tyrosine	None		6.9		
GF	None		10.1		
	Cu (II)/ascorbate	7.6 (0.76)	8.3 (0.83)	9.0 (0.90)	10.0
GGFL	None		19.3		
	Cu (II)/ascorbate	15.3 (0.79)	16.3 (0.85)	17.9 (0.93)	19.3
	PMA-stimulated PMNs	15.3	16–16.5	17.9	19.3
Leu-enkephalin	None		23.2		
	Cu (II)/ascorbate	18.4 (0.79)	19.4 (0.84)	21.5 (0.92)	23.2
	PMA-stimulated PMNs	18.4	19.2–19.5	21.5	23.2
YGGYL	None		18.7		

Chromatography was performed as described in Materials and Methods; retention times (min) were determined spectrofluorometrically at 300 nm (excitation at 280 nm).

* Values in brackets correspond to ratios of the retention time of the product to the retention time of the unmodified starting peptide.

to yield a final concentration of 10 mM. The tubes were capped with rubber stoppers and preincubated at 37° with stirring for 7 min before activation with PMA (400 ng/mL). After 30 min of incubation, the tubes were chilled and centrifuged at 4° for 20 min (27,000 *g*). Supernatant fractions were concentrated under vacuum, 1 mL methanol was added and the fractions were filtered before analysis by HPLC or mass spectrometry.

Modification of peptides by the Cu (II)/*ascorbate system.* The reaction was conducted at room temperature in phosphate buffer (10 mM), pH 5. The reaction mixture contained 3 mM ascorbate, 5 mM peptide and 2 mM cupric sulfate. After 2 hr of stirring, the reaction was stopped by the addition of EDTA and the solution was concentrated under vacuum, methanol was added and the solution was filtered and analysed by HPLC or mass spectrometry.

HPLC analysis. HPLC analysis was performed with an LKB 2150 dual pump system equipped with a Jasco 820 FP spectrofluorimetric detector. Separations were carried out on Octadecyl Si 100 Serva column (25 × 1 cm) from Serva-Feinbiochemica GmbH (Heidelberg, Germany). The mobile phases were (A) 0.1 M NaH_2PO_4 in water and (B) 100% acetonitrile. The flow rate was 1 mL/min. The elution program was as follows: a linear gradient from 0 to 20% B for 20 min followed by isocratic elution with 20% B for 10 min. Tyrosine emission was observed at 300 nm (excitation at 280 nm).

Dityrosine detection by spectrofluorimetry. Dityrosine production was characterized by measurement of the intensity of the fluorescence emission at 400–405 nm for an excitation at 222 nm and at 410–415 nm for an excitation at 315 nm [12] in sodium borate buffer, pH 9.5.

High-resolution mass spectrometry. Fast atom bombardment mass spectrometry was performed with a JEOL, JMS DX 300 mass spectrometer.

Colorimetric detection of hydroxylated tyrosine. *o*-Dihydroxy groups were estimated by the method of Ravindranath *et al.* [13]: an aliquot of the solution to be tested was evaporated to dryness under vacuum and the residue was dissolved in 0.2 mL of 5% trichloroacetic acid and 0.2 mL of 10% $NaWO_4$, 0.2 mL of 0.5% $NaNO_2$ and 0.1 mL of 0.5 N HCl were then added. The yellow complex was converted to a cherry-red chromophore by the addition of 0.4 mL of 0.5 N NaOH, within 2 min of alkali addition. The intensity of the red color was measured with a recording Cary 118 C spectrometer at 540 nm.

Lucigenin-enhanced CL assays. CL was monitored in a model 1251 luminometer LKB Wallac (Wallac Co., Turku, Finland) connected to an Apple computer in the presence of 0.5 mM lucigenin. PMA (0.1 μg/mL) was used as a stimulus. PMNs (10^6 cells) were incubated with peptides for 7 min, unless otherwise stated at 37° before the addition of PMA. Measurements were carried out once a minute until a maximal CL value was obtained. Controls were performed with cells incubated without stimuli.

RESULTS

Modification of Leu-enkephalin by the Cu (II)/*ascorbate system*

Table 1 reports the HPLC separation of the three

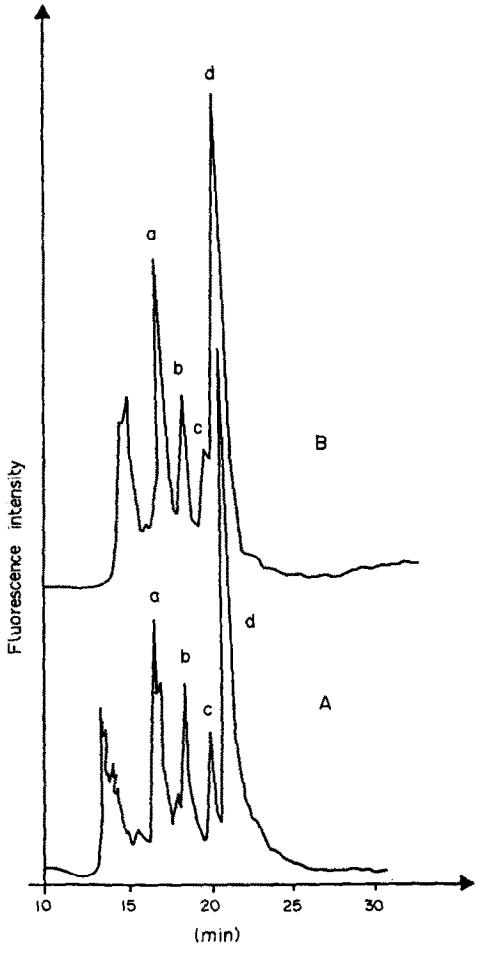


Fig. 1. HPLC chromatograms of Leu-enkephalin (d) after 30-min reaction with the Cu (II)/ascorbate system (A) or with PMA-stimulated PMNs (B). The experimental conditions are described in Materials and Methods. Peaks a, b and c correspond to *p*-, *m*-, *o*-hydroxy-phenylalanyl derivatives, respectively.

tyrosine isomers (*p*-tyrosine at 5.9 min, *m*-tyrosine at 6.2 min and *o*-tyrosine at 6.9 min). At the emission wavelength used, phenylalanine itself was detected as a small peak at 7.5 min as shown by co-elution experiments.

The incubation of millimolar concentrations of each of the phenylalanyl-containing peptides, GF, GGFL and Leu-enkephalin, with a mixture of Cu (II) and ascorbate caused the formation of three products (Fig. 1) whose retention times are shown in Table 1.

For each experiment, we compared the ratio of the retention time of each new product with the retention time of the corresponding unmodified product. All of the first eluted compounds have a constant ratio of 0.78, the second eluted compounds a ratio of 0.83 and the third a ratio of 0.92.

Then, we considered that for each tested peptide the isomeric hydroxylated compounds were eluted in the same order as the three authentic standards of tyrosine isomers: *para, meta* and *ortho*. This was confirmed by the relative areas of the peaks whose ratios were consistent with those expected from random attack of the phenylalanine by hydroxyl radicals. Mass spectra of the peptide mixtures obtained after treatment with AOS confirm the formation of new peptides containing tyrosyl residue instead of phenylalanyl residue.

The mass spectrum presented in the Fig. 2 is characterized by two sets of peaks at m/z 594, 556, 279 and 120, on the one hand, and at m/z 610, 572, 295 and 136, on the other hand, with a constant m/z difference of 16 units. The first set of peaks corresponds to Leu-enkephalin and the second set to the monohydroxylated derivative. Moreover, the absence of fragment a dihydroxylated phenylalanyl residue confirms that phenylalanine in position 4 is the only hydroxylated residue.

Modification of Leu-enkephalin by PMA-stimulated PMNs

Resting PMNs incubated with Leu-enkephalin showed no evidence of any Leu-enkephalin modification ability. As seen in Table 1, when cells were stimulated with PMA in the presence of Leu-enkephalin HPLC analysis of the 30-min supernatant fraction showed the presence of two new peaks. The 18.4-min retention time of the main peak obtained by incubation of Leu-enkephalin with activated PMNs was very close to the 18.7-min retention time of the authentic sample of YGGYL. When this compound was spiked with the incubated sample, it was eluted with the 18.4 min peak (Fig. 1).

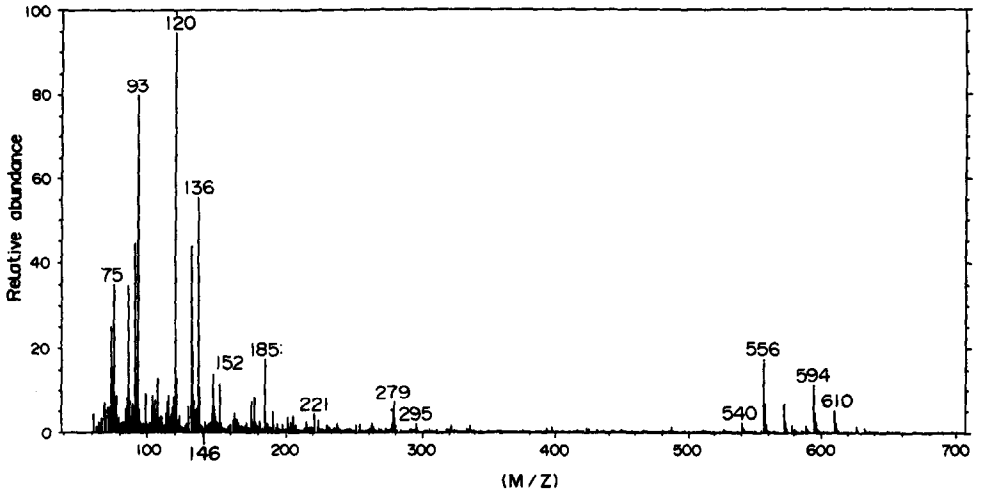
A second small broad peak with a retention time of 21.5 min could be identified as the corresponding *o*-hydroxy-phenylalanyl peptide, by comparison with the relative retention times observed for the HPLC analysis of the mixture of *o*-, *m*- and *p*-hydroxy-phenylalanyl peptides obtained by incubation of Leu-enkephalin with the Cu (II)/ascorbate system.

Similarly, when the fragment of Leu-enkephalin devoid of tyrosine, GGFL, was incubated with stimulated cells, the 30-min supernatant fraction showed a pattern with two new peaks having retention times of 15.3 and 17.9 min which corresponded to those observed previously by incubation with the Cu (II)/ascorbate system and were identified as the corresponding *o*- and *p*-hydroxy-phenylalanyl peptides.

Detection of dityrosine and o-hydroxy-tyrosine

Although no other significant peak was observed in the short retention time part of the HPLC chromatogram of the supernatant fraction of Leu-enkephalin incubation with either stimulated cells or the Cu (II)/ascorbate system, we tested this fraction for both dityrosine and *o*-hydroxy-tyrosine formation.

No dityrosine, or peptide containing dihydroxylated phenylalanyl residue were detected. These results were confirmed by fast atom bombardment mass analysis: the Leu-enkephalin mixtures resulting from exposure to activated PMNs or to the Cu (II)/ascorbate system have similar mass spectra with molecular ions which correspond to a monohydroxylation (Fig. 2). The phenylalanyl



Ion (m/z)	Composition
610	$M'+K]^+$
594	$M'+K]^+$
572	$M'+H]^+$
556	$M'+H]^+$
295	$\overset{+}{N}H_3CH(CH_2C_6H_5OH)CONHCH(CH_2CH(CH_3)_2)COOH$
279	$\overset{+}{N}H_3CH(CH_2C_6H_5)CONHCH(CH_2CH(CH_3)_2)COOH$
221	$NH_2CH(CH_2C_6H_5OH)CONHCH_2C{\equiv}O$
120	$C_6H_5-CH_2-CH=\overset{+}{N}H_2$
136	$C_6H_4(OH)-CH_2-CH=\overset{+}{N}H_2$

M = Leu-enkephalin; M′ = YGGYL.

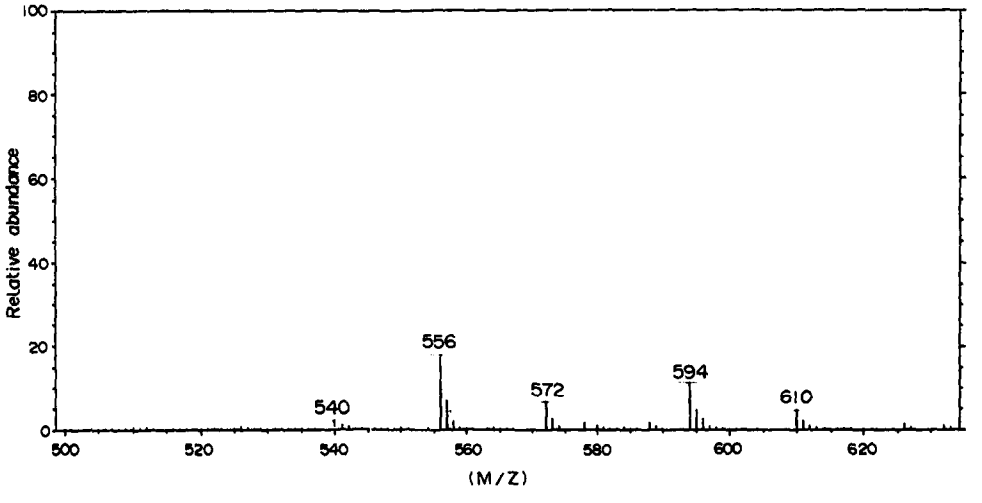


Fig. 2. Mass spectrum of the crude mixture of peptides obtained after incubation of Leu-enkephalin with PMA-stimulated PMNs. For details see Results.

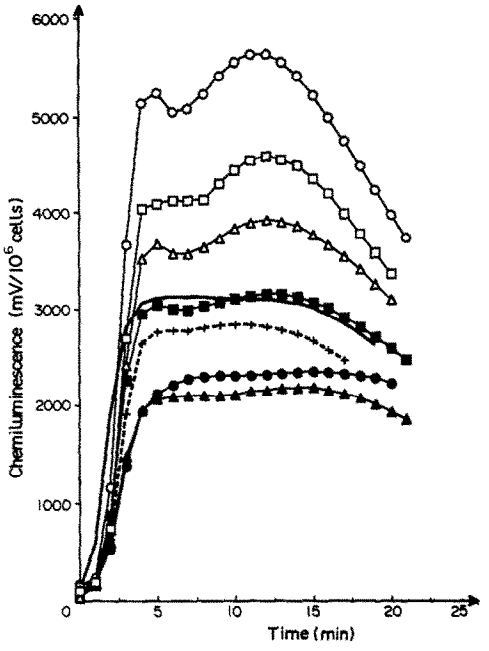


Fig. 3. Lucigenin-enhanced CL response of human PMNs stimulated by PMA to Leu-enkephalin and YGGYL. This figure presents results obtained from PMNs isolated from one donor but is representative of other donors tested. The concentrations used were: 10^{-8} M (×); 10^{-11} M (●); 10^{-12} M (▲); 10^{-14} M (■) for Leu-enkephalin; and: 10^{-10} M (□); 10^{-12} M (○); 10^{-14} M (△) for YGGYL. The black solid line corresponds to the CL response of PMA-stimulated cells previously incubated without any peptide.

residue was the site of hydroxylation as shown by fragments at 295 and 221 m/z and the mass spectra of the peptides YGG and YGGYL (results not shown) which remained unchanged before and after treatment with AOS (under the same experimental conditions as Leu-enkephalin).

These results disagree with those of Margolis *et al.* [14] and Grojenski *et al.* [15] who observed the formation of slight amounts of hydroxy-tyrosines and dityrosine by irradiation with a ^{60}Co γ source of tyrosine solutions saturated with oxygen-free N_2O for 30 min, but their experimental conditions were more drastic than ours and larger amounts of hydroxyl radicals were produced.

Effect of modified Leu-enkephalin on the chemiluminescence of the PMA-stimulated PMNs

As shown in Fig. 3, when Leu-enkephalin was incubated with PMNs 7 min before stimulation by PMA in the presence of lucigenine as CL enhancer, a lowering of the CL response was observed, whereas when synthetic peptide YGGYL, a model of hydroxylated Leu-enkephalin, was used instead of Leu-enkephalin a CL enhancement was observed. Both effects were dose-dependent at concentrations ranging from 10^{-10} to 10^{-14} M.

DISCUSSION

Our results demonstrate that the phenylalanyl residue of Leu-enkephalin can be hydroxylated by hydroxyl radicals generated by the Cu (II)/ascorbate system as well as by AOS released by PMA-stimulated PMNs; no modification of the N-terminus tyrosyl residue occurred. (The observed hydroxylations clearly occurred in association with respiratory burst activity of PMNs triggered by PMA and the hydroxyl radicals released by stimulated PMNs [5] would seem to be logical promotors of the hydroxylation. This assumption was confirmed by the inhibition caused by catalase and mannitol.)

In vivo, the production of hydroxyl radicals might occur in the proximity of opiate peptides since these peptides bind specific receptors of either the neural tissue which contain the elements for the Fenton reaction: ascorbic acid (which plays a crucial role in the proper functioning of both nervous and endocrine systems [16]) and copper (which is present in the opiate receptor and is implicated in its activation [11]), or the phagocytic cells which release AOS during the respiratory burst.

Phenylalanine has been shown to be associated with the affinity of peptides for ∂ opiate receptors [17] and with the inactivation of these peptides by peptidases [18]. Thus the interest, "*in vivo*", of the hydroxylated opiate peptides formed by reaction with hydroxyl radicals depends on their biological activities.

To test such activities, we prepared the synthetic peptide YGGYL as a model of "hydroxylated" Leu-enkephalin and compared its effect on the respiratory burst of PMA-stimulated PMNs with the effect of Leu-enkephalin, by means of lucigenin-enhanced CL assays.

As shown previously by Diamant *et al.* [2], Leu-enkephalin dose-dependently inhibited PMA-induced CL in human PMNs at concentrations ranging from 10^{-10} to 10^{-14} M (Fig. 3). However, the model peptide YGGYL promoted a dose-dependent enhancement of the CL response in PMA-stimulated PMNs, in the same concentration range. In all cases, the obtained patterns were very similar to those of PMA-stimulated PMNs which were previously incubated without peptides (black solid lines on Fig. 3). In the absence of the soluble PMA stimulus, opioid peptides did not significantly affect the basal CL response of PMNs.

The converse effects of opioid peptides at low concentrations on PMA-induced respiratory burst in human PMNs, depending on whether they are exposed or not to AOS, point towards complex links between oxygen radicals, the neural tissues and the immune system, and further studies in this field are presented in the next paper.

REFERENCES

1. Sharp BM, Keane WF, Suh HJ, Gekker G, Tsukayama D and Peterson PK, Opioid peptides rapidly stimulated superoxide production by human PMN leukocytes and macrophages. *Endocrinology* **117**: 793–795, 1985.
2. Diamant M, Henricks PAJ, Nijkamp FP and de Wied D, β-Endorphin and related peptides suppress phorbol myristate acetate-induced respiratory burst in human polymorphonuclear leukocytes. *Life Sci* **45**: 1537–1545, 1989.
3. Davis WB, Mohammed BS, Mays DC, She Z-W,

Mohammed JR, Husney RM and Sagone AL, Hydroxylation of salicylate by activated neutrophils. *Biochem Pharmacol* **38**: 4013–4019, 1989.
4. Alexander MS, Husney RM and Sagone AL, Metabolism of benzoic acid by stimulated polymorphonuclear cells. *Biochem Pharmacol* **35**: 3649–3651, 1986.
5. Samuni A, Black CDV, Krishna CM, Malech HL, Bernstein EF and Russo A, Hydroxyl radical production by stimulated neutrophils reappraised. *J Biol Chem* **263**: 13797–13801, 1988.
6. Halliwell B and Foyer CH, Ascorbic acid, metal ions and the superoxide radicals. *Biochem J* **155**: 697–700, 1976.
7. Halliwell B, Grootveld M and Gutteridge JMC, Methods for the measurement of hydroxyl radicals in biochemical systems. In: *Methods in Biochemical Analysis* (Ed. Glick D), Vol. 33, pp. 59–90. John Wiley and Sons, New York, 1988.
8. Uchida K and Kawakishi S, Ascorbate-mediated specific modification of histidine-containing peptides. *J Agric Food Chem* **37**: 897–901, 1989.
9. Shimohigeski Y, Roles of tyrosine residue of enkephalin in opiate receptor recognition. In: *Opioid Peptides: Medicinal Chemistry, NIDA Research Monograph* (Eds. Rapaka RS, Barnett G and Hauks RL), Vol. 96, pp. 291–311. US Government Printing Press, Rockville, 1986.
10. Oke AF, May L and Adams RN, Ascorbic acid distribution patterns in human brain. *Ann NY Acad Sci* **498**: 1–11, 1987.
11. Okuyama S, Hashimoto S, Aihara H, Willingham WM and Sorenson JRJ, Copper complexes of non-steroidal antiinflammatory agents: analgesic activity and possible opioid receptor activation. *Agents Actions* **21**: 130–144, 1987.
12. Amato R, Aeschbach R and Neukom H, Dityrosine: *in vitro* production and characterization. In: *Methods in Enzymology* (Eds. Wold F and Moldave K), Vol. 107, part B, pp. 377–388. Academic Press, New York, 1984.
13. Ravindranath SD, Kumar AA, Kumar RP, Vaidyanathan CS and Rao NA, Mechanism of aromatic hydroxylation. *Arch Biochem Biophys* **165**: 478–484, 1974.
14. Margolis SA, Coxon B, Gajenski E and Dizdaroglu M, Structure of a hydroxyl radical induced cross-link of thymine and tyrosine. *Biochemistry* **27**: 6353–6359, 1988.
15. Gajenski E and Dizdaroglu M, Hydroxyl radical induced cross-linking of cytosine and tyrosine in nucleohistone. *Biochemistry* **29**: 977–980, 1990.
16. Glembotski CC, The role of ascorbic acid in the biosynthesis of the neuroendocrine peptides α-MSH and TRH. *Ann NY Acad Sci* **498**: 54–62, 1987.
17. Schwartz JC, De La Baume S, Malfroy B, Patey G, Perdrisot R, Swerts JP, Fournie-Zaluski MC, Gacel G and Roques BP, Inactivation biologique des encéphalines. In: *Quo Vadis? Neuropeptides, Montpellier, France 8–9 May 1980* (Eds. Muyard JP and Roncucci R), pp. 53–68. Centre de Recherche Clin Midy Groupe Sanofi.
18. Loukas S, Varoucha D, Zioudrou C, Streaty RA and Klee WA, Opioid activities and structures of casein-derived exorphins. *Biochemistry* **22**: 4567–4573, 1983.