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Original Contribution

# Neutrophil-mediated oxidation of enkephalins via myeloperoxidase-dependent addition of superoxide

Péter Nagy *, Anthony J. Kettle, Christine C. Winterbourn

*Free Radical Research Group, Department of Pathology, University of Otago Christchurch, Christchurch 8140, New Zealand*

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ABSTRACT

Neutrophils play a major role in acute inflammation in part by generating superoxide and an array of other reactive species. These white blood cells also contribute to protection against inflammatory pain by releasing opioid peptides. The biochemical interactions of enkephalins with neutrophil-derived oxidants are not well understood. In this investigation we reveal that neutrophils use myeloperoxidase to oxidize enkephalins to their corresponding tyrosyl free radicals, which react preferentially with the superoxide to form a hydroperoxide. In methionine enkephalin, rapid intramolecular oxygen transfer from the hydroperoxide to the Met sulfur results in the formation of a sulfoxide derivative. This reaction may occur at sites of inflammation where enkephalins are released and neutrophils generate large amounts of superoxide. Hydroperoxide formation destroys the aromatic character of the Tyr residue by forming a bicyclic structure via conjugate addition of the terminal amine to the phenol ring. As the N-terminal Tyr and its amino group are essential for their opiate activity, we hypothesize that oxidative modification of this residue should affect the analgesic activity of enkephalins.



The enkephalins are neurotransmitters and neurohormones that act in the nervous and endocrine systems but are also widely distributed throughout the body. Leucine and methionine enkephalins (Leu-Enk[1], YGGFL, and Met-Enk, YGGFM, respectively) are opioid pentapeptides that bind to the same receptors as morphine. They are generated during stress and inflammation. Enkephalins are involved in the communication between the immune and the neuroendocrine systems by having analgesic and antinociceptive properties and they alleviate inflammatory pain [1,2]. White blood cells were shown to play a major role in this process by producing and excreting enkephalins at sites of inflammation [3–6].

Both Leu-Enk and Met-Enk bear an N-terminal Tyr residue, which is an absolute requirement for them to exert their opiate effects [7]. The liberation of Tyr is the major pathway for their inactivation and degradation [8,9]. In a previous study we showed that the Tyr residues of Leu-Enk and Met-Enk are modified when they are exposed to the horseradish peroxidase/superoxide/$H_2O_2$ (HRP/$O_2^{\bullet-}$/$H_2O_2$) system. Under these conditions HRP catalyzes the one-electron oxidation of the Tyr residues to give Tyr radicals (TyrO$^\bullet$, Scheme 1, Reaction 1). These can either combine to form dimers ($(Tyr)_2$, Reaction 2) or react with $O_2^{\bullet-}$ to give a hydroperoxide (Tyr-OOH, Reaction 3b) [10–14]. These hydroperoxides can hydrolyze (Tyr-OH, Reaction 4). Alternatively, in peptides that contain a Met residue (such as Met-Enk) they generate Met sulfoxide (MetS=O) via rapid intramolecular oxygen transfer (Tyr-OH–Met=O, Reaction 5) [10]. We have recently shown that protein Tyr radicals also react preferentially with $O_2^{\bullet-}$ by the same mechanism [15].

Stimulated neutrophils produce large amounts of extracellular $O_2^{\bullet-}$ and $H_2O_2$ and release myeloperoxidase (MPO) at sites of inflammation. MPO uses $H_2O_2$ to produce powerful oxidants including hypochlorous acid (HOCl) as well as organic free radicals such as Tyr radicals (Scheme 1, Reaction 1) [16]. Neutrophils also synthesize neuropeptides [6,17] and respond to them through specific opioid receptors [18]. However, only a few studies have been published on the redox biochemistry of enkephalins with neutrophil-generated reactive oxidants [10,19–26]. It has previously been shown that MPO can use Leu-Enk and Met-Enk as substrates [25]. Also, stimulated neutrophils oxidized Met-Enk to its sulfoxide and, among other minor products, to the corresponding Tyr-linked dimers [19,20]. In this contribution we studied the efficiency of oxidation of Leu-Enk and Met-Enk by MPO and the mechanisms of the oxidation by stimulated human neutrophils. We demonstrate that these leukocytes use $O_2^{\bullet-}$ and MPO to oxidize the enkephalins via radical-mediated addition and discuss the physiological plausibility of these reactions.

*Abbreviations:* Leu-Enk (YGGFL), leucine enkephalin; Met-Enk (YGGFM), methionine enkephalin; MetS=O, methionine sulfoxide; ABAH, aminobenzoic acid hydrazide; DTPA, diethylenetriamine-penta-acetic acid; HRP, horseradish peroxidase; MPO, myeloperoxidase; XO, xanthine oxidase; SOD, superoxide dismutase; LC–MS, liquid chromatography–mass spectrometry.

* Corresponding author.
*E-mail address:* peter.nagy@otago.ac.nz (P. Nagy).

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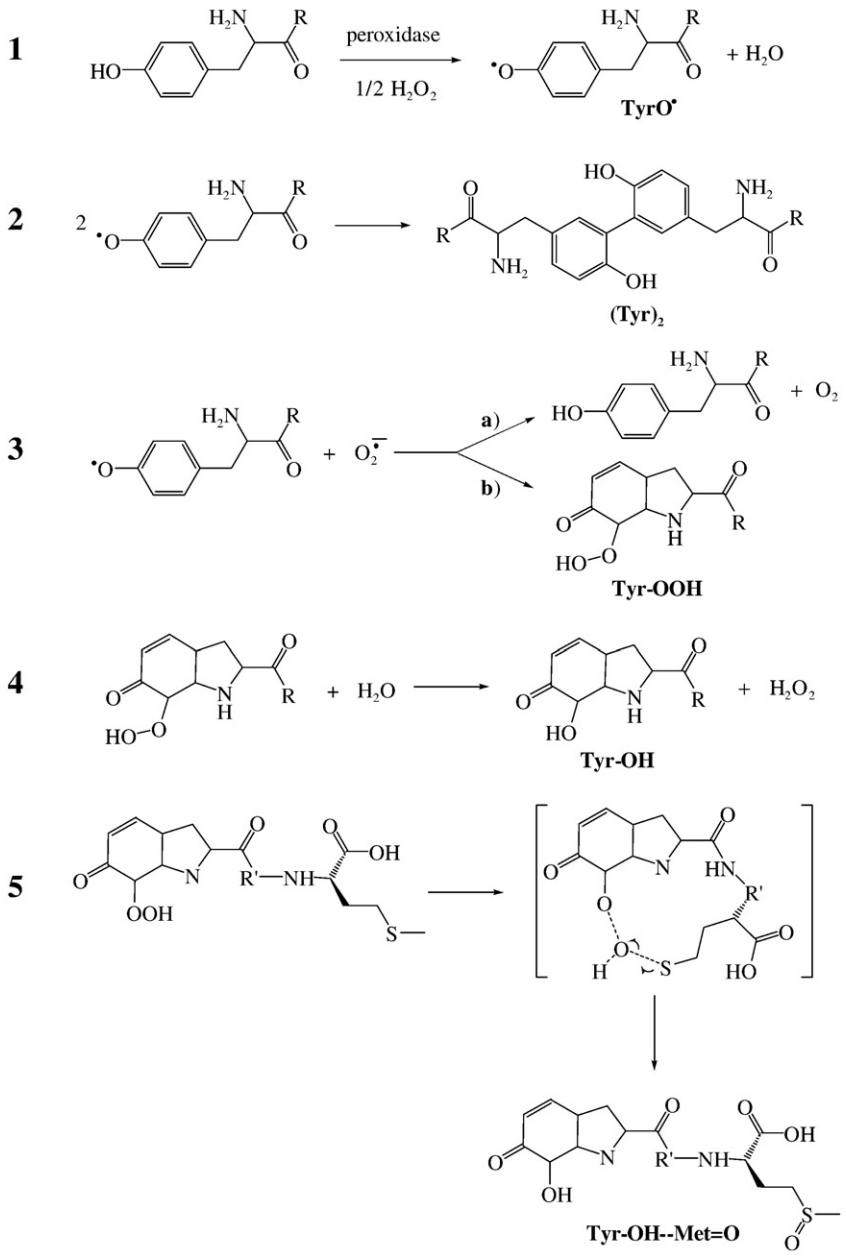


**Scheme 1.** (Reaction 1) Peroxidases catalyze the one-electron oxidation of Tyr residues in peptides to give a phenoxyl radical. (Reaction 2) Phenoxyl radicals combine to form covalently bound dimers ($k \sim 5 \times 10^8\ M^{-1}\ s^{-1}$). (Reaction 3) Phenoxyl radicals also react with $O_2^{\bullet-}$ either by electron transfer (a) or by addition (b) ($k \sim 1.5 \times 10^9\ M^{-1}\ s^{-1}$). $O_2^{\bullet-}$ addition is followed by conjugate addition of the Tyr nitrogen to the phenolic ring to give a nonaromatic bicyclic product. (Reaction 4) The hydroperoxide can hydrolyze to give the corresponding alcohol. (Reaction 5) In peptides that also contain a Met residue (such as Met-Enk) oxygen is transferred intramolecularly from the bicyclic hydroperoxide to the Met sulfur. R represents GGFM or GGFL for Leu-Enk or Met-Enk, respectively. R′ represents GGF.

## Materials and methods

### *Reagents*

Water was purified by running through a Milli-Q system (Millipore) so that its resistivity was greater than 18 MΩ-cm. All reagents and enzymes were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise indicated. Leu- and Met-Enk were >98% pure. MPO was purchased from Planta (Vienna, Austria) and its concentration was determined measuring the absorbance of its Soret peak ($\varepsilon_{430} = 89{,}000\ M^{-1}\ cm^{-1}$). The concentrations of stock solutions of $H_2O_2$ were determined spectrophotometrically ($\varepsilon(H_2O_2)_{240\ nm} = 43.6\ M^{-1}\ cm^{-1}$). Stock solutions of xanthine oxidase (XO) were prepared by dilution of an ammonium sulfate suspension with 50 mM phosphate buffer (at pH 7.4) and spinning through a G25 Sephadex column to remove the ammonium sulfate. The activity of XO was measured by the cytochrome *c* assay [27] and by quantifying $H_2O_2$ formation using the FOX assay [28] as in [10]. Enzyme and acetaldehyde stock solutions were prepared fresh daily and stored on ice.

### *Quantification of dimer formation and peroxidase-catalyzed oxidation of enkephalins*

Dimers were measured fluorimetrically (excitation 325 nm, emission 400 nm) with a Hitachi F-4500 spectrofluorimeter as described previously [13]. Fluorescence units were converted to concentrations based on calibration curves obtained by generating dimers from the relevant peptides in at least 20-fold molar excess over $H_2O_2$ in the presence of HRP. Calibration curves were developed daily for each peptide and Tyr. The rate of the catalytic reaction was followed by

monitoring dimer formation. Data points were recorded at 0.5-s intervals. Initial rates were determined by fitting the linear part of the kinetic traces (see Fig. 1b inset). Reaction conditions are given in the legend of Fig. 1.

*Neutrophil-mediated oxidation of enkephalins*

Blood was obtained by venipuncture from healthy adult donors with informed consent. The procedure was approved by the Upper South A Regional Ethics Committee. Neutrophils were isolated from heparinized peripheral blood by Ficoll–Hypaque centrifugation, dextran sedimentation, and hypotonic lysis [29]. Unless stated otherwise, neutrophils ($2\times10^6$ cells/ml) were stimulated at 37 °C in Hanks buffer with phorbol myristate acetate (PMA; 0.1 μg/ml) in the presence of 100 μM enkephalin. After 20 min, reactions were stopped by adding 20 μg/ml catalase. The cell supernatants were analyzed by liquid chromatography/mass spectrometry (LC–MS) for oxygenated products and by spectrofluorimetry for dimers. Under these conditions the neutrophils typically produced approx 200–300 μM $O_2^{\bullet-}$ (measured as SOD-inhibitable cytochrome *c* reduction) and 100–150 μM $H_2O_2$ (as determined by the FOX assay on the supernatant of cells stimulated in the presence of azide) [10,13,28].

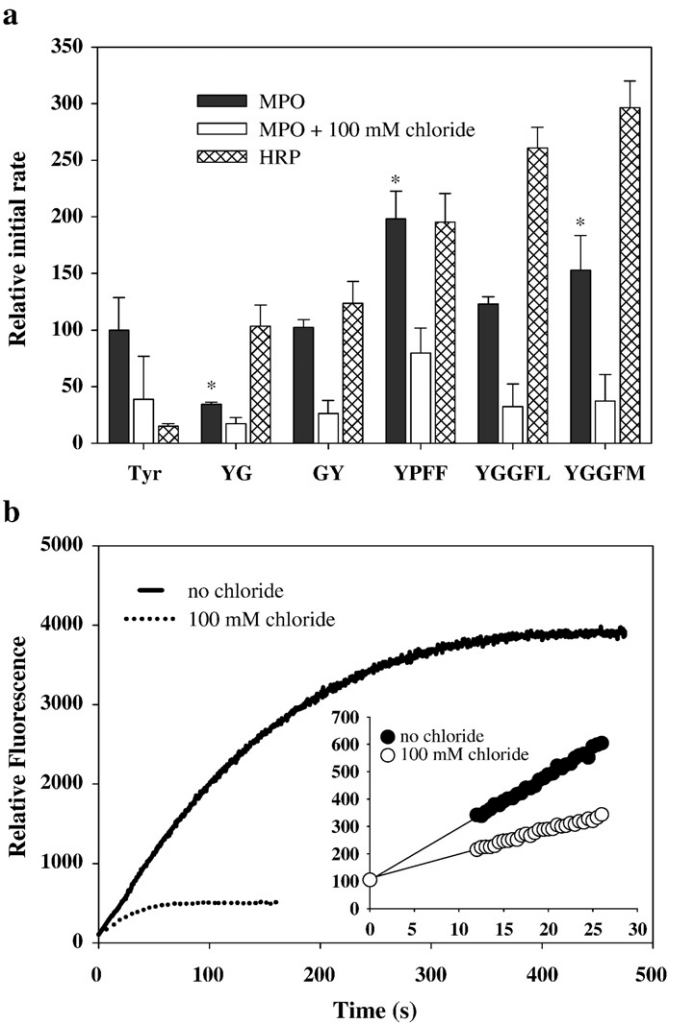


**Fig. 1.** Comparison of Tyr-containing peptides as substrates for myeloperoxidase (MPO) and horseradish peroxidase (HRP). The reactions were followed by monitoring dimer formation using spectrofluorimetry (see Materials and methods). (a) The comparison is based on their steady-state rates of dimer formation, measured over the first 25 s of the reaction as in the inset in (b) and are expressed as a percentage of the rate of oxidation of Tyr by MPO under similar reaction conditions. The initial rate of dimer formation for Tyr was 15 ± 4 nM/s. Reactions were started by adding 4 μM $H_2O_2$ to 50 mM phosphate buffer, pH 7.4, at 25 °C containing 7.5 nM peroxidase (HRP or MPO), 200 μM tyrosine or peptide, 0 or 100 mM sodium chloride, and 50 μM diethylenetriamine-penta-acetic acid (DTPA). The reactions were started by adding $H_2O_2$. Results represent the average and standard deviation of four experiments. Data were analyzed by ANOVA with the Holm–Sidak method for post hoc analysis. The rate of dimer formation catalyzed by HRP was significantly different ($p<0.001$) for all peptides compared to Tyr. $^*p < 0.001$, statistically significant different dimer formation rate catalyzed by MPO compared to Tyr. $Cl^-$ significantly ($p<0.001$) inhibited dimer formation in all cases except for YG. (b) Representative kinetic traces for Leu-Enk dimer formation in the presence and absence of 100 mM chloride. Inset shows the linear part of the kinetic traces.

*LC–electrospray ionization (ESI)–MS*

LC–ESI–MS and LC–ESI–MS/MS analyses were performed with a Thermo Finnigan LCQ Deca XP Plus ion trap mass spectrometer (San Jose, CA, USA) coupled to a Surveyor HPLC system and PDA detector. Positive ion mode was used. Data were analyzed using Finnigan Xcalibur, Thermo Finnigan Qual Browser 1.3, and High Chem Mass Frontier 3.0 programs. Fragmentation patterns were analyzed using Bioworks Browser 3.1 and peptide fragment ions were assigned and discussed based on the Roepstorff–Fohlman nomenclature. LC–MS conditions were as described previously [10].

*Quantification of Met-Enk and Leu-Enk dioxide products*

A previously published procedure was used with minor modifications [10]. Briefly LC–MS peak integrals were calibrated based on the phenylalanine content of the purified enkephalin derivatives (measured after acid hydrolysis). Ion suppression in the supernatants (after the neutrophils were removed by centrifugation) was assessed by spiking with preformed authentic samples of each analyte. Leu-Enk hydroperoxide concentrations represent the sum of detected hydroperoxide and its hydrolysis product (the corresponding alcohol derivative).

*Quantification of Met-Enk sulfoxide*

The relationship between the concentration and the MS peak intensity of Met-Enk sulfoxide was determined by adding HOCl to Met-Enk. Quantitative conversion to the sulfoxide was observed at <1:1, HOCl:Met-Enk mole ratios (Supplementary Fig. S1). The decrease in Met-Enk peak intensity ratio was accompanied by an almost identical increase in Met-Enk sulfoxide, suggesting similar ionizability of the two analytes. Ion suppression in the supernatants of the neutrophil samples was corrected for by spiking with authentic sulfoxide.

## Results

*Myeloperoxidase-catalyzed oxidation of enkephalins*

To assess whether the opioid peptides are good substrates for MPO, we compared them with free Tyr and the dipeptides Gly-Tyr (GY) and Tyr-Gly (YG) by measuring the rates of fluorescent dimer formation in the presence of $H_2O_2$ and either MPO or HRP (Reactions 1 and 2 in Scheme 1). Leu-Enk, Met-Enk, and the related peptide endomorphin 2 (YPFF) were oxidized by MPO at rates comparable to those for Tyr and GY (Fig. 1a). In contrast, HRP oxidized the opioid peptides faster than Tyr and the rate increased with the length of the peptide. The relative rates of dimer formation are in agreement for MPO with a previous report [25], but the absolute rates were found to be different. The active site of HRP has been shown to be more accessible [30–33] than that of MPO, which is in a hydrophobic cleft [34]. Therefore, it might be expected that the longer peptides would be oxidized less well by MPO and their reactivity would decrease with peptide length. However, these trends were not apparent and the results indicate that the opioid peptides are competitive with Tyr, which is regarded as a comparatively good MPO substrate [35].

In the presence of physiological chloride (100 mM), the initial rate of peptide oxidation was inhibited (Fig. 1a), but only by a maximum of 75% (50% for Tyr in accordance with [36]). Chloride is, however, the preferred substrate at these concentrations and accounted for the majority of the $H_2O_2$ consumption. Thus, the amount of dimer formed at the end of the reaction was decreased to approximately 5–10% when 100 mM $Cl^-$ was present (Fig. 1b). This, together with the rates of Tyr and Gly-Tyr oxidation, is in agreement with previous reports [35–39].

*Neutrophil-mediated oxidation of enkephalins*

When the enkephalins were exposed to neutrophils, stimulation with PMA gave two major products that were detected by LC–MS from both Leu-Enk (Fig. 2a) and Met-Enk (Fig. 2b), as well as fluorescent dimers. The two products were 16 and 32 Da heavier than the native peptides and represent the corresponding monoxide and dioxide derivatives. For Met-Enk, a trioxide was also detected (see later). The molecular structures of these species were assigned based on the chromatographic mobilities and fragmentation patterns in the LC–MS/MS traces. They were identical to those of previously characterized derivatives of Met-Enk and Leu-Enk that were generated via the XO/acetaldehyde/HRP system [10]. For Leu-Enk the dioxide peak represents the bicyclic Tyr-hydroperoxide (Tyr-OOH) and the monoxide is assigned to its hydrolysis product (Tyr-OH). For Met-Enk, the dioxide corresponds to the dioxide formed by transfer of one oxygen from the Tyr hydroperoxide to Met (Tyr-OH–Met = O). The Met-Enk monoxide peak was much more prominent than the dioxide and was not seen in the enzymatic system [10]. Its fragmentation pattern was typical for a Met-sulfoxide derivative (key fragments a4, $m/z$ 397.1; b4, $m/z$ 425.0 and $m/z$ 526.1, representing the characteristic 64-mass-unit loss of sulfoxides) and, as shown below, it was formed via oxidation of the peptide by neutrophil-derived HOCl.

The requirements for oxidation by neutrophils were investigated for Leu-Enk and Met-Enk (Figs. 3a and b, respectively). No product formation was detected in the absence of cells or the enkephalin substrate (not shown) and only small amounts in the absence of PMA. Formation of all the products was almost completely inhibited by the MPO inhibitor 4-aminobenzoic acid hydrazide (ABAH), indicating a requirement for MPO activity. It was also inhibited by catalase, showing the requirement of $H_2O_2$. In line with the proposed model (Scheme 1) the formation of Leu-Enk hydroperoxide plus monoxide, and Met-Enk dioxide, was inhibited by SOD, with a corresponding increase in dimer formation due to the phenoxyl radicals no longer reacting with $O_2^{\bullet-}$. Met-Enk sulfoxide formation was unaffected by SOD and therefore not $O_2^{\bullet-}$-dependent. This can be explained by the cells being in Hanks buffer, in which most of the MPO would have reacted with the 100 mM chloride present to generate HOCl [40]. Adding 1 mM methionine or 10 mg/ml human serum albumin (Albumex) to the neutrophil system to scavenge HOCl largely inhibited sulfoxide production, but had minimal effect on the dioxide yield (Fig. 3b). The lesser effect of albumin is possibly explained by some of the scavenged HOCl giving rise to chloramines on the albumin, which could also oxidize the Met-Enk to the sulfoxide. Thus, the high yield of Met-Enk sulfoxide can be explained by the generation of HOCl by the neutrophils and its efficient reaction with Met residues.

In the complete Met-Enk system in Fig. 3b, the major oxidation product was the HOCl-generated sulfoxide derivative (18 μM/100 μM generated $H_2O_2$). However, Met-Enk radical formation was able to compete with HOCl production to some extent, resulting in the generation of 0.4 μM (per 100 μM generated $H_2O_2$) Tyr-OH–Met = O via $O_2^{\bullet-}$ radical addition. The dimer yield was comparable to that of Tyr-OH–Met = O. Under the same conditions in the Leu-Enk system (Fig. 3b), only ~5% of the native peptide was lost per 100 μM $H_2O_2$ produced and the major detectable oxidation product was the hydroperoxide species (2 μM Tyr-OOH and 0.3 μM dimer was formed). In the Leu-Enk system HOCl is still produced in similar quantities, but it would not preferentially react with the enkephalin because it lacks the reactive Met residue.

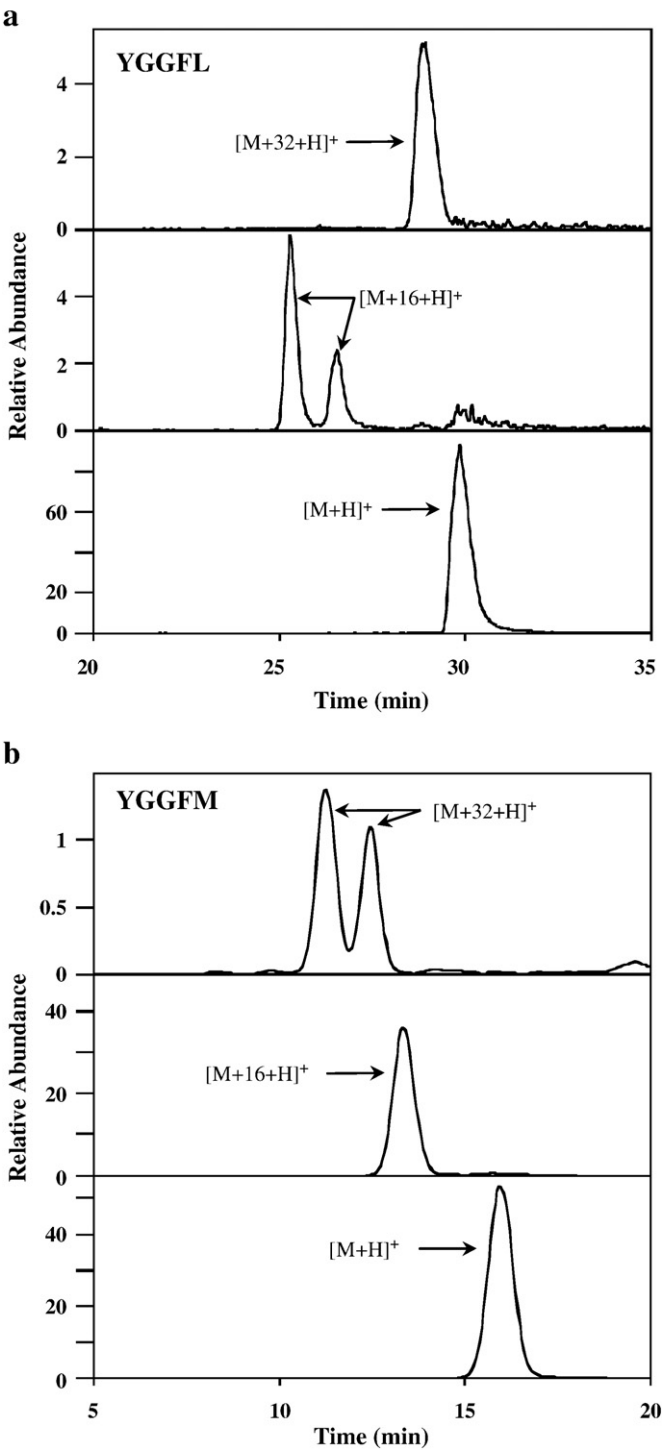


**Fig. 2.** LC–MS detection of the monoxide and dioxide products from (a) Leu-Enk and (b) Met-Enk exposed to PMA-stimulated neutrophils. Chromatograms represent the native peptides and the monoxide and dioxide derivatives (bottom up) obtained (a) by extraction from the total ion chromatograms or (b) by selective ion monitoring. Amounts of products are shown as relative abundance compared with the parent peptide (in a sample where MPO was inhibited and therefore no reaction occurred) set as 100%. For reaction conditions see Materials and methods. Chromatographic conditions are as described previously [10].

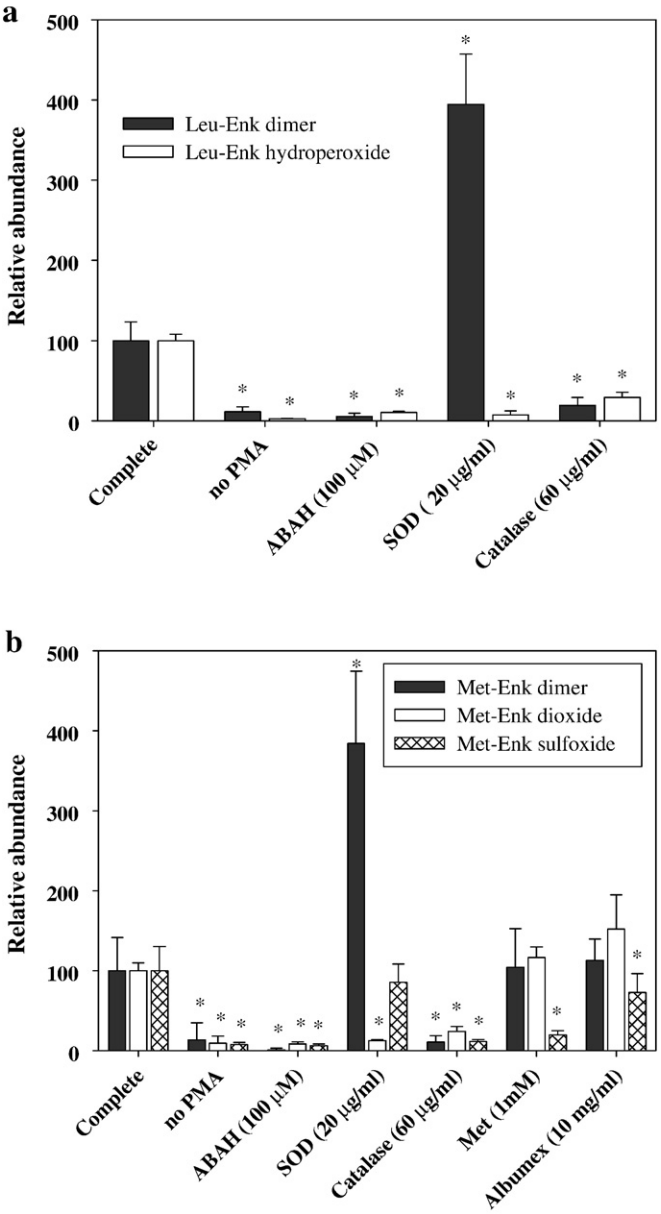


**Fig. 3.** Inhibitor profiles for (a) Leu-Enk and (b) Met-Enk oxidation by neutrophils. The complete Met-Enk system yielded $0.4 \pm 0.1$ μM dioxide, $0.4 \pm 0.2$ μM dimer, and $18 \pm 6$ μM sulfoxide per 100 μM generated $H_2O_2$. The complete Leu-Enk system yielded $2.0 \pm 0.2$ μM combined hydroperoxide and monoxide and $0.3 \pm 0.1$ μM dimer and ~5% loss of the native Leu-Enk (compared to the sample with ABAH) per 100 μM $H_2O_2$ generated by the neutrophils. Results and standard deviations represent independent experiments in which neutrophils were obtained from different donors (for (a) $n=3$ and for (b) $n=4$). Data were analyzed as described for Fig. 1. $^*p<0.001$, statistically significant inhibition (or $p=0.035$ for the inhibition of sulfoxide formation by Albumex), or enhanced dimer formation in the presence of SOD, compared to the complete systems. For reaction conditions and quantification see Materials and methods. Reactions were started by the addition of PMA and stopped by adding 20 μg/ml catalase, which was followed by centrifugation. The supernatant was analyzed by LC–MS for Met-Enk dioxide, Met-Enk sulfoxide, Leu-Enk hydroperoxide, and Leu-Enk monoxide and by spectrofluorimetry for dimers (for details see Materials and methods).

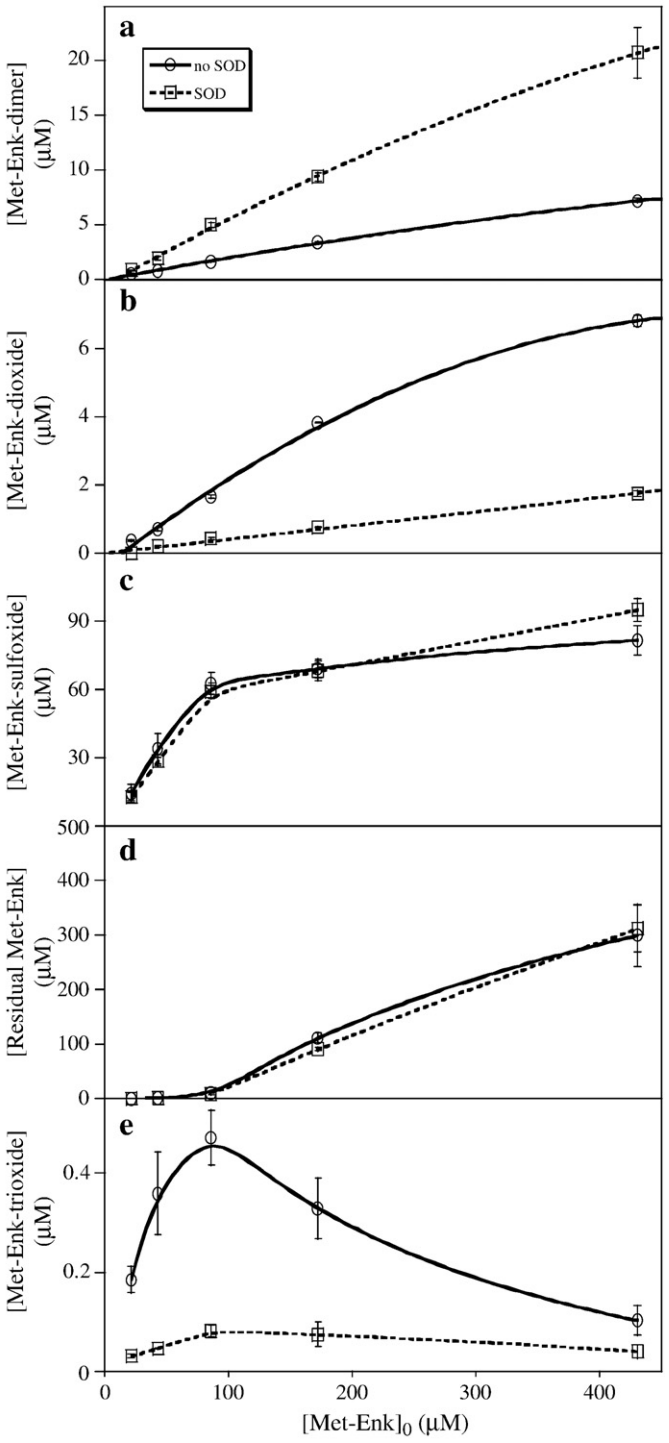


**Fig. 4.** Neutrophil-mediated oxidation products of Met-Enk at various Met-Enk concentrations. The effects of Met-Enk concentration on the yield of (a) dimer, (b) Met-Enk dioxide, (c) Met-Enk sulfoxide, (d) residual Met-Enk, and (e) Met-Enk trioxide in the presence and absence of SOD. Reactions were started by adding PMA (0.1 mg/ml) to $4 \times 10^6$ neutrophils/ml in Hanks buffer, pH 7.4, at 37 °C. After 20 min incubation the reactions were stopped by adding 20 μg/ml catalase, which was followed by centrifugation. Data are representative of three independent experiments in which similar trends were observed. Results are means and ranges of duplicates from one experiment.

Quantification was further investigated with Met-Enk over a concentration range. Fig. 4 shows a concentration-dependent increase in the formation of the dimer (Fig. 4a) and Tyr-OH–Met = O (Fig. 4b) species. SOD inhibited the formation of Tyr-OH–Met = O and increased dimer yield. Met-Enk sulfoxide was the major oxidation product (accounting for most of the lost native peptide) at all concentrations and its formation was independent of SOD (Figs. 4c and d). Although the formation of sulfoxide reached a plateau at larger Met-Enk concentrations (due to the efficient scavenging of the majority of HOCl), the percentage yield of Tyr-OH–Met = O was independent of the initial enkephalin concentration. This observation

indicates that the $O_2^{\bullet-}$-mediated oxidation of enkephalins might be plausible at low, physiologically relevant concentrations.

Under conditions where most of the Met-Enk was consumed in the neutrophil system (≤100 μM initial concentrations, see Fig. 4d), we observed the formation of two new peaks that had a mass 48 Da higher than that of Met-Enk, representing isomers (regio or stereo) of a trioxide species (run time 12.4 and 13.1 min, see Fig. 5a). We have not quantified this species, but based on peak areas, the maximum yield with 85 μM initial [Met-Enk] under the conditions of Fig. 4 corresponded to approximately one-third of the dioxide produced (compare Figs. 4b and e). Formation of both peaks was inhibited in the presence of SOD, indicating the requirement of $O_2^{\bullet-}$ (Fig. 4e). The same species was generated when the sulfoxide derivative of Met-Enk (Met-Enk-S=O) was exposed to MPO, $H_2O_2$, and $O_2^{\bullet-}$ (Fig. 5a), indicated by the identical mass, chromatographic mobility, and fragmentation pattern. In contrast to Met-Enk, the reaction produced substantial hydroperoxide activity in the FOX assay (Fig. 5b). This activity was inhibited by SOD. Structural information on this species was obtained by studying its fragmentation pattern (Fig. 6a and Supplementary Fig. S2). The fragment ion at $m/z$ 588 represents the loss of 64 mass units, which is a characteristic loss for Met sulfoxides. The b3 and b4 masses are consistent with the extra two oxygens being attached to the Tyr residue. A detailed structural characterization of this species by LC–$MS^n$ (see Supplementary Fig. S2) revealed that it is a Tyr-hydroperoxide-Met-sulfoxide derivative, in which the Tyr residue is modified in a way similar to that in Leu-Enk (for the proposed molecular structure see Fig. 6c).

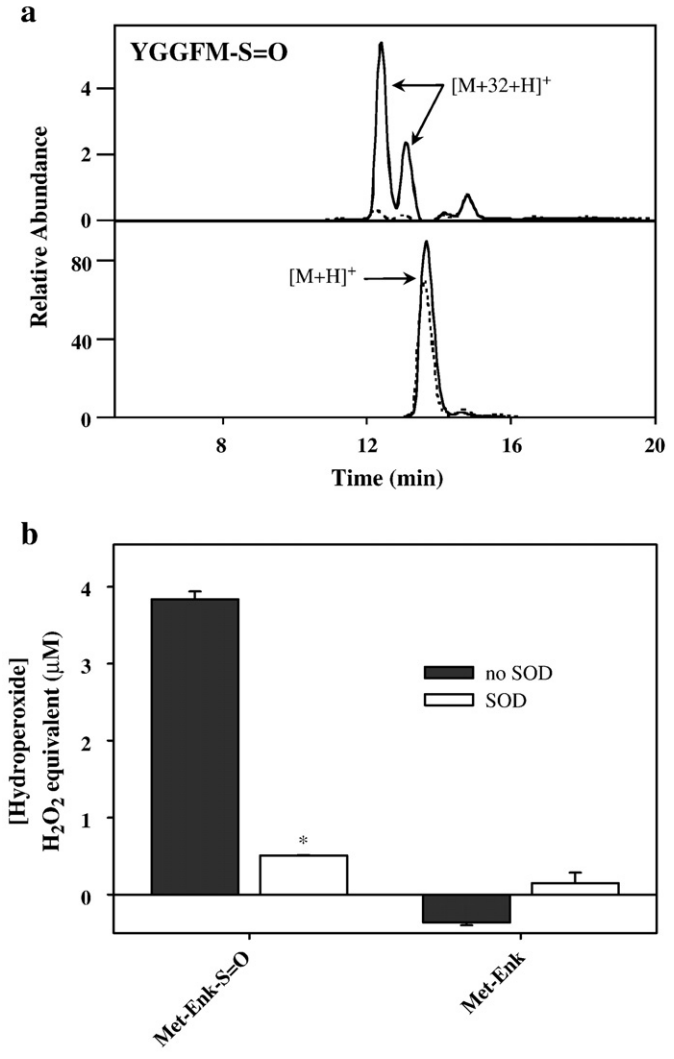


**Fig. 5.** Detection of hydroperoxide formation when Met-Enk-S=O was exposed to the MPO/XO/acetaldehyde system by (a) LC–MS and (b) the FOX assay. Samples were incubated with 140 nm MPO, 1 mM acetaldehyde, and XO (0.001 unit/ml corresponds to an initial rate of 2.8 μM/min $O_2^{\bullet-}$ and total production of 36 μM $H_2O_2$ over 30-min reaction period) and 200 μM Met-Enk-S=O in 50 mM phosphate buffer plus 50 μM DTPA (except for the FOX assay experiment). Reactions were started by the addition of XO. (a) Chromatograms represent native Met-Enk-S=O (bottom) and its dioxide derivative (top) obtained by selective ion monitoring in the presence (solid lines) and absence (dashed lines) of SOD. Amounts of products are shown as relative abundance compared with the parent peptide (in the −XO sample) set as 100%. (b) Peroxide yields are expressed as $H_2O_2$ equivalents using a calibration curve that was developed with known amounts of $H_2O_2$. However, tyrosine hydroperoxide concentrations are underestimated by the FOX assay when expressed as $H_2O_2$ equivalents [13]. Zero time blanks were subtracted and error bars represent the range of means from two experiments. Data were analyzed as described for Fig. 1. *$p<0.001$, statistically significant inhibition by SOD.

## Discussion

We have shown that MPO reacts with the opioid enkephalins and endomorphin 2 with rates comparable to those of Tyr to form the corresponding Tyr radicals, which (in the absence of $O2^{\bullet-}$) combine to give Tyr-linked dimers. In the presence of 100 mM $Cl^-$, dimer yields dropped significantly, but the initial rate of dimer formation was less affected. This minor effect of chloride on the rate of dimer formation

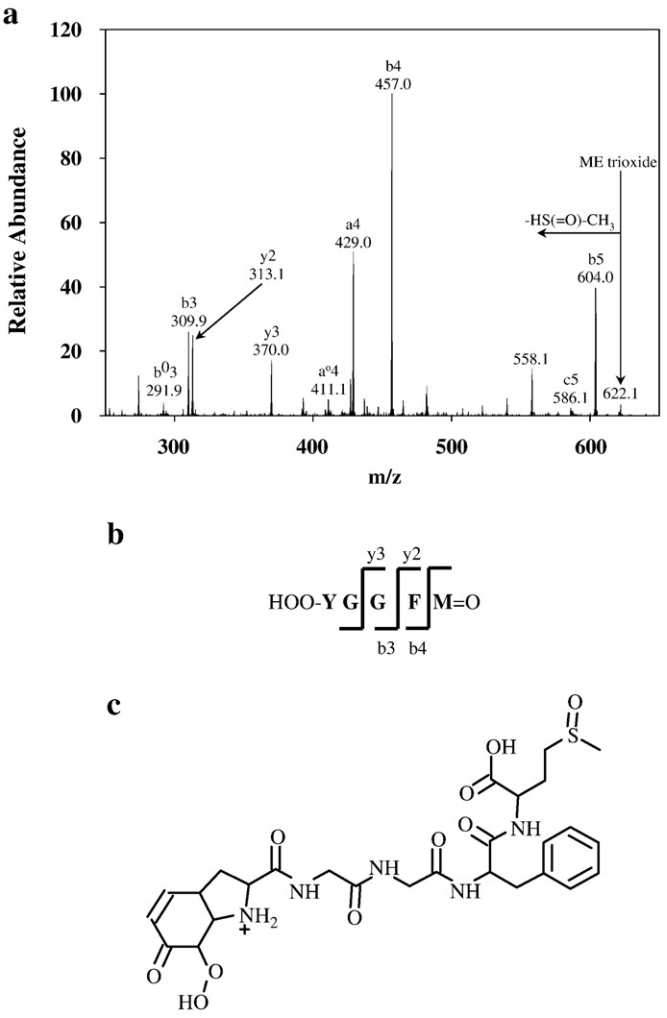


**Fig. 6.** (a) Fragmentation pattern, (b) major fragments, and (c) proposed structure of Met-Enk-trioxide. Met-Enk trioxide was prepared as described for Fig. 5 and purified by LC–MS and the fragmentation pattern was recorded using direct infusion in 1:1 MeOH:$H_2O$ and 0.1% formic acid. Peptide fragments were assigned based on the Roepstorff–Fohlman nomenclature. The loss of water is denoted by "°", where $y°x$ is $yx-H_2O$.

occurred because chloride and analogues of tyrosine have different rate-determining steps in their oxidation by the enzyme. The catalytic cycle of MPO starts by the reaction of the native enzyme with $H_2O_2$ to form Compound I. Compound I has an oxy-ferryl heme center and a porphyrin radical and it can engage in both one- and two-electron reactions. One-electron donors (such as Tyr) reduce Compound I to Compound II, which is the dominant state of the enzyme during catalytic turnover in most systems. Compound II also has an oxy-ferryl heme center and is reduced via one-electron reactions in a rate-determining step to regenerate the native enzyme [41]. In vivo numerous one-electron reductants will reduce Compound I to Compound II. Turnover of Compound II, however, is much more restricted so that it is likely that enkephalins can compete with other substrates at this stage of the enzymatic cycle for oxidation [42].

Interestingly, in contrast to HRP, the rate of oxidation of the various Tyr-containing peptides by MPO was relatively independent of their size. This suggests that they had similar access to the active site of Compound II. The one exception was the small peptide YG, which was a relatively poor substrate. A possible explanation for its tardy oxidation is that the negative charge on the carboxylate group of this substrate is optimally located to prevent access of the dipeptide to the active site. In support of this proposal, it has been reported that eliminating the negative charge on cysteine by removing its carboxylate group or esterification dramatically increases the rate constants for reaction of aliphatic thiols with both Compound I and Compound II [30].

Previous studies have shown that neutrophils can oxidize Met-Enk [19,20]. We have extended these studies and characterized new products. We have shown that neutrophils use MPO, $H_2O_2$, and $O_2^{\bullet-}$ to oxidize the enkephalins via $O_2^{\bullet-}$ addition to their Tyr radicals. Although most of the $H_2O_2$ released by neutrophils is converted to HOCl, Met-Enk and Leu-Enk competed to some extent for MPO and were directly oxidized to their Tyr radicals at physiological chloride concentrations. Leu-Enk was converted to its hydroperoxide as the major oxidation product (via Reactions 1–3). The Met residue of Met-Enk reacts with a substantial amount of the generated HOCl to give MetS = O. However, HOCl is a promiscuous oxidant that is likely to be quenched by proteins and other two-electron-scavenging small molecules (such as GSH, urate, or ascorbate) under physiological conditions [43]. We demonstrated this effect by showing that, in the presence of added methionine or albumin, Met enkephalin oxidation was largely inhibited. Met (or albumin) had no effect on the yield of Tyr-OH–Met = O, which is generated by a radical-mediated pathway (Reactions 1–5). Although radical scavengers such as ascorbate have the potential to scavenge Tyr radicals [44,45], the physiological relevance of Tyr radical formation is demonstrated by the fact that dityrosine formation is used as a clinical biomarker for oxidative stress [35,46,47]. Because Tyr radicals react preferentially with superoxide over dimerization [10,13] neutrophils might oxidize enkephalins via superoxide-mediated radical mechanism in vivo. We have shown that this mechanism operates even when most of the Met-Enk was converted to its sulfoxide (by neutrophil-generated HOCl), to give in this case a hydroperoxide–sulfoxide derivative.

A novel finding from this work is that neutrophils can oxidize methionine residues independent of hypohalous acids by using the peroxidation activity of myeloperoxidase in conjunction with $O_2^{\bullet-}$. Such reactions may not be restricted to enkephalins and could occur through protein-bound Tyr radicals reacting with $O_2^{\bullet-}$ to form hydroperoxides (as in [15]) and then oxidizing neighboring methionine residues.

Previous data suggest that neutrophils are not only contributors to pain in inflamed tissue, but could also be a major source of enkephalins that protect against neuropathic pain [6,17]. Enkephalins may also have a priming effect on neutrophil responses at physiological concentrations [18,48]. Based on our results, enkephalin oxidation by neutrophils could also occur at sites of inflammation. In fact when neutrophils are activated under inflammatory conditions, locally high concentrations of neutrophil-generated opioid peptides and oxidants could favor their biochemical interactions. Oxidation results in hydroperoxide (or alcohol) and sulfoxide formation on the Tyr and Met residues, respectively. In these reactions the Tyr residue is modified by the addition of a hydroperoxide (or alcohol) group, and cyclization through conjugate addition of the amine nitrogen destroys its aromatic character (Scheme 1, Reaction 3b). Oxidation of the Met residue to its sulfoxide does not compromise the analgesic activity of Met-Enk [49]. However, because the Tyr residue and its terminal amine group are pivotal for the activity of both Met-Enk and Leu-Enk [7,49], these oxidative Tyr modifications could dampen their opiate effect. Moreover, hydroperoxides are highly reactive species that could have adverse biological effects. If enkephalins are released locally by neutrophils together with a large amount of $O_2^{\bullet-}$, such inactivation may be possible and warrants further investigation.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.freeradbiomed.2010.05.033.

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