Endomorphins, endogenous opioid peptides, provide antioxidant defense in the brain against free radical-induced damage

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Abstract

Oxidative stress has been considered to be a major cause of cellular injuries in a variety of chronic health problems, such as carcinogenesis and neurodegenerative disorders. The brain appears to be more susceptible to oxidative damage than other organs. Therefore, the existence of antioxidants may be essential in brain protective systems. The antioxidative and free radical scavenging effects of endomorphin 1 (EM1) and endomorphin 2 (EM2), endogenous opioid peptides in the brain, have been investigated in vitro. The oxidative damage was initiated by a water-soluble initiator 2,2'-azobis(2-amidinopropane hydrochloride) (AAPH) and hydrogen peroxide (H2O2). The linoleic acid peroxidation, DNA and protein damage were monitored by formation of hydroperoxides, by plasmid pBR 322 DNA nicking assay and single-cell alkaline electrophoresis, and by SDS-polyacrylamide gel electrophoresis. Endomorphins can inhibit lipid peroxidation, DNA strand breakage, and protein fragmentation induced by free radical. Endomorphins also reacted with galvinoxyl radicals in homogeneous solution, and the pseudo-first-order rate constants were determined spectrophotometrically by following the disappearance of galvinoxyl radicals. In all assay systems, EM1 was more potent than EM2 and GSH, a major intracellular water-soluble antioxidant. We propose that endomorphins are one of the protective systems against free radical-induced damage in the brain.

Keywords: Endomorphin; Antioxidant; Comet assay; Lipid peroxidation; Free radical

1. Introduction

Endomorphin 1 (EM1) and endomorphin 2 (EM2), endogenous opioid peptides, have been found in much higher amounts in the human brain and they interact specifically and with high affinity with μ-opioid receptors [1]. The two peptides differ in one amino acid: EM1 (Tyr-Pro-Trp-Phe-NH2) and EM2 (Tyr-Pro-Phe-Phe-NH2). The major effect of the endomorphins is their antinociceptive action. They have been shown to be potent spinal, supraspinal, and peripheral antinociceptive, antihyperalgesic, and anti-allodynic agents [2–5]. Additionally, a number of studies have proved that endomorphins cause vasodilatation by stimulating nitric oxide release from the endothelium [6] and bind to μ-opioid receptors to activate G-proteins, regulate gastrointestinal transit, respiratory system and memory [2,7–10]. Recently, endomorphins have been investigated to modulate damage related to inflammatory diseases of the brain [11].

It is now commonly recognized that reactive oxygen species (ROS) are involved in a variety of physiological and pathological processes, including cellular signal transduction, cell proliferation and differentiation, and apoptosis, as well as ischemia-reperfusion injury, inflammation, and many neurodegenerative disorders [12–14]. Excess production of ROS can potentially damage different macromolecules such as proteins, DNA and lipids, leading to a variety of chronic health problems, such as cancer, aging and Parkinson’s disease [15–18]. The brain and nervous system may be especially prone to radical damage because, first, brain has a high content of easily peroxidizable unsaturated fatty acids; second, brain has a high content of both iron and ascorbate; and third, brain requires very high amounts of oxygen per unit weight. Furthermore, brain is not highly enriched antioxidant protective defenses and this then adds to its otherwise readily poised potential for oxidative dam-
age [17,18]. Therefore, the existence of antioxidants with an ability to scavenge radicals, and inhibit lipid peroxidation, DNA and protein damage in brain protective systems should be of critical importance.

It has been reported that the brain monoamines and their metabolites can inhibit lipid peroxidation and protect from oxidative damage in the brain [19]. Recent studies have demonstrated that enkephalins (leu-enkephalin, met-enkephalin) and their derivatives (5-S-cysteinylleu-enkephalin, 2-S-cysteinylleu-enkephalin and [d-Ala²,d-Leu⁵]enkephalin) have free radicals scavenging activities and the capacity to reduce ROS-induced lipid peroxidation [20–22]. We present herein the antioxidant effect of endomorphins on a water-soluble initiator 2,2′-azobis(2-amidinopropane hydrochloride) (AAPH)- and hydrogen peroxide (H₂O₂)-mediated lipid peroxidation, DNA strand breakage and protein fragmentation, and their free radical scavenging effect. It is suggested that EM1 and EM2, endogenous opioid peptides, may provide some antioxidant activities in protecting from oxidative damage in the brain.

2. Materials and methods

2.1. Materials

EM1 and EM2 were synthesized in our laboratory [23]. The purity of the compounds was determined by HPLC (>95%) and their structures were verified by mass spectrometry and amino acid analysis. Linoleic acid, agarose, low-melting point agarose, butylated hydroxytoluene (BHT), and L-glutathione (GSH) were purchased from Sigma. AAPH and galvinoxyl were purchased from Aldrich. pBR 322 DNA was obtained from MBI. Bovine serum albumin (BSA), acrylamide and bis-acrylamide were from BBI. All other chemicals were of the highest quality available.

2.2. Determination of linoleic acid hydroperoxides

Aliquots of the reaction mixture in an open vessel were taken out at appropriate time intervals and subjected to HPLC analysis using a Gilson liquid chromatograph with a ZORBAX ODS reversed phase column (6 × 250-mm Du Pont instruments) and eluted with methanol—water (95:5 v/v). The flow rate was set at 1.0 ml/min. A Gilson model 116 UV detector was used to monitor the total linoleic acid hydroperoxides at 235 nm. The concentration of the hydroperoxides was determined by integration of the peak area which was calibrated by iodometric determination of the hydroperoxides using molar extinction coefficient of 2.19 × 10⁴ at 350 nm. Every experiment was repeated three times and the results were reproducible within 10% experimental deviation.

2.3. Determination of oxidative DNA strand breakage

Conversion of the supercoiled form of plasmid DNA to the open-circular and further linear forms has been used as an index of DNA damage [24]. To assay DNA strand breakage induced by peroxyl radical and inhibited by EM1 and EM2, supercoiled pBR 322 plasmid DNA (0.1 μg) was incubated in 25 μl of 10 mM Tris–HCl buffer (pH 7.4) with AAPH, with or without different concentration of EM1 and EM2. After incubation for 1 h at 37 °C, 2 μl of loading buffer consisting of 0.25% xylene cyanole, 0.25% bromphenol blue tracking dye, and 30% (v/v) glycerol was added and the resulting mixtures were subjected to 1% agarose gel stained with ethidium bromide. Then, the samples were electrophoresed in a horizontal slab gel apparatus in TAE buffer (40 mM Tris, 20 mM sodium acetate and 2 mM EDTA) at 5 V/cm for 1 h. The gel was photographed under a transilluminator.

2.4. Single-cell alkaline electrophoresis (comet assay)

Human mononuclear cells were isolated from fresh blood obtained from healthy volunteers as described previously [25]. Comet assay was performed as described by Singh et al. [26]. In brief, mononuclear cells (2 × 10⁶ cells) treated with H₂O₂ and/or EM1, EM2 and GSH were washed with PBS and then mixed thoroughly with 1% low-melting point agarose dissolved in PBS at 39 °C. The mixture was transferred immediately onto a glass microscope slide pre-coated with 0.5% normal melting point agarose dissolved in PBS at 39 °C. The mixture was placed on top of ice for 10 min. After removing the cover slip, the slide was immersed slowly in alkaline cell lysis solution (2.5 M NaCl, 0.1 M EDTA, 1% N-lauroylsarcosine, 1% Triton X-100, 10% DMSO, 10 mM Tris, pH 10) at 4 °C for 1 h to remove cellular membranes and proteins. Slides then were denatured in a horizontal electrophoresis tank containing 0.3 M NaOH and 1 mM EDTA for 20 min. Next,
electrophoresis was carried out at a constant potential of 25 V/300 mA for 20 min. Afterwards, each slide was gently immersed three times in neutralization buffer (0.4 mM Tris–HCl, pH 7.5) for 5 min. After applying 100-μl ethidium bromide solution (5 μg/ml) to stain and covering with cover slips, the slides were viewed with a fluorescence microscope and 150 randomly selected cell nucleoids were scored on a slide, and gave the overall comet score ($A$), in which the tail is longer than half of length of head. The percentage DNA damage was calculated from the ratio of the measurements as follows: ($A/150$) × 100%. The scoring method is consistent with computer imaging analysis [27].

2.5. Determination of protein oxidation

Protein oxidation was assayed as described previously [28] with minor modifications. Oxidation of BSA (40 μg/ml) in PBS was initiated by AAPH and inhibited by EM1, EM2 and GSH. After incubation for 24 h at 37 °C, 0.02% (w/v) BHT was added to the reaction mixture to prevent the formation of further peroxyl radical. Then, the samples were mixed with an equal volume of 2× SDS-PAGE sample buffer (100 mM Tris–HCl, pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.01% bromophenol blue), heated at 100 °C for 5 min and loaded onto a 10% SDS polyacrylamide gel. The gels were stained with 0.05% Coomassie brilliant blue R-250. The gel was photographed.

2.6. Reaction with galvinoxyl radical

The reaction kinetics of galvinoxyl (5 μM) with EM1, EM2 and GSH (100 μM) in ethanol solution were monitored spectrophotometrically at 429 nm [29] with a Hitachi model 557 UV spectrometer (Hitachi High Technologies) at 37 °C.

2.7. Statistical analysis

Unless otherwise indicated, each result described in this paper is representative of at least three separate experiments.

### Table 1

<table>
<thead>
<tr>
<th>Compounds</th>
<th>$R_{inh}$ (10⁻⁶ M min⁻¹)</th>
<th>$R_{p}$ (10⁻⁶ M min⁻¹)</th>
<th>$t_{inh}$ (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4.90</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSH (80 μM)</td>
<td>0.69</td>
<td>4.40</td>
<td>31.0</td>
</tr>
<tr>
<td>EM2 (80 μM)</td>
<td>1.97</td>
<td>4.28</td>
<td>14.2</td>
</tr>
<tr>
<td>EM1 (40 μM)</td>
<td>1.19</td>
<td>4.25</td>
<td>42.0</td>
</tr>
<tr>
<td>EM1 (80 μM)</td>
<td>0.33</td>
<td>4.60</td>
<td>84.5</td>
</tr>
</tbody>
</table>

* The reaction conditions and the initial concentration of the substrates are the same as described in the legends of Fig. 1 for reactions conducted in SDS micelles. Data are the average of three determinations which were reproducible with deviation less than ± 10%.

The inhibition of AAPH-initiated peroxidation of linoleic acid by endomorphins and GSH in SDS micelles is shown in Table 1. A set of representative kinetic curves of the total hydroperoxides formation during the peroxidation of linoleic acid in SDS micelles is shown in Fig. 1. It is seen from Fig. 1 that the concentration of the hydroperoxides increased fast and linearly with time in the absence of antioxidants upon AAPH-initiation, demonstrating fast peroxidation of the substrate. The hydroperoxide formation was inhibited by addition of
EM1, EM2 and GSH in the inhibition period. After the inhibition period the rate of hydroperoxide formation turned faster, which is close to the original rate of propagation, demonstrating the exhaustion of the antioxidant. In the inhibition period the concentration of the hydroperoxides increased approximately linearly with time and $R_{\text{inh}}$ also reflects the antioxidative potential of the antioxidant. Fig. 1 also shows the antioxidant effects of EM1, EM2 and GSH on AAPH-induced linoleic acid peroxidation. The kinetic data deduced from Fig. 1 are listed in Table 1. It is clearly seen from Fig. 1 and Table 1 that addition of EM1 to SDS micelles significantly increased the inhibition period $t_{\text{inh}}$ and decreased the $R_{\text{inh}}$, and the inhibition period depended on the concentration of EM1. On the basis of $t_{\text{inh}}$ and $R_{\text{inh}}$, the antioxidant activity follows the sequence EM1>GSH>EM2 in SDS micelles.

### 3.2. Inhibition of AAPH-mediated DNA strand breakage by EM1 and EM2

The plasmid DNA nicking assay was used as an initial approach toward determining whether EM1 and EM2 could protect against AAPH-induced DNA damage. Fig. 2A shows gel electrophoretogram of pBR322 DNA treated with different concentrations of AAPH at 37 °C for 1 h. As observed, the plasmid DNA was mainly of the supercoiled form in the absence of AAPH (Fig. 2A, lane 1). With the addition of AAPH (1.25–40 mM), the DNA supercoiled form decreased gradually and converted into the open circular and linear form (Fig. 2A, lanes 2–7). Fig. 2B indicates the protective effects of EM1, EM2 and GSH against 10 mM AAPH-induced DNA damage. It is clearly seen from Fig. 2 that addition of EM1, EM2 and GSH significantly increased supercoiled form of pBR322 DNA, and the generation of supercoiled form was dependent upon concentrations of EM1 and EM2. It is suggested that EM1 and EM2 can protect against AAPH-induced DNA damage and EM1 is more effective than EM2.

### 3.3. Inhibition of $H_2O_2$-induced oxidative DNA damage by EM1 and EM2

We investigated the inhibitory effects of EM1 and EM2 on $H_2O_2$-induced DNA damage in human peripheral blood mononuclear cells by the comet assay (Fig. 3). It was found that mononuclear cells showed increased DNA strand breakage after treated with different concentration of $H_2O_2$ in PBS for 10 min, and the percentage of DNA damage was about 95% in the presence of 50 μM $H_2O_2$. However, with the addition of EM1 and EM2, the DNA strand breakage was inhibited in a concentration-dependent manner. EM1 is more effective than EM2.

### 3.4. Inhibition of AAPH-induced protein fragmentation by EM1 and EM2

As shown in Fig. 4A, when BSA was incubated with AAPH at 37 °C for 24 h, a concentration-dependent degradation pattern of BSA was observed using SDS polyacrylamide gel electrophoresis. Treatment of albumin with
EM1, EM2 and GSH in the presence of 20 mM AAPH decreased the extent of protein fragmentation (Fig. 4B and C). Compared to the AAPH-treated albumin band in lane 2, a discernible increase in the intensity of protein bands was noted in a concentration-dependent manner. On the basis of the intensity of protein bands, the protective activity follows the sequence of EM1>GSH>EM2, similar to that observed in SDS micelles.

3.5. Free radical scavenging activity of EM1 and EM2 as studied by reaction with galvinoxyl

The kinetic decay of galvinoxyl radicals in homogeneous solution has been used to evaluate the chemical activity of antioxidants of biochemical interest [30]. In the present study, the interaction of EM1, EM2 and GSH with galvinoxyl was studied in ethanol solution. In the absence of antioxidants, the UV spectrum of galvinoxyl was stable under the experimental conditions, and no appreciable decay of its UV spectrum could be observed within several hours. When EM1, EM2 and GSH were added to the ethanol solution of galvinoxyl, the UV spectrum of galvinoxyl diminished gradually with time. A set of representative experimental results is shown in Fig. 5, demonstrating that EM1, EM2 and GSH directly scavenged galvinoxyl radicals. The decay was found to be exponential and the pseudo-first-order rate constants ($k_{\text{obs}}$) for the reactions can be obtained from the slope of the linear plot of the logarithm of the concentration of galvinoxyl versus reaction time (Fig. 6). The results are summarized in Table 2. It is seen from Fig. 6 and Table 2 that the reaction activity of EM1, EM2 and GSH with galvinoxyl follows the sequence of EM1>GSH>EM2, similar to the sequences for their anti-linoleic acid hydroperoxides activities and DNA and protein damage inhibitory activities.

4. Discussion

There is little doubt that oxygen-related free reactions are pervasive in living systems and that cellular or tissue oxidative damage results in brain aging and neurodegenerative disorders. Removal of excess ROS or suppression of oxygen free radical generation by antioxidants may be effective in preventing oxidative cell death [31,32]. In this study we used AAPH, a water-soluble initiator, which decomposes at physiological temperature producing alkyl radicals followed by fast reaction with oxygen to give alkyl peroxyl radicals [33,34] to initiate the linoleic acid peroxidation (Eqs. (1)–(5)).

Initiation:

$$\text{AAPH} \rightarrow 2\text{R}^* + \text{N}_2$$  \hspace{1cm} (1)

$$\text{R}^* + \text{O}_2 \rightarrow \text{ROO}^*$$  \hspace{1cm} (2)
ROO· + LH → ROOH + L·

Propagation:
L· + O₂ → LOO·
LOO· + LH → LOOH + L·

where R is the alkyl radical generated from the thermal decomposition of AAPH and LH represents a lipid molecule with an abstractable hydrogen, i.e., linoleic acid. In the presence of an antioxidant molecule, AH, either the initiating peroxyl radical and/or the propagating lipid peroxyl radical can be trapped and a new antioxidant radical, A⁻, produced (Eqs. (6) and (7)). If the A⁻ is a stabilized radical (e.g., α-tocopherol radical or ascorbate radical) which can promote the rate-limiting hydrogen abstraction reactions (Eqs. (6) and (7)) and undergo fast termination reactions (Eqs. (8) and (9)), the peroxidation would be inhibited.

ROO· + AH → ROOH + A⁻
LOO· + AH → LOOH + A⁻
A⁻ + LOO⁻ → LOOA
2A⁻ → A – A + Other products

The primary peroxidation products of linoleic acid are hydroperoxides. They showed characteristic UV absorption at 235 nm, which was used to monitor the formation of the total hydroperoxides by HPLC [35,36]. The kinetics of linoleic acid peroxidation initiated by azo-compounds and its inhibition by chain-breaking antioxidants have been discussed in detail in previous papers [37,38]. The rate of the chain propagation, \( R_p \), in Eq. (5), the inhibited rate of propagation by antioxidants, \( R_{inh} \), in Eq. (7), and the inhibition period, \( t_{inh} \), can be easily obtained from HPLC experiment. In the present work, we can find from Fig. 1 and Table 1 that on the basis of \( t_{inh} \) and \( R_{inh} \), the antioxidant activity follows the sequence EM1>GSH>EM2 in SDS micelles. It is suggested that EM1 can trap the AAPH-derived radical (ROO·) in the bulk water phase and the propagating linoleic acid peroxyl radicals (LOO·) on the surface of the micelle and behave well as chain-breaking antioxidants against AAPH-induced linoleic acid peroxidation in SDS micelles.

Free radicals can attack DNA, causing sugar fragmentation, base modification, and DNA strand breakage. AAPH-derived peroxyl radical at 37 °C can abstract a hydrogen atom from the C-4' atom of DNA molecules and cause strand breakage [39]. The plasmid DNA nicking assays are relatively simple, yet sensitive and quantitative assays based on the differential mobility of supercoiled, circular and linear forms of plasmid DNA in agarose gel electrophoresis [24]. The comet assay is a visual fluorescent technique for measurement of DNA strand breaks in individual cells. It is generally believed that it is a simple, reliable, reproducible and sensitive technique for assessing DNA damage in individual cells and detecting intercellular differences in DNA damage [40,41]. It is clearly seen from Figs. 2 and 3 that EM1 and EM2 have protective effects on AAPH-induced plasmid DNA strand breakage and \( \mathrm{H}_2\mathrm{O}_2 \)-induced DNA damage in human peripheral blood mononuclear cells, and EM1 is more effective than EM2. Furthermore, we also used BSA as a model protein to test the ability of EM1 and EM2 to inhibit protein fragmentation induced by AAPH. The inhibitory activity follows the sequence of EM1>G-SH>EM2.

Galvinoxyl is a stable phenoxy radical that exhibits characteristic UV absorption at 429 nm in ethanol solution. This allows easy measurement of the depletion of galvinoxyl radicals in the presence of antioxidants [29]. It is seen from Fig. 6 and Table 2 that the reaction activity of EM1, EM2 and GSH with galvinoxyl follows the sequence of EM1>G-SH>EM2, similar to the sequences for their antilinoleic acid hydroperoxides activities and DNA and protein damage inhibitory activities.

The results presented in this paper provide evidence that endomorphins, endogenous opioid peptides in the brain, can protect lipid peroxidation, DNA and protein damage induced by free radicals. They are also effective scavengers for galvinoxyl radicals. These facts suggest that endomorphins can react with the AAPH-derived radicals. In all assay systems, the antioxidant effect of EM1 is significantly more potent than that of EM2 and GSH, a major intracellular water-soluble antioxidant. The difference of EM1 and EM2 is primarily Trp and Phe at position 3. Trp does not posses phenolic hydrogens and only has indole ring, similar to melatonin which has been reported able to protect against lipid peroxidation induced by ROS and is potent both hydroxyl and peroxyl radicals scavenger. Its antioxidant mechanism is complex and not completely clear, and probably involves abstracting hydrogen and electron transfer reaction [42–44]. Therefore, it is suggested that the active group of EM1 trapping free radicals might be phenol hydrogen of Tyr and indole hydrogen of Trp and the latter should be very important.

Although human body in general has evolved several defense mechanisms to counteract oxidative stress, the brain appears to be more susceptible to this damage than other organs [18,45]. It is widely believed that one of the most important events during pathogenesis of neurodegenerative diseases is depletion of GSH in the brain. Hence, it is plausible to increase GSH levels. However, GSH cannot easily penetrate the blood–brain barrier due to the presence of the cysteine SH group and is not efficiently absorbed into neuronal cells in the brain [46]. Therefore, novel therapeutics based on blocking neuron damaging neuroinflammatory processes show great promise and the existence of antioxidants may be essential in brain protective
systems. Recent studies have demonstrated that endogenous opioid peptides are released from cells during inflammation and stress, and reach high levels at these sites, where the concentration of endorphins may be close to that found in our experiments [20,21]. We propose that an important function of endorphins is to scavenge radicals, and inhibit lipid peroxidation, DNA and protein oxidative damage so they provide antioxidant defense in the brain against oxidant- and free radical-induced damage. As a consequence, the neuroprotective activities of endorphins may provide new insights into therapeutics of neurodegenerative diseases and a new understanding for oxidant stress in the brain.

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