ACCELERATED COMMUNICATION

Secretary Peptide Hormones Are Biochemical Antioxidants: Structure-Activity Relationship

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Received May 7, 2001; accepted November 1, 2001

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

The secretory peptides luteinizing hormone-releasing hormone, enkephalin, angiotensin, and oxytocin are biochemical antioxidants in aqueous medium. These hormones scavenge free peroxyl radicals, prevent the oxidation of low-density lipoprotein, and inhibit lipid peroxidation in brain membranes. Their capacity to directly suppress free radical-mediated reactions is demonstrated by electron-spin resonance spectroscopy. Electrospray ionization-mass spectrometry analysis of the free radical-quenching reaction reveals distinct oxidation products, including peptide dimers. Moreover, secretory peptide hormones can scavenge reactive nitrogen species derived from nitric oxide and peroxynitrite. An analysis of the structure-activity relationship indicates that their antioxidant activity is derived from the occurrence of solvent-exposed tyrosine and tryptophan residues, which is consistent with the mass spectrometry results. Significant effects in vitro can be observed at nanomolar concentrations, which makes these peptides comparable in potency with classic antioxidants having low molecular mass. Secretory peptide hormones may constitute an important part of the antioxidant defense system, and the sequences of the described antioxidant peptides may be unique lead structures for the rational design of novel antioxidant drugs having an improved pharmacological profile.

Free radicals play an important role in cellular physiology. They are produced by diverse enzyme systems, either constitutively or in response to various stimuli, ranging from neurotransmitters and other extracellular signaling molecules to shifts in the intracellular ratio of single substrates of energy metabolism (Lander, 1997). Free radicals may lead to distinct cellular responses such as differentiation or cell division, but excessive production of free radicals or other electrophilic species may result in an adverse cellular and physiological state termed "oxidative stress," which is reflected by the loss of function of important cellular macromolecules (Sies, 1986; Irani et al., 1997; Anderson et al., 1999). Oxidative stress can ultimately lead to necrotic or apoptotic cell lysis and cell death.

The extracellular compartment is likewise subjected to oxidative alterations in structure and function, which may lead to detrimental biochemical and physiological consequences. Examples of this are the oxidation of low-density lipoprotein (LDL), causally involved in the atherosclerotic process (Heinecke, 1999); oxidative changes in the aggregation behavior of eye lens proteins, leading to cataracts (Christen, 1999); or oxidative cross-links of collagen as observed in diabetes, arthritis, or aging in general (Beckman and Ames, 1998; Baynes and Thorpe, 1999). The extracellular space usually exhibits lower turnover rates than the intracellular compartment; nevertheless, a number of global and local secretory antioxidant defense systems of the extracellular space are known and are also potentially affected by certain states of disease (Sies, 1993; Cross et al., 1998). These antioxidants range from plasma proteins such as albumin (Halliwell, 1998) to compounds with low molecular weight such as melatonin, a secreted pineal hormone acting as a regulator of the circadian rhythm (Vanecek, 1998) and as a unique endogenous neuroprotective antioxidant (Reiter, 1998).

Peptides with low molecular mass can exhibit a disparate

ABBREVIATIONS: LHRH, luteinizing hormone-releasing hormone; PBN, N-tert-butyl-α-phenylnitrone; luminol, 5-amino-2,3-dihydro-1,4-phthalazinedione; AIBN, 2,2′-azo-bis-(2-methylpropionamide); ACCH, 1,1′-azo-bis-(1-cyanocyclohexane); SIN-1, 3-morpholinosydnonimine; LDL, low-density lipoprotein; ESR, electron-spin resonance; UV/VIS, UV/visible; FSHRF, follicle-stimulating hormone-releasing factor; amu, atomic mass units; Hb, hemoglobin; RNS, reactive nitrogen species.
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redox biochemistry; apart from the well-known examples of thiol-containing cofactors serving as shuttles of reducing equivalents (glutathione) (Sies, 1999), numerous peptides can initiate pro-oxidative events in cell-dependent (Behl et al., 1994) and -independent ways (Dikalov et al., 1999). The exact mechanisms of peptide pro-oxidant action, however, are not completely understood (Schubert et al., 1995; Dikalov et al., 1999). A prime example of this is Alzheimer’s disease-associated amyloid β protein (Behl, 1997).

Our recent finding that peptide stretches cut from the transmembrane domains of integral membrane proteins act as cytoprotective agents and redox regulators in lipid bilayers (Moosmann and Behl, 2000) prompted us to investigate whether other endogenously occurring peptide structures, especially secretory peptide hormones, could also exhibit antioxidant activities in their respective topological compartments. Finally, we were interested in elucidating the structure-activity relationship of peptide antioxidant action, and we investigated the biochemical antioxidant potential of peptide antioxidants compared with classic low-molecular-weight antioxidants.

Materials and Methods

Peptides and Chemicals. All peptide hormones and derivatives thereof were from Bachem (Bubendorf, Switzerland), if not otherwise stated. Luteinizing hormone-releasing hormone [LHRH(7–10)], N-tert-butyl-a-phenylnitrone (PBN), 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol), and all biochemicals were from Sigma Chemical (Deisenhofen, Germany). The highest grade available was ordered for each: tert-Butyl-hydroperoxide, 2,2’-azo-bis-(2-methylpropionamide) (AIBN), 1,1’-azo-bis-(1-cyanocyclohexane) (ACCH), and all solvents were supplied by Aldrich Chemical (Deisenhofen, Germany). Peroxynitrite, spermine NONOate, and 3-morpholinosydnonimine (SIN-1) were from Calbiochem (Schwalbach, Germany). Bovine serum albumin was purified by column chromatography using Sephadex G-25 from Amersham Biosciences (Uppsala, Sweden).

Protein Oxidation. Porphyridium cruentum B-phycoerythrin (10 nM) was oxidized using AIBN (500 μM) at 37°C as a source of free peroxyl radicals essentially as published previously (Mooradian, 1993). The destruction of the protein was measured by monitoring the temporal decrease in intrinsic protein fluorescence by flash fluorometry (excitation window, 340 ± 50 nm; emission window, 572 ± 6 nm; counting delay, 25 μs; counting window, 30 μs) with a 1420 multilabel counter (PerkinElmer Wallac, Freiburg, Germany). Under the conditions used, no significant photobleaching occurred. When testing the lipophilic antioxidants α-tocopherol and 17β-estradiol, the final ethanol concentration was 0.5%.

LDL Oxidation. The oxidation of human blood plasma LDL was done exactly as described previously (Moosmann and Behl, 1999). In brief, fresh human LDL (0.1 mg/ml protein) was oxidized catalytically by 10 μM CuSO4 at 37°C. Reaction products of LDL decomposition (conjugated dienes) were measured photometrically at 234 nm with a DU 640 spectrophotometer (Beckman, Unterschleissheim, Germany).

As a second method of LDL oxidation, experiments were performed using AIBN to induce conjugated diene formation. LDL (0.2 mg/ml protein) was supplemented with 15 μM EDTA, and then the respective peptides were added. Thermal decomposition of AIBN (5 mM) at 37°C initiated LDL oxidation in a metal-independent way. At selected time points, aliquots were diluted (1:4) with phosphate-buffered saline and were immediately measured at 234 nm. The results were normalized to control values resulting from measurements without LDL.

Brain Lipid Peroxidation. Native cortical rat brain membranes were prepared from adult female Sprague-Dawley rats and were assayed essentially as described previously (Moosmann and Behl, 1999). Samples (0.5 mg/ml protein) were incubated with ascorbate (50 μM) at 37°C to initiate the oxidative decomposition of the lipid membranes. Low-level chemiluminescence, indicative of peroxidation reactions in progress, was monitored by single photon counting with an LS 6500 scintillation counter (Beckman).

ESR Spectrometry. Electron-spin resonance (ESR) spectra were acquired on an ER-200 n-SRC spectrometer (Bruker, Rheinstetten, Germany) supplied with a TE 102 cavity. The settings were: center field, 3360 G; field sweep, 100 G; scanning time, 5 s; modulation frequency, 100 kHz; peak-to-peak modulation amplitude, 2 G; microwave power, 5 mW; and receiver gain, 2.5 × 106.

The samples were prepared by coinubcation of the spin trap PBN (50 mM), the corresponding concentration of the peptide hormones, and the radical initiator ACCH (100 mM) in acetonitrile/ethanol/water (1:2:1). The samples were incubated at 80°C for 6 h; afterward, they were transferred to glass capillaries having an internal diameter of 0.9 mm. The typical sample volume was 40 to 50 μl. All recordings were performed at room temperature.

Mass Spectrometry. Electrospray ionization-mass spectra were measured with an API 150EX liquid chromatograph/mass spectrometer (Applied Biosystems, Weiterstadt, Germany). For sample application, 5 μl of each sample was injected into a high-performance liquid chromatography loop (internal volume, 10 μl). The loop was unloaded into the spectrometer by flushing the capillary with acetonitrile/water (1:1) supplemented with 0.1% formic acid at a flow rate of 10 μl/min. The samples were identical with those used in the ESR experiments.

Nitric Oxide Scavenging. Nitric oxide was liberated from spermine NONOate (10 μM) in nitrogen-flushed, afterward-degassed phosphate-buffered saline at 22°C. Induced chemiluminescence of luminal (20 μM) was monitored after 3 h to quantify the activity of nitric oxide-derived species in aqueous solution with or without added peptide hormones.

Peroxynitrite Scavenging. Peroxynitrite was either used directly as a bolus or generated in situ by thermal decomposition of SIN-1. Bolus peroxynitrite in alkaline solution was rapidly mixed with a solution of phosphate-buffered (20 mM, pH 7.4) B-phycoerythrin (20 nM) to yield a peroxynitrite concentration of 100 μM. The final pH of the buffer did not change significantly; treatment of phycoerythrin with a corresponding sodium hydroxide solution with or without added nitrate had no effect on protein fluorescence measured as described above. Using SIN-1 as a source of peroxynitrite, the effect of 1 μM SIN-1 on phycoerythrin fluorescence during a 4-h incubation at 37°C was monitored. Chemically, SIN-1 can liberate nitric oxide and superoxide, but under the conditions used, it behaves as a source of peroxynitrite (Singh et al., 1999). Hemoglobin (5 μM) from human plasma was incubated with SIN-1 (10 μM) at 37°C for 3 h with or without peptide hormones. The resulting changes in absorption were measured by UV/VIS spectrometry. SIN-1–treated LHRH was prepared by incubation of a 3-fold molar excess of SIN-1 with LHRH (10 mM) at 37°C overnight.

Results

Peptide Hormones Act as Biochemical Antioxidants. We have identified a novel class of endogenous antioxidants: short, soluble tyrosine- or tryptophan-containing peptide hormones and mediators such as LHRH, [Leu] and [Met]enkephalin, angiotensin I and II, vasopressin, and oxytocin. These secretory peptides show the capacity to block the oxidative destruction or modification of soluble proteins, lipoproteins, and lipid membranes (Fig. 1, Table 1). The left column in Fig. 1 shows the potential of LHRH, [Leu]enkephalin, angiotensin II, and oxytocin to prevent the oxidation of the globular protein phycoerythrin by the radical initiator AIBN. Enkephalin, angiotensin, and oxytocin protect this
globular protein with half-maximal effective concentrations of approximately 2 μM; LHRH exhibits half-maximal protection at a concentration of less than 200 nM. The central column in Fig. 1 illustrates the capacity of secretory peptide hormones to prevent the metal-catalyzed oxidation of human plasma low-density lipoprotein. All four hormones significantly delay the oxidation of LDL at a concentration of 20 μM. Because the peptides’ antioxidant action was not related to the molar ratio of copper versus peptide, and because differential UV/VIS spectrometry did not indicate a direct interaction of the peptide hormones with the pro-oxidant catalyst copper (data not shown), we conclude that the formation of a copper complex is not the major origin of the LDL-protective effect. This is confirmed by the fact that all the peptides also show significant protection against metal-independent, AIBN-induced LDL oxidation (Fig. 1, central column, inlays).

With respect to brain lipid peroxidation, LHRH, again, is the most effective structure (Fig. 1, right column), but the other hormones also show significant but less pronounced effects at low micromolar concentrations. This difference might be accounted for by the higher lipophilicity of LHRH compared with the other terminally charged peptides (Table 1).

Antioxidant Potential of the Secretory Peptide Hormones Is Based on Their Tyrosine and Tryptophan

![Fig. 1. Antioxidant properties of peptide hormones. Left, peptide hormones protect globular proteins from destruction by peroxyl radicals liberated from AIBN. •, control; ○, 200 nM; ▲, 2 μM; ▽, 20 μM concentrations of LHRH (a), [Leu]enkephalin (d), angiotensin II (g), and oxytocin (j). In the case of LHRH (a), a concentration of 200 nM is already higher than the compound’s EC50. Quadruplicate determinations are shown. Center, human plasma LDL is protected from copper-catalyzed oxidation. •, control; ▲, 20 μM; △, 100 μM concentrations of LHRH (b), [Leu]enkephalin (e), angiotensin II (h), and oxytocin (k). Center inlays, all the peptides also exhibit significant antioxidant effects in LDL oxidation as induced by AIBN. Experiments were performed in duplicate. Right, secretory peptides protect neuronal membranes from oxidative destruction. •, LHRH; ▲, [Leu]enkephalin; △, angiotensin II; ▽, oxytocin. The hydrophobic molecule LHRH is very effective as a membrane antioxidant; the other peptides exhibit partial protection. Triplicate determinations were performed. Results are given as mean ± S.E.M.](image-url)
TABLE 1
Sequences of some tyrosine- and tryptophan-containing peptide hormones exhibiting potent antioxidant properties

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>LHRH (human)</td>
<td>PyrHWSYGLRPG-amide</td>
</tr>
<tr>
<td>LHRH (salmon)</td>
<td>PyrHSMKFWPG-amide</td>
</tr>
<tr>
<td>FSHRF</td>
<td>PyrHSMNKKPG-amide</td>
</tr>
<tr>
<td>[Leu]enkephalin</td>
<td>YGGFL</td>
</tr>
<tr>
<td>[Met]enkephalin</td>
<td>YGGFM</td>
</tr>
<tr>
<td>Oxytocin</td>
<td>CYIQNCPLG-amide (oxidized form)</td>
</tr>
<tr>
<td>Vasopressin</td>
<td>CFQNPCFRG-amide (oxidized form)</td>
</tr>
<tr>
<td>Angiotensin I</td>
<td>DRVYHFPFWL</td>
</tr>
<tr>
<td>Angiotensin II</td>
<td>DRVYHFPF</td>
</tr>
</tbody>
</table>

Pyr, pyroglutamate. Tyrosine and tryptophan residues are in boldface type.

**Content.** Using truncated LHRH versions, Fig. 2 shows that the antioxidant activity of LHRH as a peroxyl radical scavenger depends on its tryptophan(3) and tyrosine(5) residues. Although LHRH(3–10) (Fig. 2b) equals LHRH(2–10) (Fig. 2a) and LHRH (Fig. 1a), the antioxidant effect of LHRH(4–10), lacking the tryptophan residue, is clearly reduced (Fig. 2c). Removal of the tyrosine residue from the remaining fragment, as in LHRH(7–10), leads to the loss of any antioxidant activity (Fig. 2d). Analogously, the effect of [Leu]enkephalin is exclusively dependent on its tyrosine(1) residue’s phenolic group: des-Tyr-[Leu]enkephalin is almost completely devoid of any effect (Fig. 2e). A similar loss of effect is observed when the tyrosine hydroxyl group is sulfated (Fig. 2f). An example of a peptide containing only tryptophan, but no tyrosine residues, which nevertheless performs as a potent antioxidant, is follicle-stimulating hormone-releasing factor (FSHRF) (Table 1; data not shown). Thus, tyrosine and tryptophan can act as independent carriers of antioxidant activity in secretory peptide hormones and short peptides in general. A similar structure-activity relationship and the necessity of tyrosine or tryptophan residues could be observed in the other assays of biochemical antioxidant activity (data not shown).

**Peptide Antioxidants Are Direct-Spin Quenchers.** To elucidate the mechanisms of the biochemical antioxidant effects of the peptide hormones in more detail, we performed a series of electron-spin resonance experiments and, in parallel, analyzed the fate of the peptide hormones by electrospray ionization-mass spectrometry. The results of these experiments with [Leu]enkephalin as a model hormone are shown in Fig. 3. The radical initiator ACCH, incubated with the spin trap PBN, leads to a strong paramagnetic signal in ESR spectroscopy (Fig. 3b). The addition of a small amount of the peptide in this experimental setting (5 mM peptide, 50 mM PBN, 100 mM ACCH) leads to a significantly decreased intensity of the paramagnetic signal (Fig. 3, d versus b), whereas the peptide itself becomes almost completely oxidized (Fig. 3c). The native peptide, with its mass of 556.4 amu, makes up only a minor proportion of the ions, whereas radical-oxidized species predominate, possibly because of the large ACCH/peptide ratio. The major species can be assigned as singly and doubly cyanated enkephalin (582.0 and 608.2 amu, respectively), resulting from the reaction with the initiator cyanocyclohexyl radical, and as a compound originating from the reaction of the peptide with the spin trap (644.0 amu; Fig. 3, c and g). An increased concentration of the peptide (50 mM) quenches further the PBN spin-trap signal (Fig. 3e). Because PBN, a classic nitrene spin trap, and [Leu]enkephalin were used in identical concentrations in this experiment, [Leu]enkephalin seems to be a rather potent spin quencher, outweighing the scavenging properties of PBN. An interpretation of the mass spectrometry peaks of this experiment is given in the legend.
Peptide hormones are direct spin quenchers and free radical sinks. The oxidation of spin traps by free radicals is prevented by peptide hormones, whereas the peptides themselves become converted to oxidized monomeric and dimeric forms. Left, mass spectra of different samples of the spin trap PBN (50 mM) oxidized with ACCH with or without [Leu]enkephalin as a radical scavenger. Note the different axis scalings. Right, the identical samples as analyzed by electron-spin resonance spectroscopy, all depicted in the same scale. a and b, the incubation of PBN with the free radical initiator alone does not result in any significant amounts of products with a higher mass than 400 amu (a); the spin trap becomes significantly oxidized (b). c and d, 5 mM [Leu]enkephalin partially suppress the formation of PBN spin adducts (d); the peptide (556.4 amu) becomes almost completely oxidized (582.0 and 608.2 amu) by cyanide radicals (c). e and f, a higher concentration of peptide results in a further suppression of PBN radicalization (f), whereas only a part of this larger amount of peptide becomes oxidized (e). Different dimeric forms of the peptide can be traced. g and h, without initiator, no free radicals occur (h), whereas [Leu]enkephalin partially reacts with the spin trap (644.1 amu) and dimerizes (1111.5 amu) (g). The labeled mass spectrometry peaks can be interpreted as the following structures [peptide, [Leu]enkephalin; dimer, bis([Leu]enkephalin)]; 556, peptide; 582, peptide-CN; 597, peptide-C(CH$_3$)NH; 608, peptide(-CN)$_2$; 644, peptide-N(OH)C(CH$_3$)$_3$; 1111, dimer; 1137, dimer-CN; 1153, dimer-C(CH$_3$)NH; 1199, dimer-N(OH)C(CH$_3$)$_3$; 1216, dimer(-CN)$_4$. The bar in h indicates 10G.
to Fig. 3. It is remarkable that although the immediate peptide dimer does not occur (1111.5 amu) in this experimental setting, all the dimers incorporating one native peptide and one of the detected oxidized monomers can be found (1137.4, 1153.8, and 1199.5 amu). We conclude that the reactivation of a peptide molecule with an initiator radical leads to the activation of this peptide to react with a second peptide molecule. Therefore, the dimerization process, which can only occur at the tyrosine residue of [Leu]enkephalin, chemically interlocks with the prior modification by free radicals (e.g., by cyanylation). The sites of these reactions most consequently be in close proximity, and the idea that the ortho positions of the tyrosine residue act as primary radical sinks seems likely. This notion is also supported by the inefficiency of des-Tyr-[Leu]enkephalin as a spin quencher (data not shown). Finally, peptide hormones in vitro also seem to undergo dimerization in response to free radical attack, which is a well-known relaxation pathway of various phenolic antioxidants, such as flavonoids. As a consequence of these experiments, we assume that the biochemical antioxidant properties of the investigated peptide hormones rely on their capacity to directly react with free radicals.

**Reactive Nitrogen Species (RNS) Interactions with Antioxidant Peptides.** Because of their tyrosine and tryptophan moieties, secretory peptide hormones also scavenge RNS, especially peroxynitrite (Fig. 4). Figure 4a shows that LHRH, [Leu]enkephalin, angiotensin II, and oxytocin are scavengers of nitric oxide-derived RNS exhibiting comparable potency. They significantly suppress the induced luminescence of luminol upon treatment with the nitric oxide–liberating compound spermine NONOate. The interaction of these peptide hormones with reactive nitrogen species also extends to peroxynitrite. When using SIN-1 as a slowly decomposing source of peroxynitrite, the protective effects of the peptide hormones were almost complete (Fig. 4b). But also when bolus peroxynitrite was used as an oxidant for the globular fluorescent protein phycoerythrin, the peptide hormones showed a clear protective potential in a micromolar concentration (Fig. 4c), which again was dependent on their tyrosine and tryptophan content (data not shown). In a competition assay for SIN-1–derived reactive nitrogen species, LHRH was able to significantly compete with hemoglobin, a known efficient scavenger of these species (Fig. 4d). This further supports the conclusion that peptide hormones can act as potent biochemical antioxidants under a variety of conditions.

**Peptidic Antioxidants Compared with Standard Antioxidant Structures.** To assess the merely structural potential of peptidic antioxidants, the sequence of LHRH was taken as a lead structure and was compared with four well-established biochemical antioxidants: two lipophilic compounds, α-tocopherol and 17β-estradiol, and two hydrophilic structures, melatonin and serum albumin (Fig. 5). Compared with the pineal hormone melatonin, LHRH showed a slightly higher efficacy in scavenging peroxyl radicals. A higher antioxidant activity of LHRH was also found when comparing it with bovine serum albumin in an identical molar concentration, despite the fact that bovine serum albumin has a molecular mass of 66 kDa and contains 2 tryptophan and 20 tyrosine residues (Fig. 5, a–c). This could be explained by the fact that in globular aqueous proteins with an intact threedimensional structure, aromatic residues are usually buried

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**Fig. 4.** Peptide hormones can scavenge reactive nitrogen species. The oxidative effects of reactive nitrogen species are suppressed by secretory peptides. **a,** stimulatory effect of nitric oxide donors on luminol luminescence; A, control; B, LHRH; C, [Leu]enkephalin; D, angiotensin II; E, oxytocin. Peptide concentration was 100 μM. All the peptides exert significant effects as scavengers of reactive nitrogen species. **b,** loss of phycoerythrin fluorescence indicative of protein structural alterations after 4 h of exposure to SIN-1, a source of peroxynitrite; compound assignment and concentrations are the same as in a. **c,** bolus peroxynitrite scavenging by 100 μM peptide hormones, measured as in b. The peptides are capable of suppressing the detrimental actions of SIN-1–derived as well as of bolus peroxynitrite. **d,** UV/VIS spectra of human blood plasma hemoglobin (Hb); ○, untreated Hb; □, SIN-1–treated Hb; ▣, SIN-1–treated Hb with 200 μM LHRH; ■, SIN-1–treated Hb with 5 mM LHRH. □ corresponds to an approximately equal weight/volume concentration of LHRH (200 μM ~ 200 mg/l) and Hb (5 μM ~ 320 mg/l), indicating that LHRH can compete for peroxynitrite, in part, even with hemoproteins. d, inset, difference spectrum of SIN-1–treated LHRH (100 μM) versus decomposed SIN-1 and LHRH, indicating the noninterference of LHRH with the Hb assay and the chemical reaction of the aromatic residues of LHRH with peroxynitrite.
inside the protein, being out of solvent access. A second important aspect could be the higher diffusibility and the numerous degrees of freedom of the tyrosine and tryptophan residues in small peptide hormones, being potentially critical for their antioxidant action. This latter point is emphasized by the fact that LHRH also effectively prevents brain lipid peroxidation (Fig. 1c), which is not achieved by the large protein albumin (data not shown). A superior activity of LHRH as a peroxyl radical scavenger was also found using lipophilic antioxidants as competitors, such as α-tocopherol and 17β-estradiol (Fig. 5, e and f). Although α-tocopherol seems to be widely ineffective in the concentrations used, 17β-estradiol shows protective effects, but to a less pronounced degree than LHRH.

Discussion

The idea that some hormone actions may actually rely on their radical chemistry is rather old (Borg, 1972), but it has been largely expanded during the last several years, mainly by use of the examples of melatonin (Reiter, 1995) and estrogen (Behl et al., 1997). In both cases, antioxidant activities, especially in the central nervous system, have been elucidated.

We report that these two hormones that have a second function as antioxidants are not solitary examples but can be extended by some peptide hormones such as LHRH, angiotensin, vasopressin, and oxytocin. LHRH and FSHRF already exhibit significant antioxidant effects at concentrations of 20 nM, and tyrosine-only peptides show clear effects in the middle nanomolar range in vitro. Circulating plasma levels of most peptide hormones are lower [e.g., [Met]enkephalin: 0.2 nM (Clement-Jones et al., 1980)]; nevertheless, the in vivo concentrations in critical anatomical regions subjected to oxidative stress are completely consistent with the observed antioxidant concentrations ([Met]enkephalin: striatum, 800 nM (Millan et al., 1981); hypothalamus, 1 μM (Przewlocki et al., 1982)], and they can locally reach much higher values ([Met]enkephalin: pituitary, 10 μM; β-endorphin: pituitary, 200 μM (Przewlocki et al., 1982)]. Furthermore, the antioxidant concentrations in vitro are in the same range as the concentrations that elicit specific hormonal responses in vitro models. FSHRF, for example, stimulates follicle-stimulating hormone release starting at a concentration of 1 nM and reaching saturation at 1 μM (Yu et al., 1997). Luteinizing hormone release by LHRH requires a concentration of 2 to 20 nM in vitro (Yu et al., 1997). Finally, a crucial point to remember is that the observed antioxidant effects of peptide hormones behave additively (data not shown). Therefore, we estimate that the investigated as well as potentially other secretory peptides together contribute significantly to the biological antioxidant defense system.

It is intriguing that when one compares the antioxidant potential of peptidic antioxidants with that of numerous established antioxidant structures, LHRH and some other peptides are more effective than tocopherol, 17β-estradiol, 4-docosylphenol, probucol, and melatonin with respect to peroxyl radical scavenging, but are also at least equally effective with respect to the inhibition of LDL oxidation (Fig. 5 and data not shown). In addition, LHRH, being N- and C-terminally modified leading to a relatively hydrophobic structure containing

Fig. 5. The antioxidant potential of LHRH compared with melatonin, albumin, tocopherol, and estradiol. The antioxidant lead structure LHRH outweighs in efficiency several well-established hydrophilic and lipophilic antioxidants as a peroxyl radical scavenger. The protective effects of LHRH (a) on phyceoehtrin fluorescence, as in Fig. 1a, as well as those of melatonin (b) and bovine serum albumin (c), in identical molar concentrations. Comparing the negative slopes of the 200 nM curves of albumin and melatonin with that of LHRH, the latter clearly has the highest antioxidant capacity. d through f, LHRH was compared with two lipophilic antioxidants, which required a final ethanol concentration of 0.5% in the medium, leading to the different shape of the control curves in these graphs. d, LHRH; e, α-tocopherol; f, 17β-estradiol. LHRH is also significantly more potent than these two antioxidants in scavenging peroxyl radicals derived from AIBN. ●, control; ○, 200 nM; ▼, 2 μM; ▼, 20 μM. Quadruplicate determinations were performed.
only one charged residue, is an efficient inhibitor of lipid peroxidation (Fig. 1c) despite being readily water-soluble. Its pharmacological half-maximal effective concentration is comparable with that of many established lipid-phase antioxidants. Thus, together with the knowledge of an apparently rather permissive structure-activity relationship of peptide antioxidant action, LHRH may serve as a straightforward template for the synthesis of novel peptidic pharmacological antioxidants without any hormonal effects. The special efficiency of LHRH may further arise from a direct redox interaction of the tyrosine and tryptophan residues within each molecule, being in van der Waals distance, as is often observed in proteins (Pruzt et al., 1980). From a pharmacological perspective, tyrosinyl and tryptophanyl peptides may be a unique, biologically compliant way of incorporating phenolic and indolic antioxidant moieties into prospective drug molecules.

Apart from this, we believe our results bear two further implications. First, a variety of antioxidants having low molecular mass exist in humans which can be built completely of endogenous precursor molecules (i.e., amino acids). This is in contrast to many other standard antioxidants (e.g., ascorbate, tocopherol, or the quinones), which have to be supplied directly as exogenous dietary components. There also may be other antioxidant peptides or protein degradation products without primary endocrinological function whose formation and/or secretion may be specifically up-regulated under conditions of oxidative stress. In addition, the finding that endogenous peptide hormones also have an antioxidant activity sheds further light on the intricate redox biochemistry of peptides, which may be especially relevant for oxidative stress-associated conditions such as Alzheimer’s disease. Intriguingly, a special role for tyrosine and its redox chemistry has been proposed for atherosclerosis (Leeuwenburgh et al., 1997) as well as for Alzheimer’s disease (Hensley et al., 1998).

Second, peptide hormones may represent target structures for free radical-signaling molecules. A plethora of systems emitting radical species of presumed signal character are known, but the number of specific targets identified so far is limited (Lander, 1997). The following arguments support this idea: 1) Deliberate targets for radical signaling molecules should be able to “amplify” their chemical modification. Therefore, the target structures characterized up to now comprise, for example, ion channels (Lipton et al., 1998) or proteins and enzymes that are critically involved in intracellular signaling cascades (Lander, 1997; Buchczyk et al., 2000). This requirement is also met by hormones. 2) Many peptide hormones meet the described structural requirements for their tyrosine residues to be preferably nitrated. By comparing tyrosine nitration efficiencies in proteins, it has been found that only solvent-accessible residues, especially those on flexible loop structures or near glycine and proline residues, are targets for nitration (Souza et al., 1999). These demands are clearly realized in peptide hormones (Table 1). Specifically, peptide hormones having 10 amino acids or fewer seem to be too short to effectively bury their aromatic residues inside a permanent secondary structure. 3) There is an involvement of radical signaling molecules in the cellular regulatory actions of peptide hormones, such as angiotensin II, that specifically stimulates superoxide production in vascular smooth muscle cells (Griendling et al., 1994).

On the other hand, it has been shown to undergo oxidative tyrosine nitration in vitro, which inhibits its vasoactive properties (Ducrocq et al., 1998). This could be interpreted in terms of the peptide hormone, being an antioxidant itself, limiting directly any unwanted damaging side effects of its own second messenger (e.g., after the reaction of the superoxide with nitric oxide yielding peroxynitrite). In terms of regulation, one could interpret angiotensin’s reactivity as part of a negative feedback loop. A second example is LHRH: nitric oxidergic neurons have been visualized directly next to LHRH terminals in the hypothalamus, stimulating pulsatile LHRH release (Rettori et al., 1993). Whether a direct functional interaction between these compounds also occurs in vivo needs to be determined.

In summary, we described a novel class of endogenous biochemical antioxidants, secretory peptide hormones, which expands our knowledge of the antioxidant actions of endocrine compounds such as estrogen and melatonin, and we outlined a structure-activity relationship that might be of use for rational antioxidant drug design. Antioxidants formed from the lead structure of these antioxidant peptides may display a unique pharmacological profile; i.e., they could be designed with respect to their biological half-life and their degradation by proteases. Furthermore, they might be targeted to special compartments of the body (e.g., across the blood-brain barrier) by being substrates to specific peptide transporters (Gao et al., 2000) or to the L-system large neutral amino acid carrier, a well-characterized transporter of tyrosine- and tryptophan-based chemicals into the brain (Smith, 1993), which shows relatively relaxed structural demands. Therefore, peptidic antioxidants may represent a promising new target for pharmacological antioxidant research.

Acknowledgments

We are indebted to K. Beyer for help with the ESR experiments.

References


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