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Short communication

Melatonin affords protection against kainate-induced in vitro lipid peroxidation in brain

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Abstract

Melatonin protection against in vitro kainic acid-induced oxidative damage in rat brain is shown. Brain disrupted cell homogenates were incubated with different concentrations of kainate and with or without different concentrations of melatonin. The concentration of malonaldehyde and 4-hydroxyalkenals was measured as an index of lipid peroxidation. When administered together with kainate, melatonin produced a concentration-dependent decrease in kainate-induced lipid peroxidation ranging from 20% to 100%. Moreover, when added to the reaction mixture alone, melatonin decreased the basal level of lipid peroxidation compared to controls.

Keywords: Melatonin; Kainate; Lipid peroxidation; Brain; (Rat)

1. Introduction

Kainic acid is a neurotoxin whose effects seem to be mediated by a subclass of non-NMDA excitatory amino acid receptors (Coyle, 1983). When injected in vivo, directly into the corpus striatum, kainic acid induces lesions resembling those detected in Huntington's disease while its effects in the limbic system provide models for certain epilepsies (Coyle, 1983). Brain damage following the intracerebral administration of kainate shows a pattern of degeneration that is characterized by death of neuronal perikarya and sparing of axons of passage. Little is known about the proximate cause of neuronal degeneration. Intracellular accumulation of calcium ions and depletion of ATP levels are commonly believed to be key factors in kainate toxicity although it is not clear if they are the causes or consequences of the excitotoxin-induced damage (Dykens et al., 1987).

Recently, free radical generation was found to be associated with excitatory amino acid-induced brain injury. Ischemic damage which involves free radicals, and edema within the central nervous system induce lipid peroxidation, induction of superoxide dismutase and a decrease in glutathione levels (Ikeda and Long, 1990). Moreover, partial protection from ischemic damage is afforded by antioxidant agents, such as vitamin E, iron chelators and corticosteroids (Willmore and Triggs, 1984; Miyamoto et al., 1989).

Direct evidence of free radical involvement in kainic acid-induced brain damage comes from in vitro and in vivo studies in which free radical generation was induced in cultures of cerebellar granular cells incubated with kainate (Dykens et al., 1987) and in gerbil brain, following its systemic administration (Sun et al., 1992). Also, reactive oxygen species were detected when kainate was added to isolated synaptoneurosomes derived from rat cerebral cortex (Bondy and Lee, 1993).

The pineal hormone melatonin takes part in many important physiological functions including the control of seasonal reproduction as well as influences on the immune and circadian rhythms (Reiter et al., 1994). For many years, melatonin was believed to be exclusively produced in the pineal gland. However, the discovery of its ubiquitous presence in virtually all vertebrates as well as in unicellular organisms, such as the dinoflagellate (Reiter et al., 1994), shows that it is produced outside of the pineal

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gland as well. The widespread actions of melatonin became clear when it was found melatonin has intracellular actions that are independent of receptor binding. Both in vitro and in vivo studies have shown melatonin to be a potent hydroxyl and peroxyl radical scavenger; indeed, its potency in this regard exceeds that of other well known radical scavengers including glutathione, mannitol and vitamin E (Reiter et al., 1994, 1995; Melchiorri et al., 1995).

In the present study, we examined the potential protective effect of melatonin on rat brain lipid peroxidation following the in vitro exposure of disrupted brain cell homogenates to kainic acid. Tissue concentrations of malonaldehyde and 4-hydroxyalkenals were used as index of lipid peroxidation (Esterbauer and Cheeseman, 1990).

2. Materials and methods

2.1. Chemicals

All reagents were of the highest quality available. Kainic acid and melatonin were purchased from Sigma (St. Louis, MO). The Bioxytech LPO-586 kit for lipid peroxidation was purchased from Cayman Chemical (Ann Arbor, MI).

2.2. Animals

Adult male Sprague-Dawley rats (body weight 230–250 g) were obtained from Harlan (Houston, TX) and housed in Plexiglas cages with 3 animals per cage. The animal room was windowless with automatically regulated temperature (22 ± 2°C) and lightning (light on 07.00 h and off 21.00 h; 14 h light/10 h dark). The rats received standard laboratory chow and water ad libitum.

2.3. Methods

Melatonin was dissolved in absolute ethanol (when added to the brain homogenate; the final alcohol concentration was 1%) and added as one of six different concentrations (0.1, 1, 2, 3, 4 and 5 mM) to the incubation mixture. Kainate was dissolved in HCl-Tris buffer (20 mM, pH 7.4) and added in 3 different concentrations (1, 6 and 12 mM) to the homogenate.

2.4. Tissue preparation and assays

Rats were anesthetized and subjected to intracardiac perfusion with ice-cold saline in order to eliminate the excess of iron that could be released from intracellular storage sites and may otherwise artificially increase free radical formation. Brains were collected and immediately frozen on solid CO_2. Each experiment was performed on 11 rats and was repeated 3 times.

2.5. Lipid peroxidation assay

Eleven brains were pooled and homogenized in ice-cold Tris-HCl buffer (20 mM, pH 7.4) with a polytron homogenizer to produce a 1/10 homogenate. Aliquots of the homogenates were incubated in a shaking water bath for 20 min at 37°C with different concentrations of kainic acid (1, 6 or 12 mM) and with or without six different concentrations of melatonin (from 0.1 to 5 mM). Moreover, different concentrations of melatonin were added to the homogenate without kainic acid. The reaction was stopped by cooling the samples in ice for 10 min. Thereafter, the samples were centrifuged at $13\,000 \times g$ for 5 min, the supernatant was collected and immediately tested for lipid peroxidation by measuring the concentration of MDA and 4-HDA. These constituents are formed, for the most part, from the peroxidation of unsaturated fatty acids and are widely used as an index of lipid peroxidation (Esterbauer and Cheeseman, 1990). The Bioxytech LPO-586 kit was used for these measurements; this kit takes advantage of a chromogenic reagent which reacts with malonaldehyde and 4-hydroxyalkenals at 45°C yielding a stable chromophore with maximal absorbance at a wavelength of 586 nm. This wavelength and the low temperature of incubation (45°C) used in this procedure minimize interferences and undesirable artifacts.

2.6. Protein assay

Proteins were determined using the Lowry procedure, with albumin as standard.

2.7. Statistical analysis

All data were analyzed by one-way analysis of variance (ANOVA). If the F values were significant, the Student-Newman-Keuls test was used to compare the treated and control groups. The level of significance was accepted at $P < 0.05$.

3. Results

Exposure of brain homogenates to different concentrations of kainic acid caused a concentration-dependent increase in the levels of malonaldehyde and 4-hydroxyalkenals compared to those in control samples (Fig. 1). Because of the high levels of lipid peroxidation induced by 12 mM kainate, this concentration was chosen for subsequent studies. When kainic acid (12 mM) was added to the reaction mixture together with different concentrations of melatonin (ranging from 0.1 to 5 mM) the increase in lipid peroxidation was reduced in a concentration-dependent manner (Fig. 2). Moreover, melatonin alone, in a concentration-dependent manner, significantly decreased the basal level of

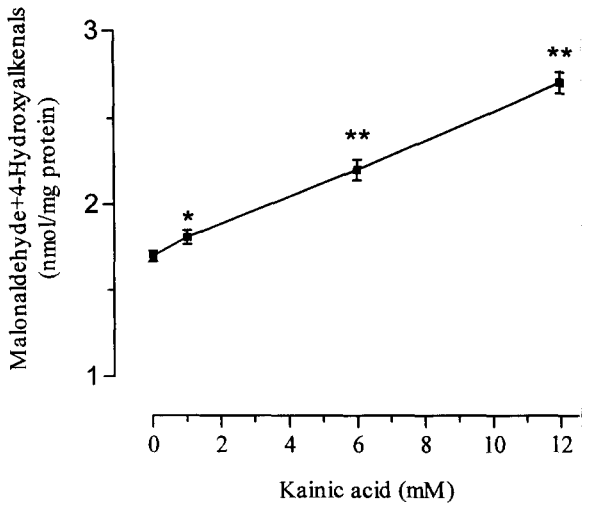

Fig. 1. Effect of different concentrations (1, 6 and 12 mM) of kainate on malonaldehyde + 4-hydroxyalkenals concentrations in rat brain homogenates following 20 min incubation. Values are means of 3 experiments. Significant differences were determined by ANOVA followed by Student-Newman-Keuls test: ** $P < 0.001$ and * $P < 0.005$ vs. control.

lipid peroxidation compared to that in control samples (Fig. 2).

4. Discussion

Results from these studies show that kainate-induced lipid peroxidation, as measured by the increase in the concentrations of malonaldehyde and 4-hydroxyalkenals, in disrupted cell homogenates from rat brain is markedly inhibited by melatonin. When added together with the excitotoxin, melatonin conferred a concentration-dependent protection against kainate-induced peroxidation of membrane phospholipids. These data are consistent with previous reports which show that kainate-induced death of cerebellar neurons is prevented either by inhibiting the enzyme xanthine oxidase, a cellular source of superoxide anions, or by the addition of free radical scavengers to the culture medium (Dykens et al., 1987). Although the mechanism of kainate toxicity is still unclear, it is generally accepted that neuronal degeneration after kainic acid administration is associated with a depletion of ATP levels and accumulation of $[Ca^{2+}]_i$. The increase in $[Ca^{2+}]_i$ may either activate enzymes such as phospholipase A_2 which in turn hydrolyzes the phospholipids to release polyunsaturated fatty acids leading to the production of free radicals, or $[Ca^{2+}]_i$ may trigger Ca^{2+}-activated protease to convert xanthine dehydrogenase to xanthine oxidase with the simultaneous formation of free radicals (Dykens et al., 1987). Furthermore, kainic acid was shown to stimulate glutamate release (Ferkany and Coyle, 1983) which in turn may amplify free radical formation. Brain is particularly vulnerable to oxidative stress. It contains large amounts of phospholipids rich in polyunsaturated fatty acids; these are selective targets for reactive oxygen species. The brain also possesses lower concentrations of antioxidants such as catalase and vitamin E. The oxidative insult to brain cell membranes results in the peroxidation of phospholipids and their breakdown into highly reactive carbonyl fragments, most importantly, malonaldehyde and 4-hydroxyalkenals (Esterbauer and Cheeseman, 1990). Thus, the measurement of the concentrations of these aldehydes provides a convenient method for estimating oxidative damage to cell membranes (Esterbauer and Cheeseman, 1990).

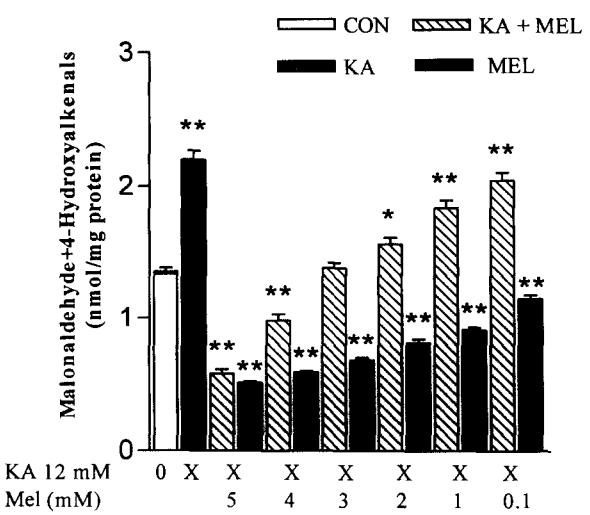

Fig. 2. Effect of melatonin and kainate (KA) + melatonin (Mel) on malonaldehyde + 4-hydroxyalkenals concentrations in rat brain homogenates following 20 min incubation. Values are means of 3 experiments. X indicates the presence of kainate. Significant differences were determined by ANOVA followed by Student-Newman-Keuls test: ** $P < 0.001$ and * $P < 0.005$ vs. control (0).

Several findings support the antioxidant activity of melatonin. In vitro and in vivo experiments have shown that the pineal hormone is a potent hydroxyl and peroxyl radical scavenger (Reiter et al., 1994; Melchiorri et al., 1995; Sewerynek et al., 1995). In a cell-free system, melatonin was proven to scavenge the hydroxyl radicals generated by the photolysis of H_2O_2 in a dose-dependent manner. Also, indirect evidence of antioxidant activity of melatonin comes from in vivo studies. The pineal hormone has been shown to prevent the DNA damage induced by the chemical carcinogen safrole as well as the oxidative injury to the lungs and liver induced by the herbicide paraquat (Reiter et al., 1994; Melchiorri et al., 1995). The protection conferred by melatonin against oxygen toxicity appears to be general and unspecific, and occurs regardless of the particular free radical-generating chemical given or tissue in which the damage appears. Thus, melatonin ability to protect against oxidative damage is likely due, at least in part, to its free radical scavenging activity. It is generally believed that the methyl group at position 5 of the indole nucleus is required for the scavenging activity of melatonin while the acetyl group on the side chain provides a synergistic effect, thereby accounting for its

antioxidant action (Reiter et al., 1994). Additionally, the pineal hormone is highly lipophilic and quite hydrophilic as well (Reiter et al., 1995) and thus it may potentially protect against oxygen toxicity occurring in all subcellular compartments. In the present studies, the reduction of kainate toxicity, following the administration of melatonin, is thus probably due to the indolamine free radical scavenging ability although an action of melatonin at the kainate receptor or at the inhibitory GABA receptor cannot be excluded.

Relatively high concentrations of melatonin (0.1–5 mM) were used to confer protection against kainate toxicity. As in previous papers (Bose et al., 1992), a relatively high concentration of kainate (11.7 mM) was needed to induce large increases in MDA and 4-HDA concentrations. This probably explains why high concentrations of melatonin (0.1–5 mM) were required to significantly reduce lipid peroxidation.

At present, few data exist related to the toxicology of melatonin after acute and chronic administration in humans. However, hematological and biochemical determinations in patients treated with the indolamine have failed to reveal any alterations due to melatonin administration (Voordouw et al., 1992). Since melatonin readily crosses the brain-blood barrier, pharmacological doses of melatonin may provide neuroprotection against oxygen toxicity.

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References

Bondy, S.C. and D.K. Lee, 1993, Oxidative stress induced by glutamate receptor agonists, Brain Res. 610, 229.

Bose, R., L. Schnell, C. Pinsky and V. Zitko, 1992, Effects of excitotoxins on free radical indices in mouse brain, Toxicol. Lett. 60, 211.

Coyle, J.T., 1983, Neurotoxic action of kainic acid. Short review, J. Neurochem. 41,1.

Dykens, J.A., A. Stern and E. Trenkner, 1987, Mechanism of kainate toxicity to cerebellar neurons in vitro is analogous to reperfusion tissue injury, J. Neurochem. 409, 1222.

Esterbauer, H. and K.H. Cheeseman, 1990, Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonenal, Methods Enzymol. 186, 407.

Ferkany, J.W. and J.T. Coyle, 1983, Kainic acid selectively stimulates the release of endogenous excitatory acidic aminoacids, J. Pharmacol. Exp. Ther. 225, 399.

Ikeda, Y. and D.L. Long, 1990, The molecular basis of brain injury and brain edema: the role of oxygen free radicals, Neurosurgery 27, 1.

Melchiorri, D., R.J. Reiter, A. Attia, M. Hara, A. Burgos and G. Nisticò, 1995, Potent protective effect of melatonin on in vivo paraquat induced toxicity, Life Sci. 56, 83.

Miyamoto, M., T.H. Murphy, R.L. Schaar and J. Coyle, 1989, Antioxidants protect against glutamate-induced cytotoxycity in a neuronal cell line, J. Pharmacol. Exp. Ther. 250, 1132.

Reiter, R.J., D.-X. Tan, B. Poeggeler, A. Menendez-Pelaez, L.-D. Chen and S. Saarela, 1994, Melatonin as a free radical scavenger: implication for aging and age-related diseases, Ann. N.Y. Acad. Sci. 719, 1.

Reiter, R.J., D. Melchiorri, E. Sewerynek, B. Poeggeler, L.R. Barlow-Walden, J. Chuang, G.G. Ortiz and D. Acuna-Castroviejo, 1995, A review of the evidence supporting melatonin's role as an antioxidant, J. Pineal Res. 18, 1.

Sewerynek, E., D. Melchiorri, L.D. Chen and R.J. Reiter, 1995, Melatonin reduces both basal and bacterial lipopolysaccharide-induced lipid peroxidation in vitro, Free Rad. Biol. Med. 19, 903.

Sun, A.Y., Y. Cheng, Q. Bu and F. Oldfield, 1992, The biochemical mechanism of the excytotoxicity of kainic acid, Mol. Chem. Neuropathol. 17, 51.

Willmore, L.J. and W.J. Triggs, 1984, Effects of phenytoin and corticosteroids on seizures and lipid peroxidation in experimental posttraumatic epilepsy, J. Neurosurg. 60, 467.

Voordouw, B.C.G., R. Eusner, R.E.R. Verdonk, B.T.H. Alberda, F.H. De Jong, A.C. Drogendijk, B.C.J.M. Fauser and M. Cohen, 1992, Melatonin and melatonin-progestin combinations alter pituary-ovarian function in women and can inhibit ovulation, J. Clin. Endocrinol. Metab. 74, 108.