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6-Hydroxymelatonin protects against quinolinic-acid-induced oxidative neurotoxicity in the rat hippocampus

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Abstract

Melatonin, a naturally occuring chemical mediator, although assigned a diverse range of functions, has attracted interest because of its ability to function as a free radical scavenger. Its major hepatic metabolite and photoproduct, 6-hydroxymelatonin (6-OHM), also shares this property. Since singlet oxygen and quinolinic acid (QUIN) are critically involved in the pathology of neurotoxicity, the objective of this study was to investigate the ability of 6-OHM to scavenge singlet oxygen and evaluate its ability to scavenge superoxide anions and reduce QUIN-induced neurotoxicity in the hippocampus in-vivo. The results show that 6-OHM is an efficient inhibitor of singlet oxygen formation as indicated by the rate constants and quantum yields reported for 6-OHM and zinc phthalocyanine (ZnPc), respectively. 6-OHM, appears to reduce QUIN-induced superoxide anion generation in the hippocampus, which provides some evidence of the neuroprotective effects of 6-OHM.

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Introduction

Free radicals have been postulated to be important mediators of tissue injury in several neurodegenerative models (Bautista & Spitzer 1990; Shuter et al 1990; Yoshikawa et al 1994). The brain is particularly susceptible due to its high utilization of oxygen and its relatively low concentration of antioxidant enzymes and free radical scavengers (Reiter 1995, 1998b). One of the most vulnerable regions of the brain to quinolate-induced neurotoxicity is the hippocampus (Schwarcz & Koler 1983). Rios & Santamaria (1991) have described the lipoperoxidant effect of quinolinic acid in-vitro and in-vivo in brain tissue. Given that the lipid peroxidation is believed to be related to free radical formation, and thus to cellular damage, this suggests that quinolinate toxicity could be partially mediated by free radical scavengers (Stone 1993).

Melatonin (*N*-acetyl-5-methoxytryptamine), a naturally occuring chemical mediator secreted from the pineal gland and found in all body fluids thereafter, has been assigned a diverse range of functions, including the control of neuroendocrine events (Reiter 1993). Melatonin possesses both hydrophilic and lipophilic characteristics (Shida et al 1994; Costa et al 1995) and thus easily penetrates all biological membranes such as the blood–brain barrier. However, it has been the discovery of melatonin as a free radical scavenger and that fact that it has been shown to be more potent then α-tocopherol, mannitol and glutathione in scavenging free radicals, that has generated the most interest in recent years (Reiter 1998a). Shown to be a scavenger of the hydroxyl radical ($^{\cdot}$OH), generated from hydrogen peroxide via the Fenton reaction (Tan et al 1998, 2001), melatonin also prevents singlet oxygen toxicity (Cagnoli et al 1995) and scavenges the peroxynitrite anion (Tan et al 2001), both of which damage neural tissue.

Melatonin interacts with free radicals to form several stable metabolites, such as 6-hydroxymelatonin (6-OHM), N^1-acetyl-N^2-formyl-5-methoxykynuramine, *N*-acetyl-5-methoxy-kynuramine and cyclic 3-hydroxymelatonin. These agents produced are in turn also effective free radical scavengers (Reiter et al 2000; Tan et al 1998, 2001). Melatonin is also rapidly metabolized in the liver to its main metabolite, 6-OHM (Matuszak et al 1997). 6-OHM is similar to melatonin, although the presence of the

OH group makes it more polar, but it is still able to easily penetrate the blood–brain barrier. In addition, 6-OHM is only metabolized in the liver, unlike melatonin, which is rapidly metabolized both in the brain and liver (Matuszak et al 1997). It has been reported by Pierrefiche et al (1993) that melatonin exhibits less antioxidant activity than this principal hepatic metabolite, 6-OHM, whose structure differs from that of melatonin only due to the presence of a hydroxyl group at position six (Tse et al 1991). Antioxidant properties have been confirmed by Maharaj et al (2003), who showed that 6-OHM is able to protect rat brain homogenate against iron-induced lipid peroxidation in-vitro. Thus, the aim of this study was to investigate firstly the ability of 6-OHM to scavenge singlet oxygen generated by photosensitization and secondly to determine whether 6-OHM is able to scavenge superoxide anions and reduce neurotoxicity in the hippocampus in-vivo, induced by quinolinic acid.

Materials and Methods

Chemicals

6-Hydroxymelatonin (6-OHM), quinolinic acid (QUIN), 2-thiobarbituric acid (TBA), 1,1,3,3-tetramethoxypropane (98%), butylated hydroxytoluene (BHT), nitroblue diformazan (NBD), nitroblue tetrazolium (NBT) and cloral hydrate were purchased from Sigma Chemicals Co. (St Louis, MO). Methanol (HPLC grade) was purchased from BDH Laboratory Supplies (Poole, UK). Trichloroacetic acid (TCA), glacial acetic acid and resorcinol were purchased from Saarchem (PTY) Ltd (Krugersdorp, South Africa). IsoluteJ C_{18} solid-phase extraction (SPE) columns were obtained from International Sorbent Technology (Mid Glamorgan, UK) and were of the highest chemical purity. Zinc phthalocyanine (ZnPc) and 1,3-diphenylisobenzofuran (DPBF) were purchased from Sigma Aldrich.

Animals

Three-week-old adult male Wistar rats, 250–300 g, were used in this study. All rats were housed in a well-ventilated animal room under a 12-h light–dark cycle (lights on 0600–1800 h). Rats had free access to food and water. The experimental protocol was approved by the Rhodes University Animal Ethics Committee.

Drug treatment

Rats were divided into three groups (I–III) of six. Each rat was anaesthetized with cloral hydrate (400 mg kg^{-1} daily, i.p.). QUIN (120 nmol) dissolved in phosphate-buffered saline (PBS) (Wu et al 1992; Heron & Daya 2001), pH 7.4, (120 nmol in 2 μL) was infused bilaterally into the hippocampi employing a rat brain stereotaxic apparatus (Stoelting, IL) with stereotaxic coordinates derived from the bregma and, according to the König & Klippel (1963) Atlas, were 4.0 mm caudal to the bregma, 2.5 mm lateral to the saggital suture and 3.2 mm ventral of the dura. Two microlitres of PBS were infused bilaterally into the hippocampi, in sham control rats (group I). Group II were infused with QUIN, while group III were infused with QUIN and received subcutaneous injections of 6-OHM (10 mg kg^{-1}) (Pierrefiche et al 1993; Matuszak et al 1997) 20 min before receiving the QUIN and thereafter, each day for 7 days, respectively. The rats in groups I and II received the vehicle for the drugs (sweet oil). On the seventh day after intrahippocampal injections, all rats were killed by cervical dislocation and the brains were removed and the hippocampi of 5 rats were rapidly excised. The brains and hippocampi were frozen in liquid nitrogen and stored at -70°C until needed.

Instrumentation

For lipid peroxidation analysis, samples were analysed on a modular, isocratic high-performance liquid chromatographic (HPLC) system. The chromatographic system consisted of a Spectraphysics Iso Chrom LC Pump, a Linear UV-Vis 200 Detector and a Perkin Elmer 561 recorder. Samples were introduced into the system using a Rheodyne fixed loop injector fitted with a 20-μL loop. Evaporation of the methanol following sample extraction was performed using an N-EVAP analytical evaporator. The excitation pulse source for singlet oxygen studies was provided by an Nd-Yag laser (providing 47 mW, 9 ns pulses of laser light at 532 nm). An energy meter was used to measure the pulse energy of the laser light. The absorbance spectra were recorded on a Varian UV-Vis-NIR spectrophotometer.

Chromatographic conditions

Analytical separation for lipid peroxidation was achieved using a C_{18} (Waters Spherisorb, 5 μm, 250 $\times$ 4.6 mm i.d.) column, fitted with an in-line pre-column filter. The mobile phase composition for the analysis was 14% methanol in Milli-Q water and was degassed using a 0.45-μm membrane filter before use. The mobile phase flow rate was 1.2 mL min^{-1}. The detector sensitivity was set at 0.1 AUFS (absorbance units full scale) and the thiobarbituric acid–malondialdehyde (TBA-MDA) complex was detected at 532 nm. Resorcinol (0.1 mg mL^{-1} in water) was used as an external standard. The HPLC method has been validated by Anoopkumar-Dukie et al (2001).

Singlet oxygen generation

The foundation for the analysis was based on the production of singlet oxygen upon irradiation by a sensitizer and a quencher, and the quencher subsequently allowed to react with the singlet oxygen that was accordingly produced (Maree 2002). For this study, zinc phthalocyanine (ZnPc) was chosen as the singlet oxygen generator, while 1,3-diphenylisobenzofuran (DPBF), which is highly reactive to singlet oxygen, was used as the quencher, and its disappearance was easily monitored using the disappearance of its absorption maximum at 417 nm. Only the first 20% decrease

is applicable to obtain reliable or accurate first-order kinetic data. To test the ability to scavenge singlet oxygen, 6-OHM was added to the above reference mixture. Some competition is therefore expected between the known scavenger DPBF and the apparent scavenger 6-OHM. The photochemical experiments were carried out in a 4-mL UV cell with an optical pathlength of 1 cm. The absorbances of the quencher (DPBF), the sample and sensitizer solutions were set to approximately 1 by dilution. The DPBF, 6-OHM and ZnPc sample solutions were made up in dimethyl sulfoxide (DMSO). Air-saturated solutions were used for all experiments and the measurements were carried out at room temperature. The decrease in the DPBF absorption band was monitored while ensuring that the bands belonging to the ZnPc remained unchanged (the light intensity was kept low enough to avoid this decrease, as any change would indicate photobleaching or photodegradation of the sample). ZnPc ($\Phi_\Delta = 0.67$ (Dawson & Windsor 1968) in DMSO) was used as both the singlet oxygen generator and the reference solution. Samples were irradiated at a wavelength of 672 nm (corresponding to the lambda max of the Q-band of ZnPc).

Homogenate preparation

The hippocampi were homogenized in 0.1 M PBS, pH 7.4, to give a 10% w/v homogenate. This was necessary to prevent lysosomal damage of the tissue (Anoopkumar-Dukie et al 2001).

Lipid peroxidation determination

The method used in this experiment was a modification of the method used by Anoopkumar-Dukie et al (2001). Briefly, the assay of lipid peroxidation was performed on each treated brain and the protocol followed according to Anoopkumar-Dukie et al (2001). Final results are expressed as nmol (mg tissue)$^{-1}$.

Nitroblue tetrazolium assay

A modification of the assay used by Ottino & Duncan (1997) was used in this set of experiments. Final results are expressed as μmol (mg protein)$^{-1}$. Protein estimation was performed using the method described by Lowry et al (1951).

Statistical analysis

The results for each experiment were analysed using a one-way analysis of variance followed by the Student–Newman–Keuls multiple range test. The level of significance for both tests was accepted at $P < 0.05$ (Zar 1974).

Results and Discussion

Singlet oxygen generation

The rate constants obtained for 6-OHM and ZnPc were calculated to be $y = -1.89 \times 10^{-3}x + 1.0104$ (R^2 0.9881; s.d. for the slope $\pm 2.08 \times 10^{-3}$) and $y = -4.76 \times 10^{-3}x + 1.0218$ (R^2 0.997; s.d. $\pm 3 \times 10^{-3}$), respectively. As expected, 6-OHM was found to decrease the rate of disappearance of DPBF quite substantially when compared with the ZnPc reference solution. These results confirm the ability of 6-OHM to scavenge singlet oxygen. The singlet oxygen quantum yield thus determined for this system was calculated to be $\Phi_\Delta = 0.26$, which is a substantial decrease from the $\Phi_\Delta = 0.67$ for ZnPc, further evidence of the ability of 6-OHM to scavenge singlet oxygen.

Lipid peroxidation

The intrahippocampal injection of QUIN caused a significant increase in lipid peroxidation products as assessed by the formation of TBA-RS (TBA reacting substances) in rat hippocampal homogenate (Table 1). This increase, when compared with the control treated rats, was greater than 70%. However, in QUIN-plus-6-OHM-treated rats there was a significant reduction in the level of lipid damage in the hippocampus induced by the neurotoxin, QUIN. There is a 50% decrease relative to levels measured in hippocampi of rats that received QUIN only.

Nitroblue tetrazolium assay

It is clear that the intrahippocampally injected QUIN caused a significant increase in superoxide anion generation in comparison with control treated rats (Table 1). The increase in superoxide anion production caused by QUIN, when compared with the control treated rats, was greater than 50%. Treatment of the rats with 6-OHM caused a significant reduction in QUIN-induced superoxide anion generation, with a 68% reduction relative to levels measured in the hippocampi of rats treated with QUIN only. It is also clearly evident from Table 1 that 6-OHM reduces the level of superoxide anion generation to below that of the control treated rats, with a 30% reduction in comparison with the levels measured in the control treated rats.

Table 1 The effect of 6-hydroxymelatonin (6-OHM) on quinolinic acid (QUIN)-induced lipid peroxidation and superoxide anion generation in rat hippocampal homogenate in-vivo

	Lipid peroxidation (nmol TBA-RS/mg tissue)	Nitroblue tetrazolium assay (μmol (mg protein)$^{-1}$)
Control	0.0057 ± 0.00093	16.864 ± 0.789
QUIN	0.0183 ± 0.00055#	37.345 ± 1.508#
6-OHM + QUIN	0.0103 ± 0.00063*	14.474 ± 0.96*$^{@}$

Each value represents the mean ± s.e.m., n = 6. # $P < 0.001$, QUIN vs control for each respective assay; *$P < 0.01$, 6-OHM + QUIN vs QUIN for each respective assay; $^{@}P < 0.05$, 6-OHM vs control for the nitroblue tetrazolium assay, Student–Newman–Keuls Multiple Range Test.

Discussion

Free radical destruction of neurons has been linked to a number of neurological diseases and to the normal aging processes of the central nervous system (Gilad et al 1997; Bonilla et al 1999). The nervous system is relatively poorly protected from, and is also highly susceptible to, the constant bludgeoning by active oxygen free radicals (Cabrera et al 2000). This relates to the fact that the brain uses a disproportionately large amount of O_2 for its size and contains high amounts of non-haem iron, ascorbic acid and easily oxidizable lipids (Reiter 1995). Additionally, the use of antioxidants to combat free radical damage in the central nervous system is not always successful since many of them do not easily cross the blood–brain barrier (e.g., vitamin E) (Cabrera et al 2000). On the contrary, melatonin readily crosses the blood–brain barrier and protects neurons from oxidative destruction (Reiter 1998a, b; Reiter et al 2000).

The results of this report show that 6-OHM is able to scavenge $^1O_2$, which is known to be neurotoxic (Cagnoli et al 1995), as evidenced by both the rate constants and quantum yields for 6-OHM and ZnPc, respectively. Thus, since 6-OHM is able to scavenge $^1O_2$ in this purely chemical system, it may be able to protect the body against the cytotoxic and deleterious effects produced by $^1O_2$ generation. This research serves as a basis of proving the ability of 6-OHM, the photoproduct and primary metabolite of melatonin, in scavenging the photocytotoxic species, $^1O_2$. It is most likely that the $^1O_2$ generated by laser photolysis is very high, and that the damaging effects that we found in our in-vitro system is much greater than the eventual biological damage induced by the amount of $^1O_2$ generated in-vivo. Thus, it can be speculated that since melatonin and 6-OHM are able to scavenge the high concentration of $^1O_2$ produced, these compounds would be effective in quenching the biological concentrations of $^1O_2$ produced and thus be effective in counteracting the damaging action of photodynamic injury. However, to demonstrate the ability of 6-OHM to scavenge $^1O_2$ in-vivo, further research needs to be done using a lipid or protein source, such as brain tissue.

It is evident from the results that 6-OHM is able to reduce QUIN-induced superoxide anion generation in the hippocampus. This reduction is significantly lower than the values obtained for the control treated rats. These results are in accordance with the paper published by Yoshida et al (2003), who showed that 6-OHM was able to decrease cisplatin-induced superoxide anion generation to lower than that of control baseline levels. The ability of 6-OHM to reduce superoxide anion generation below that of the control group is attributed to its ability to scavenge the $^1O_2$ species. In addition, 6-OHM is possibly acting similarly to melatonin to enhance the effects of the antioxidant enzyme, superoxide dismutase, which is the body's own defence mechanism against $O_2^{\cdot}$ (Reiter 1995, 1998). However, this needs to be further investigated. 6-OHM also proved to be effective in providing significant protection against a QUIN-induced rise in lipid peroxidation products in the rat hippocampal homogenate. These results further support earlier findings (Maharaj et al 2002; Yoshida et al 2003) that 6-OHM is a potent free radical scavenger and antioxidant. It may thus be concluded that there is evidence of the neuroprotective effects of 6-OHM against singlet oxygen and QUIN-induced oxidative damage. These findings suggest the use of 6-OHM to combat neurodegenerative diseases, such as Alzheimer's disease and Huntington's disease, where elevated QUIN level is implicated.

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