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MELATONIN STIMULATES BRAIN GLUTATHIONE PEROXIDASE ACTIVITY

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Abstract—Exogenously administered melatonin causes a 2-fold rise in glutathione peroxidase activity within 30 min in the brain of the rat. Furthermore, brain glutathione peroxidase activity is higher at night than during the day and is correlated with high night-time tissue melatonin levels. Glutathione peroxidase is thought to be the principal enzyme eliminating peroxides in the brain. This antioxidative enzyme reduces the formation of hydroxyl radicals formed via iron-catalyzed Fenton-type reactions from hydrogen peroxide by reducing this oxidant to water. Since the hydroxyl radical is the most noxious oxygen radical known, induction of brain glutathione peroxidase might be an important mechanism by which melatonin exerts its potent neuroprotective effects.

Melatonin acts as a primary non-enzymatic antioxidative defense against the devastating actions of the extremely reactive hydroxyl radical (Reiter *et al.*, 1994, 1995b). Since the highly reactive and toxic hydroxyl radical cannot be detoxified enzymatically, such as the superoxide anion radical and hydrogen peroxide, low molecular weight antioxidants such as melatonin are the only endogenous protective defense against oxidative stress and damage induced by this electrophilic intermediate (Poeggeler *et al.*, 1993, 1994).

Oxidative stress in general, and hydroxyl radicals in particular, inactivate superoxide dismutase, catalase and glutathione peroxidase, the enzymes involved in detoxifying reactive oxygen intermediates such as superoxide anion radicals and hydrogen peroxide (Nistico *et al.*, 1992; Pigeolet and Remacle, 1991; Raes *et al.*, 1987; Tabatabaie and Floyd, 1994). Glutathione peroxidase (EC 1.11.1.9) plays a key role in the enzymatic defense system against oxygen free radicals generated in the presence of peroxides by reducing hydrogen peroxide and lipid peroxides with the concomitant oxidation of glutathione (Michiels *et al.*, 1994; Raes *et al.*, 1987). However, compared to superoxide dismutase and catalase, glutathione peroxidase is also much more vulnerable to peroxide-induced and hydroxyl radical-mediated inactivation and degradation (Pigeolet and Remacle, 1991).

In aged animals, superoxide dismutase activity is increased without a concomitant, compensatory increase in glutathione peroxidase (Haan *et al.*, 1992). Hydrogen peroxide accumulates and is converted partially to the highly reactive hydroxyl radical in a Fenton-type reaction catalyzed by transition metals (Fig. 1). Therefore hydroxyl radical formation is enhanced in aged animals and hydroxyl radical mediated oxidative stress and damage finally lead to neuronal cell degeneration and death (Beal, 1993; Haan *et al.*, 1992; Poeggeler *et al.*, 1993). It has been proposed that neuronal damage and death is the pacemaker of the aging process (Poeggeler *et al.*, 1993).

Glutathione peroxidase protects neurons from oxidative stress and damage (Mirault *et al.*, 1994). An enhanced glutathione peroxidase activity could slow

$$H_2O_2 + 2GSH \xrightarrow{GSH\text{-}Px} 2H_2O + GSSG$$

$$H_2O_2 + O_2^{\cdot-} \xrightarrow{Fe^{2+/3+}} {\cdot}OH + OH^- + O_2$$

Fig. 1. Reduction of hydrogen peroxide to water catalyzed by glutathione peroxidase and reduction of hydrogen peroxide to the highly hydroxyl radical in the presence of iron chelates.

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the progress of neurodegenerative diseases, which are associated with lower levels of glutathione peroxidase activity as well as enhanced oxidative stress due to the highly reactive and toxic hydroxyl radical (Beal, 1993; Haan *et al.*, 1992; Mirault *et al.*, 1994). The level of glutathione peroxidase inactivation and degradation by hydroxyl radical mediated oxidation can reach a critical level above which too many reactive oxygen intermediates accumulate in neurons as well as glial cells resulting in neuronal degeneration and death (Haan *et al.*, 1992; Mirault *et al.*, 1994; Pigeolet and Remacle, 1991; Tabatabaie and Floyd, 1994). If the non-enzymatic antioxidative defense mechanisms against the highly reactive hydroxyl radical are inefficient, antioxidant enzymes will be destroyed leading to a low defense potential and a possible destruction of cell components by this toxic intermediate (Haan *et al.*, 1992; Mori *et al.*, 1994; Pigeolet and Remacle, 1991; Tabatabaie and Floyd, 1994).

The synthetic specific hydroxyl radical scavenger dimethylurea enhances glutathione peroxidase activity (Milner *et al.*, 1993). Indole alkaloids with antioxidative properties increase glutathione peroxidase activity and protect neuronal cells from oxidative damage due to reactive oxygen intermediates (Favit *et al.*, 1993). Melatonin is a potent, endogenous neuroprotective agent which protects the brain against oxidative damage (Hardeland *et al.*, 1993; Poeggeler *et al.*, 1993, 1994; Reiter *et al.*, 1993, 1995). Therefore, we investigated the effects of the endogenous hydroxyl radical scavenger melatonin on brain glutathione peroxidase.

EXPERIMENTAL PROCEDURES

Materials

Melatonin was administered (500 μg/kg, i.p.) at 1200 h to adult 12 week old female Harlan Sprague–Dawley rats (Harlan S–D, Indianapolis, IN). Rat brain was obtained by decapitation (Holson, 1992) 30 and 180 min after melatonin injection. Daytime control animals were sacrificed at 1200 h and night-time controls at 2400 h. The frontal cortex was homogenized 1 : 12 (w : v) in ice cold 50 mmol/l Tris (hydroxymethyl)aminomethane hydrochloride, 200 mmol/l potassium chloride pH 7.6. This and all chemicals were purchased from Sigma Chemical Co., St Louis, MO, except potassium cyanide which was obtained from Fluka Chemical Corp., Ronkonkoma, N.Y. The homogenate was centrifuged at 15,500 ***g*** for 15 min at 4°C, excess fat was removed, and the supernatant decanted and recentrifuged at 100,000 ***g*** for 60 min at 4°C. This supernatant was taken and diluted 1 : 2 with 50 mmol/l potassium chloride, pH 7.6. The samples were stored at −80°C. After the first centrifugation, a fraction of the supernatant (3 μl) was taken for protein determination by the microassay procedure using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA).

Glutathione peroxidase assay

Glutathione peroxidase was measured indirectly by a coupled reaction with glutathione reductase using cumene hydroperoxide as substrate. To determine glutathione peroxidase activity, a spectrophotometric system by manual method, modified for brain tissue, was used as previously described (Jaskot *et al.*, 1983). For direct assay, 10 μl of prepared brain homogenate was used.

Melatonin assay

Melatonin content in the brain and in the serum was determined with radioimmunoassay after chloroform extraction (Champney *et al.*, 1984). The tissue was homogenized 1 : 10 in 10 mM phosphate buffer, pH 7.8, and extracted 1 : 1 with chloroform in ice cold water for 15 min. The serum samples were also extracted with the same volume of chloroform, the chloroform extract of the brain tissue and the serum samples were evaporated overnight in the darkness and the dried extract was redissolved in the same volume of radioimmunoassay buffer. Recovery of exogenous standard melatonin was 69% on average for brain tissue (rat brain frontal cortex) and 66% on average for the serum samples. Serial dilution and parallel inhibition of endogenous and exogenous melatonin was established for both brain and serum extracts. Melatonin content was determined as previously described (Fraser *et al.*, 1983).

Statistical analysis

Data are expressed as mean ± SEM. Statistical analysis of the data was performed using a Student's *t*-test.

RESULTS

Melatonin, administered at a pharmacological dose of 500 μg/kg i.p. during the day, elicited an immediate and long lasting increase in brain glutathione peroxidase activity (Fig. 2). Glutathione peroxidase activity is increased 2-fold at 30 min after administration of melatonin (Fig. 2); this increase persists for at least 180 min (Fig. 2) after the i.p. injection of the indoleamine. Nocturnal brain glutathione peroxidase activity is 2-fold higher than diurnal glutathione peroxidase activity (Fig. 3).

Brain melatonin levels are increased at least 10-fold 30 min after melatonin administration (Fig. 2), while brain melatonin levels are only 2-fold higher after 180 min (Fig. 2) compared to non-injected controls. Night-time melatonin levels are higher than daytime melatonin levels (Fig. 3). Circulating melatonin levels are 1000 times higher 30 min after administration of the indoleamine (Table 1), but are reduced to supraphysiological levels 3 h later (Table 1). Circulating serum melatonin levels are much higher in the night than during the day (Table 1).

Melatonin tissue levels are 1000 times higher than the circulating levels in the serum (Figs 2, 3 and Table

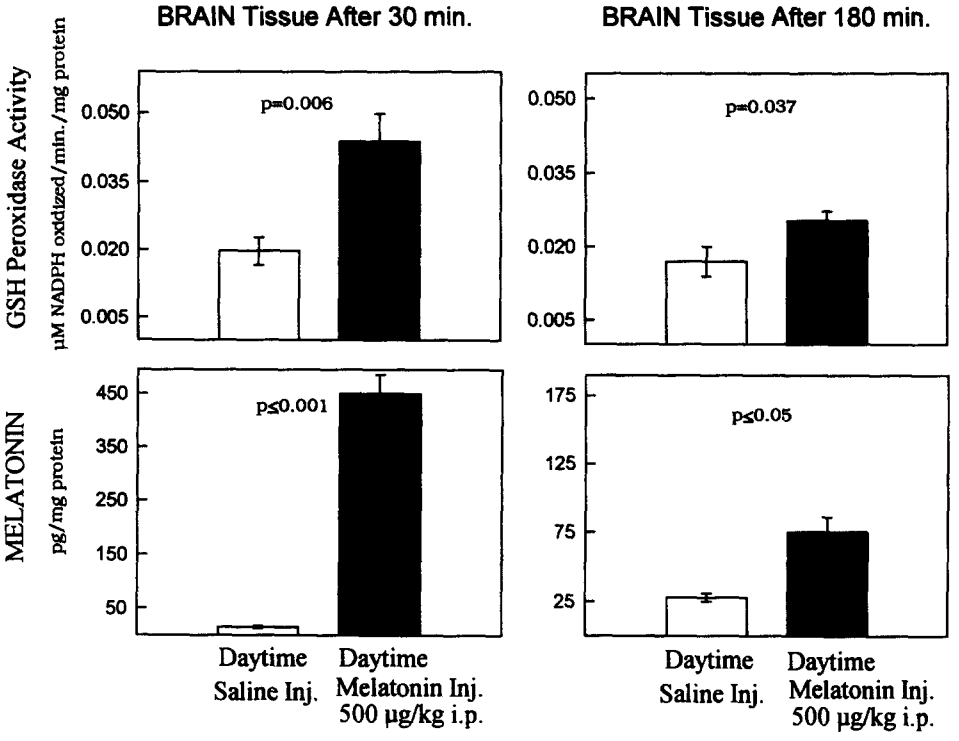

Fig. 2. Effects of melatonin (500 μg/kg i.p., at 1200 h) on brain glutathione peroxidase activity and melatonin immunoreactivity in the brain. The tissue was collected 30 and 180 min after the administration of the indoleamonine ($n = 8$).

1). High levels of endogenous melatonin in the rat brain are associated with high activity of brain glutathione peroxidase (Figs 2 and 3). However, even the 2-fold increase after exogenous melatonin administration and the physiological, nocturnal increase in circulating and tissue melatonin levels are sufficient to induce a maximal response (Figs 2 and 3). Much higher circulating and tissue melatonin levels do not induce a further stimulation of brain glutathione peroxidase activity (Figs 2 and 3).

DISCUSSION

Melatonin boosts the most important endogenous enzymatic antioxidative defense mechanism in the brain, glutathione peroxidase activity (Figs 2 and 3). The increase in glutathione peroxidase activity is substantial and long lasting (Fig. 2). As shown herein, glutathione peroxidase can be induced by the administration of pharmacological amounts of melatonin. However, the diurnal rhythm in brain glutathione peroxidase activity might be related to the endogenous circadian rhythm of physiological tissue concentrations of the indoleamine.

It is apparent that melatonin administration not only increases the detoxification and elimination rate of the highly reactive and toxic hydroxyl radical by direct hydroxyl radical scavenging, but it also reduces the generation and formation of hydroxyl radicals by reducing the accumulation of peroxides through induction of glutathione peroxidase (Figs 2 and 3). Thus, the potent antioxidative effects of melatonin are caused by at least two different mechanisms, which might, however, be interdependent (Beloqui and Cederbaum, 1986; Hardeland *et al.*, 1993; Poeggeler *et al.*, 1993; Reiter *et al.*, 1994, 1995). It has been demonstrated that the glutathione–glutathione peroxidase system suppresses hydroxyl radical generation and prevents oxidative damage and destruction due to this highly reactive radical (Beloqui and Cederbaum, 1986).

Other indolic compounds with glutathione peroxidase stimulating activity such as the ergot alkaloid dihydroergocryptine are also able to prevent age-dependent and glutamate-induced neuronal degeneration due to peroxide accumulation and hydroxyl radical generation (Favit *et al.*, 1993). The potent enzyme inducing effects of melatonin and structurally related

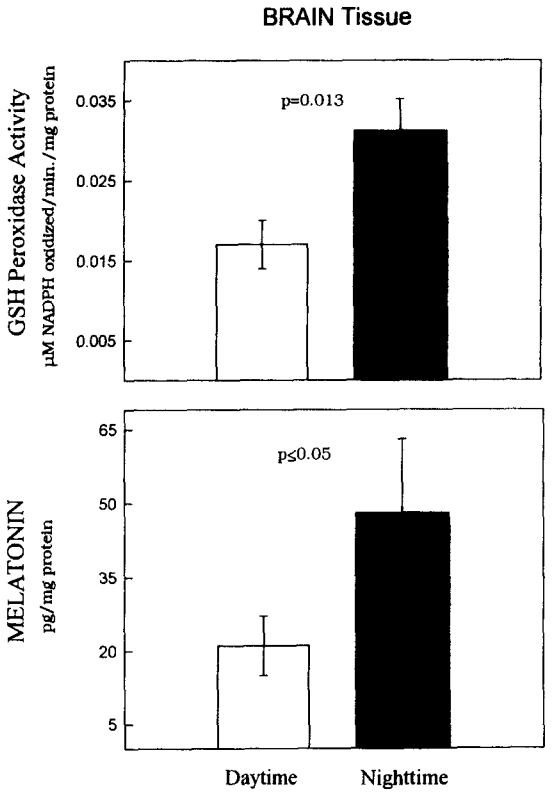

Fig. 3. Diurnal rhythm of glutathione peroxidase activity and melatonin immunoreactivity in the brain. The tissue was collected at 1200 and 2400 h ($n = 8$).

indoles might also contribute significantly to the neuroprotective activity of these compounds and to the potent anti-aging effects elicited by melatonin and its naphtalenic analogs (Hoffen *et al.*, 1993; Pierpaoli and Regelson, 1994; Poeggeler *et al.*, 1993; Reiter *et al.*, 1995).

Hydrogen peroxide accumulates in the brain of aged animals because superoxide dismutase gene expression and activity are increased without a concomitant, compensatory increase in the activity of catalase or glutathione peroxidase to detoxify this reactive oxygen intermediate (Haan *et al.*, 1992). Aged animals are melatonin deficient and more sensitive to oxidative stress (Poeggeler *et al.*, 1993; Reiter *et al.*, 1994, 1995).

Recently a comparison was made between the short-lived house mouse (*Mus musculus*) and the long-lived white-footed mouse (*Peromyscus leucopus*). The glutathione peroxidase activity in the brain of white-footed mice is much higher than the enzyme activity in house mice (Sohal *et al.*, 1993). The long-lived rodents have a much higher rate of hydrogen peroxide detoxification and a lower rate of hydrogen peroxide generation than do short-lived animals (Sohal *et al.*, 1993). Likewise, the level of hydroxyl radical-mediated oxidative damage is significantly lower in the long-lived mice than in their short-lived counterparts (Sohal *et al.*, 1993).

The efficiency of hydrogen peroxide elimination is a major factor in determining longevity and life span potential in mammals (Haan *et al.*, 1992; Sohal *et al.*, 1993). Aged rodents lose the ability to detoxify hydrogen peroxide efficiently (Haan *et al.*, 1992). Hydroxyl radical-mediated oxidative damage to neurons increases exponentially with age, possibly in part related to the fact that aged animals and humans lose their melatonin-mediated antioxidative protection (Beal, 1993; Poeggeler *et al.*, 1993; Reiter *et al.*, 1994, 1995).

Melatonin and structurally related aromatic compounds with high resonance stability and electroreactivity are a class of chemoprotectants that act by several mechanisms, including radical scavenging, protection against thiol depletion and calcium overload as well as enzyme induction (Chen *et al.*, 1993, 1994; Hardeland *et al.*, 1993; Poeggeler *et al.*, 1993, 1994; Reiter *et al.*, 1993–1995; Tan *et al.*, 1993a, 1994). To date the putative interactions between the specific melatonin receptors, their activation and antioxidative protection, as controlled by glutathione peroxidase gene expression have not been investigated (Reiter *et al.*, 1995).

It is extremely difficult to distinguish between specific receptor mediated mechanisms, e.g. enzyme induction and non-receptor mediated mechanisms such as radical scavenging, e.g. enzyme protection (Beloqui and Cederbaum, 1986; Reiter *et al.*, 1994). Neither is it known whether the radical scavenging and receptor-mediated actions are exerted independently or whether they are highly interdependent (Reiter *et al.*, 1995). The use of specific receptor agonists and antagonists as well as melatonin analogs with similar molec-

Table 1. Effects of melatonin (500 μg/kg i.p., at 1200 h) on daytime serum melatonin levels and diurnal rhythm of circulating melatonin levels. The serum was collected 30 min and 180 min after the administration of the indoleamine, and at 2400 h ($n = 8$)

	Serum melatonin (pg/ml)		
	Mean	SEM	*P*-Value
Daytime	20.50	2.50	
30 min after injection	15,800.00	3,600.00	≤0.001
180 min after injection	78.00	12.00	≤0.050
Nighttime	126.00	18.00	≤0.001

ular structure will enable us to distinguish between the direct and indirect antioxidative actions of melatonin and other structurally related molecules.

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