Department of Biochemistry, Faculty of Science, Annamalai University, Annamalainagar, Tamil Nadu, India

Effects of melatonin on the levels of antioxidants and lipid peroxidation products in rats treated with ammonium acetate

P. J. LENA, P. SUBRAMANIAN

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Perumal Subramanian, Department of Biochemistry, Faculty of Science, Annamalai University, Annamalainagar-608 002, Tamil Nadu, India
psub@rediffmail.com

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The antioxidant potential of melatonin (MLT) on hyperammonemia (induced by ammonium acetate treatment) were studied in rats. The levels of circulatory ammonia, urea and non-protein nitrogen increased significantly in ammonium acetate treated rats and decreased significantly in rats treated with melatonin and ammonium acetate. In brain tissues, the same pattern of alterations across groups was observed in the levels of thiobarbituric acid reactive substances and lipid profile variables (free fatty acids, triglycerides, phospholipids, and cholesterol). Further, enzymatic (superoxide dismutase, catalase and glutathione peroxidase) and non-enzymatic (reduced glutathione) antioxidants in brain tissues decreased significantly in ammonium acetate treated rats and increased significantly in rats treated with melatonin and ammonium acetate. These biochemical alterations could be due to the ability of melatonin to (i) scavenge a variety of radicals and reactive oxygen species (ii) induce antioxidative enzymes which reduce steady state levels of reactive oxygen species and (iii) stabilize cell membranes which assist them in reducing oxidative damage and thus could prevent oxidative stress in rats.

1. Introduction

Ammonia is a catabolic product of protein and nitrogenous compounds that is formed in mammals and humans. At high levels, ammonia is neurotoxic; it affects the functions of the central nervous system, and leads to coma and death (Plum et al. 1976). Hyperammonemia, caused by insufficient removal of ammonia in the liver (Meijer et al. 1990) or portacaval shunting (Butterworth et al. 1987), leads to increased ammonia levels in the brain (Butterworth et al. 1987), which is responsible for the development of hepatic encephalopathy (Butterworth et al. 1995). Ammonia intoxication impairs mitochondrial function (Kosenko et al. 1997) which could lead to decreased ATP synthesis and increased formation of free radicals (Kosenko et al. 2000). The major toxic effects of ammonia likely involve changes in cellular pH and the depletion of certain citric acid cycle intermediates, in particular α-ketoglutarate. Sustained hyperammonemia in mice leads to increased lipid peroxidation in liver and brain, reflecting an oxidative stress condition (Connor and Costell 1990; Dakshayani et al. 2002). Melatonin (*N*-acetyl-5-methoxytryptamine) is the main secretory product of the pineal gland. It is present in virtually all organisms ranging from bacteria (Manchester et al. 1995) to mammals (Poeggeler et al. 1991). Further, Melatonin is an endogenous free radical scavenger (Tan et al. 1993) and a broad spectrum antioxidant (Reiter et al. 1999). It detoxifies a variety of free radicals and reactive oxygen intermediates including the hydroxyl radical, peroxynitrite anion, singlet oxygen and nitric oxide (Reiter et al. 1999). Melatonin, which shows extreme diffusibility through membranes, is important for its scavenging action, since it could enter all cells and every subcellular compartment.

Systematic investigations of the levels of lipid peroxidation products and the levels of enzymatic and non-enzymatic antioxidants under the conditions of hyperammonemia are lacking. The present study deals with the levels of thiobarbituric acid reactive substances (TBARS-the products of lipid peroxidation) and the levels of catalase, superoxide dismutase and glutathione peroxidase (enzymatic antioxidants) and reduced glutathione (non-enzymatic antioxidant) in the brain tissue under the conditions of hyperammonemia and during melatonin treatment in rats. Furthermore, the levels of lipid profile variables (free fatty acids, triglycerides, phospholipids and cholesterol) in all the groups were investigated.

2. Investigations and results

There were no significant changes in the body weights (mean ± SD) of the experimental animals compared to controls. Concentrations of ammonia, urea and non-protein nitrogen (Table 1) increased significantly in group 2 rats and group 3 rats showed low levels (Table 1). Rats in group 4 showed no significant differences in the levels when compared with group 1 rats. Similar patterns on the levels of TBARS in the brain tissues (Table 2) were found in various groups.

Administration of ammonium acetate significantly decreased the levels of catalase, superoxide dismutase and glutathione peroxidase in the brain tissue of group 2 (Ta-

Table 1: Changes in the levels of ammonia, urea and non-protein nitrogen in the experimental groups

Variables	Group 1	Group 2	Group 3	Group 4
Ammonia (μM/L)	88.27 ± 7.82	331.11 ± 17.04[a]	166.76 ± 16.86[b]	83.92 ± 7.25[ns]
Urea (mg/100 ml)	10.68 ± 0.65	22.60 ± 1.19[a]	13.29 ± 1.04[b]	11.62 ± 0.96[ns]
Non-protein nitrogen (mg/100 ml)	23.57 ± 1.06	52.51 ± 1.24[a]	32.62 ± 1.77[b]	22.83 ± 1.31[ns]

Statistical significance was evaluated using ANOVA followed by Least Significant Difference (LSD) test. Group 2 is compared with group 1 ([a] $p < 0.05$). Group 3 is compared with group 2 ([b] $p < 0.05$). Group 4 is compared with group 1; [ns] – not significant

Table 2: Changes in the levels of TBARS and antioxidants in brain

Variables	Group 1	Group 2	Group 3	Group 4
TBARS (nM/100 g tissue)	1.50 ± 0.10	2.85 ± 0.11[a]	1.85 ± 0.08[b]	1.52 ± 0.11[ns]
Catalase (μmol of H_2O_2 consumed/min/mg protein)	3.63 ± 0.27	2.21 ± 0.13[a]	2.70 ± 0.13[b]	3.68 ± 0.07[ns]
SOD (50% inhibition of NBT reduction/min/mg protein)	3.09 ± 0.13	1.36 ± 0.05[a]	2.66 ± 0.15[b]	3.15 ± 0.18[ns]
Glutathione peroxidase (μg of GPx consumed min/mg protein)	4.36 ± 0.28	1.65 ± 0.20[a]	3.07 ± 1.37[b]	4.40 ± 0.38[ns]
Reduced glutathione (mg/g tissue)	11.25 ± 0.93	5.51 ± 0.74[a]	8.23 ± 0.63[b]	12.63 ± 0.90[ns]
Free fatty acids (mg/100 g tissue)	20.79 ± 1.79	36.68 ± 2.42[a]	25.94 ± 1.94[b]	19.33 ± 1.58[ns]
Triglycerides (mg/100 g tissue)	348.41 ± 31.05	435.27 ± 35.00[a]	374.24 ± 33.76[b]	344.95 ± 24.14[ns]
Phospholipids (mg/100 g tissue)	779.85 ± 85.29	1016.89 ± 70.20[a]	855.94 ± 35.83[b]	793.13 ± 85.54[ns]
Cholesterol (mg/100 g tissue)	1103.56 ± 58.04	2046.94 ± 79.42[a]	1246.97 ± 35.35[b]	1111.06 ± 73.76[ns]

Statistical significance was evaluated using ANOVA followed by Least Significant Difference (LSD) test. Group 2 is compared with group 1 ([a] $p < 0.05$). Group 3 is compared with group 2 ([b] $p < 0.05$). Group 4 is compared with group 1; [ns] – not significant

ble 2) compared with controls. Group 3 showed significantly increased levels of these enzymatic antioxidants and no significant changes were observed in the levels of these enzymatic antioxidants in group 4. Similar changes between the groups were observed regarding the levels of non-enzymatic antioxidant-reduced glutathione (Table 2).
Levels of free fatty acids, triglycerides, phospholipids and cholesterol in the brain tissue were significantly increased in group 2 (Table 2) compared with controls; these levels were decreased in group 3 and non-significantly different in group 4 when compared with controls.

3. Discussion

Ammonia is removed either in the form of urea in periportal hepatocytes and/or as glutamine in perivenous hepatocytes (Nelson and Cox 2000) Elevated levels of ammonia, urea and non-protein nitrogen in ammonium acetate treated rats may be due to the tissue damage caused by ammonia induced free radical generation, leading to oxidative stress and tissue damage (Kosenko et al. 2000; Dakshayani et al. 2002; Vidya et al. 2003). Melatonin is an effective free radical scavenger (Reiter et al. 1999), which by its antioxidant potential decreases the levels of ammonia, urea and non-protein nitrogen. Under hyperammonemic conditions, elevated levels of ammonia result in the production of free radicals such as hydroxyl radicals, superoxide radicals, peroxyl radicals, alkoxyl radicals and reactive nitrogen species. Melatonin interacts with nitric oxide to form 1-nitroso melatonin (Blanchard et al. 2000). This product might diminish the levels of ammonia, urea and non-protein nitrogen, thereby reducing oxidative stress and tissue damage.
Elevated levels of TBARS have been observed in the brain tissue of ammonium acetate treated rats indicating the increased levels of lipid peroxidation. It is a well established fact that ammonia intoxication enhances lipid peroxidation and generates free radicals (Kosenko et al. 2000; Dakshayani et al. 2002; Vidya et al. 2003). The levels of TBARS in ammonium acetate and melatonin treated rats were significantly decreased when compared to group 2 rats. This suggests that melatonin could offer protection against lipid peroxidation (Lastra et al. 1997).
The non-enzymatic antioxidant glutathione is a scavenger of hydroxyl radicals and singlet oxygen (Halliwell and Gutteridge 1999). It has been reported that ammonia intoxication induces depletion of glutathione and an increase in lipid peroxidation (Kosenko et al. 1999). Reports have also shown that ammonia intoxication leads to the increased formation of nitric oxide which results in the oxidation of glutathione (GSH) to glutathione disulphide (GSSG) and to mixed glutathione disulphides (GSSR) resulting in depletion of GSH and increased free radical formation (Luperchio et al. 1996). Group 3 rats compared to group 2 rats showed elevated levels of glutathione. This is because, under hyperammonemic conditions, melatonin increases the levels of glutathione, an important intracellular antioxidant, by stimulating its rate- limiting enzyme, γ-glutamylcysteine synthase (Urata et al. 1999).
In our study, the decreased activities of antioxidant enzymes (SOD, CAT, GPx) in the ammonium acetate treated group may be due to the inhibition of these enzymes by nitric oxide. It is known that ammonia-induced inhibition of antioxidant enzymes is mediated by the activation of NMDA receptors that leads to increased intracellular calcium levels, which in turn activate neuronal nitric oxide synthase, leading to the formation of nitric oxide which inhibits the activities of antioxidant enzymes (Kosenko et al. 2000). Under hyperammonemic conditions, melatonin causes an increase in the gene expression and activities of the antioxidant enzymes such as glutathione peroxidase, glutathione reductase and superoxide dismutase (Barlow et al. 1995; Antolin et al. 1996) which results in the elevated levels of these enzymes in group 3 rats. The elevated levels of these enzymes might protect against oxidative damage caused by the free radical formation (Reiter et al. 2003).
Melatonin directly scavenges hydrogen peroxide to form N^1-acetyl-N^2-formyl-5-methoxy kynuramine, which by the action of catalase forms N^1-acetyl-5-methoxy kynuramine (Tan et al. 2000). These biogenic amines could also scavenge hydroxyl radicals and reduce lipid peroxidation.

Table 3: Biochemical determinations and methods

Parameter	Studies carried out in blood/plasma/serum/brain tissue	Method
Total protein	Brain	Lowry et al. (1951)
Ammonia	Plasma	Wolheim (1984)
Urea	Blood	Varley et al. (1991)
Non-protein nitrogen	Blood	Varley et al. (1991)
Thiobarbituric acid reactive substances	Brain	Nichans and Samuelson (1968)
Reduced glutathione	Brain	Ellman (1959)
Superoxide dismutase	Brain	Kakker et al. (1984)
Catalase	Brain	Sinha (1972)
Glutathione peroxidase	Brain	Rotruck et al. (1973)
Free fatty acids	Brain	Falholt et al. (1973)
Phospholipids	Brain	Zilversmit et al. (1950)
Triglycerides	Brain	Foster and Dunn (1973)
Cholesterol	Brain	Zlatkis et al. (1953)

Ammonium acetate may deplete the levels of α-KG and other Krebs cycle intermediates (Yamamoto 1989) and thus elevate the levels of acetyl coenzyme A. The elevated levels of acetyl coenzyme A may increase the levels of lipid profile variables (free fatty acids, triglycerides, phospholipids and cholesterol) as observed in our study. The decreased α-KG levels in rats treated with ammonium acetate might have led to the accumulation of fatty acids and might be reversed during treatment with melatonin, since melatonin was found to reduce these levels (Baydas et al. 2002).

Our results suggest that melatonin could control the oxidative stress caused by hyperammonemia by (i) directly scavenging a variety of radicals and reactive oxygen species (ii) inducing antioxidative enzymes which reduce steady state levels of reactive oxygen species and by (iii) stabilizing cell membranes which assist them in reducing oxidative damage.

4. Experimental

Adult male Wistar rats (180–220 g), obtained from National Centre for Laboratory Animal Sciences, Hyderabad, were maintained in polypropylene cages in a controlled environment (22–24 °C) under 12 : 12 h light dark cycles. Standard pellet diet (Kamadhenu Agencies, Bangalore, India) and water were provided *ad libitum*. All studies were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (National Institute Guide, 1985). Melatonin was purchased from Sisco Research Laboratories Private Limited, Mumbai, India. Ammonium acetate and all other chemicals used in this study were of analytical grade.

The animals were divided into four groups of six rats each and all were fed with the standard pellet diet. Group 1 animals served as controls. Group 2 animals were administered with ammonium acetate intraperitoneally (100 mg/kg) every day for 45 days (Hilgier et al. 1990). Group 3 animals were treated with ammonium acetate as group 2 animals along with melatonin (5 mg/kg) intraperitoneally (Liu and Ng 2000). Group 4 animals received melatonin (5 mg/kg) intraperitoneally throughout the experiment.

The experiment was terminated after 45 days and all animals were killed by cervical decapitation. Blood samples were collected from each group of rats. Biochemical analyses were performed in blood and plasma samples. Further, the brain tissues were taken in ice cold containers for biochemical determinations. Biochemical determinations were done by the methods mentioned in Table 3.

The data were analysed using an analysis of variance (ANOVA) and the group means were compared by Least Significant Difference (LSD) test. The results were considered statistically significant if the p-value was 0.05 or less.

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