Journal of Cellular Biochemistry 102:729–735 (2007)

Melatonin Reduces Protein and Lipid Oxidative Damage Induced by Homocysteine in Rat Brain Homogenates

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Abstract Numerous data indicate that hyperhomocysteinemia is a risk factor for cardio- and cerebrovascular diseases. At least in part, homocysteine (HCY) impairs cerebrovascular function because it generates large numbers of free radicals. Since melatonin is a well-known antioxidant, which reduces oxidative stress and decreases HCY concentrations in plasma, the aim of this study was to investigate the effect of melatonin in preventing HCY-induced protein and lipid oxidation in rat brain homogenates. Brain homogenates were obtained from Sprague–Dawley rats and were incubated with or without HCY (0.01–5 mM) or melatonin (0.01–3 mM). Carbonyl content of proteins, and malondialdehyde (MDA) and 4-hydroxyalkenals (4-HDA) concentrations in the brain homogenates were used as an index of protein and lipid oxidation, respectively. Under the experimental conditions used, the addition of HCY (0.01–5 mM) to the homogenates enhanced carbonyl protein and MDA+4-HDA formation. Melatonin reduced, in a concentration-dependent manner, protein and lipid oxidation due to HCY in the brain homogenates. These data suggest that preserving proteins from oxidative insults is an additional mechanism by which melatonin may act as an agent in potentially decreasing cardiovascular and cerebrovascular diseases related to hyperhomocysteinemia. J. Cell. Biochem. 102: 729–735, 2007. © 2007 Wiley-Liss, Inc.

Key words: melatonin; homocysteine; protein oxidation; lipid peroxidation; antioxidant therapy; brain

Free radicals are defined as any species capable of independent existence that contains one or more unpaired electrons in their outer orbital. When free radicals interact with other molecules and when they exceed the defense capacity of the endogenous antioxidant systems, indiscriminate damage occurs in macromolecules, such as proteins and lipids; this damage compromises cellular functions. The central nervous system (CNS) is a tissue highly susceptible to free radical injury for several reasons: first, in the CNS membrane, lipids are rich in polyunsaturated fatty acid side chains, which are particularly sensitive to free radical attack; second, the CNS has a poorly developed endogenous antioxidant defense system, for example, catalase is particularly low; third, certain areas of the brain produce H_2O_2 and they are rich in iron and copper, two metals which readily stimulate hydroxyl radical (•OH) formation via the Fenton reaction; and finally, the CNS has a high metabolic rate which initiates the generation of large numbers of damaging free radicals [Sinet et al., 1980; Beard et al., 1993; Reiter, 1998].

Homocysteine (HCY) is an intermediate aminoacid, which results from the conversion of methionine to cysteine. Hyperhomocysteinemia is a risk factor for coronary atherosclerotic vascular diseases, stroke, venous thrombosis, and it has been associated with Alzheimer's disease and vascular dementia [Kang et al., 1992; Stampfer et al., 1992; Loscalzo, 1996; Rodrigo et al., 2003; Faraci and Lentz, 2004]. Elevated HCY concentrations in the blood are detected in 40% of patients with coronary,

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Received 4 September 2006; Accepted 9 February 2007
DOI 10.1002/jcb.21327

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cerebrovascular, or peripheral atherosclerosis [Clarke et al., 1991]. Although its physiopathological mechanisms are complex and not fully understood, much evidence suggests that hyperhomocysteinemia induces vascular and brain damage because of the highly reactive thiol group in HCY that it is readily oxidized leading to the formation of homocystine, HCY-mixed disulfides, and HCY thiolactone. During these oxidative processes, several reactive species are generated [Loscalzo, 1996; Moselhy and Demerdash, 2003, 2004; Perna et al., 2003; Topal et al., 2004; Perez-de-Arce et al., 2005].

Melatonin (N-acetyl-5-methoxytryptamine) is the main product of the pineal gland and, additionally, it is produced in several organs in vertebrates [Tan et al., 1999]. Melatonin concentrations in the serum exhibit a pronounced circadian rhythm, with the highest levels during the night-time and lowest concentrations during the daytime. In the last five decades, intensive research has shown that melatonin is involved in the modulation of a variety of endocrine, neural and immune processes [Reiter, 2004]. Numerous publications have proven that melatonin protects the brain from many chemical insults both in vivo and in vitro [Lapin et al., 1998; Wakatsuki et al., 1999; Cabrera et al., 2000; Ortega-Gutiérrez et al., 2001; Baydas et al., 2003; Lee et al., 2005].

We previously demonstrated that melatonin inhibits HCY-induced oxidative damage in brain homogenates [Osuna et al., 2002]. The purpose of the current work was to examine the potential role of melatonin in preventing HCY-induced protein oxidative damage in the brain and compare these effects with the ability of melatonin to reduce lipid peroxidation. Protein carbonyl content and malondialdehyde (MDA) and 4-hydroxyalkenals (4-HDA) concentrations in the brain homogenates were used as indices of oxidative damage of proteins and lipids, respectively.

MATERIALS AND METHODS

Chemicals

All reagents were of analytical grade and obtained from commercial sources. Homocysteine and melatonin were purchased from Sigma–Aldrich (Madrid, Spain). Other chemicals used were of the highest quality available. The Bioxytech LPO-586 kit for lipid peroxidation was obtained from Calbiochem (La Joya, CA).

Animals and Homogenates

The handling and animal procedures were made in strict accordance with the recommendations of the European Economic Community Committee (86/609/CEE) for the care and use of laboratory animals. Male Sprague–Dawley rats weighing 225–250 g were purchased from Harlan Ibérica S.A. (Barcelona, Spain) and received standard chow and water ad libitum. After being acclimated for 2 weeks under a light/dark cycle of 12/12 h (lights on at 7:00 a.m.) the animals were anesthetized with sodium thiopental administered intraperitoneally (50 mg/kg) and perfused through the heart with an ice-cold saline solution (0.9% NaCl) to minimize the excess of intravascular transition metals that could artificially increase oxidative damage. Immediately after perfusion, the brains were quickly removed, washed in saline, and homogenized (1:10 w/v) with a Polytron-like stirrer in 20 mM Tris-HCl buffer (pH 7.4).

Induction of Oxidative Damage in Brain Homogenates

In a first study, protein and lipid oxidation was induced by incubation of brain homogenates ($n = 6$ each) in a shaking water bath for 2 h at 37°C using six different concentrations of HCY (0.01, 0.05, 0.1, 0.5, 1, 5 mM). This study was performed to determine the concentration of HCY required to induce an appropriate amount of protein and lipid oxidation. In a second study, to determine the optimal incubation time, aliquots of brain homogenates ($n = 6$ each) were incubated with 1 mM HCY for 0, 10, 30, 60, 90, 120, and 180 min. In a third study ($n = 6$), several concentrations of melatonin (0.01, 0.1, 0.5, 1, and 3 mM) were used in combination with 1 mM HCY. Melatonin was dissolved in absolute ethanol and then diluted with buffer; the final concentration of alcohol was $<1\%$ in the brain homogenates. The same volume of ethanol was added to all homogenates regardless of treatment.

After incubating the samples as described above, the oxidative reaction was stopped by placing the aliquots on ice for 10 min. They were then centrifuged at $3{,}000 \times g$ for 10 min at 4°C. Thereafter, the supernatants were assayed for their levels of protein carbonyl and MDA+4-HDA concentrations.

Analytical Procedures

Carbonyl contents were measured as an index of oxidative protein damage using the method described by Levine et al. [1990]. Carbonyl rests interact with 2,4-dinitrophenhylhydrazine, yielding a colored complex with a peak absorbance at 375 nm in the ultraviolet spectrum. Results are expressed as nmol carbonyl per mg protein.

MDA+4-HDA levels were used as an index of the oxidative breakdown of lipids in the brain homogenates [Janero, 1990]. In the assay, MDA+4-HDA react with N-methyl-2-phenylindole, yielding a stable chromophore with a peak maximal absorbance at 586 nm; 1,1,3,3-tetramethoxypropane was used as a standard. Results are expressed as nmol MDA+4-HDA per mg protein.

The protein concentrations in the incubation media were assessed using the method of Bradford [1976], where bovine serum albumin served as a standard.

Statistical Analysis

Results are expressed as means $\pm$ standard errors. Student's paired data t-test was used for comparison of the means. Values were accepted as being statistically different if a P-value was ≤ 0.05.

RESULTS

HCY Concentration-Response Studies

The initial study showed that HCY induces oxidative stress in proteins and lipids in the rat brain homogenates. HCY concentrations greater than 0.01 mM and 0.1 mM, respectively, significantly increased carbonyl content in the proteins and the MDA+4-HDA levels in the homogenates (Fig. 1). An HCY concentration of 1 mM was selected for the following studies since it provided high levels of both protein and lipid oxidative damage.

Time Course Studies

The second study was performed to assess the optimal incubation time to generate an appropriate amount of oxidative damage induced by 1 mM HCY. As showed in the Figure 2, protein carbonyl content as well as lipid peroxidation increased in a time-dependent manner. A 2 h incubation was selected for following studies.

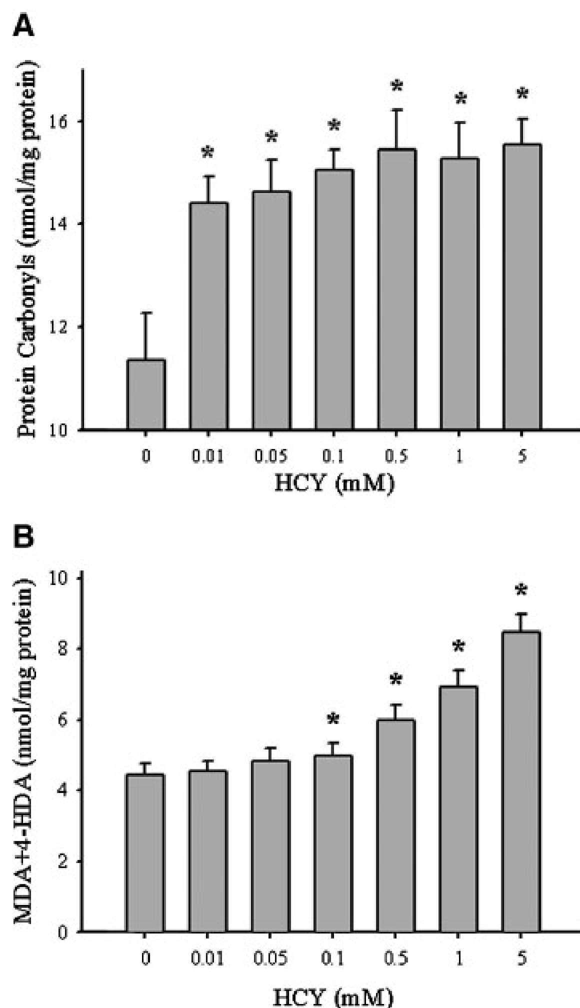

Fig. 1. Incubation of rat brain homogenates with homocysteine (HCY) at 37°C for 2 h increased carbonyl content in proteins (**A**) and malondialdehyde (MDA) and 4-hydroxyalkenals (4-HDA) concentrations (**B**). Values are means $\pm$ standard errors (n = 6). *$P \leq 0.05$ versus brain homogenates without HCY.

Melatonin Co-Treatment Studies

The final study tested whether melatonin reduces oxidative damage to proteins and lipids in rat brain homogenates induced by 1 mM HCY incubated for 2 h. Melatonin's inhibitory effects on HCY-induced carbonyl content in proteins and MDA+4-HDA levels increased in a concentration-dependent manner, as illustrated in Figure 3. Melatonin (0.5 mM) or greater significantly reduced the carbonyl content in proteins of brain homogenates below those treated with 1 mM HCY alone. Melatonin (3 mM) lowered the oxidative protein injury even to levels below those in the controls. The concentration of melatonin required to prevent

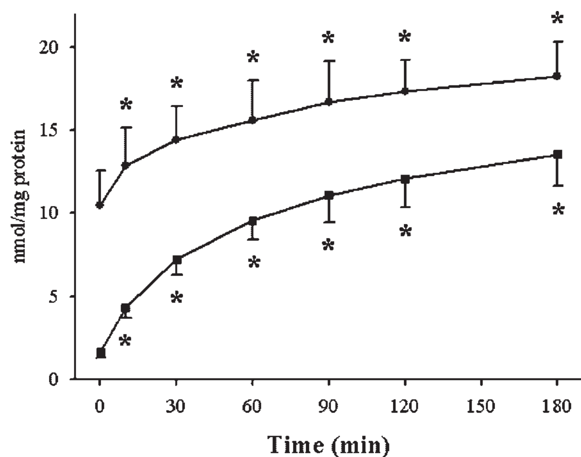

Fig. 2. Time-dependent increase in carbonyl content in proteins (●) and malondialdehyde (MDA) and 4-hydroxyalkenals (4-HDA) concentrations (■) in rat brain homogenates following incubation with 1 mM homocysteine (HCY). Results are means ± standard errors of six independent experiments. *$P \leq 0.05$ versus control homogenates without incubation.

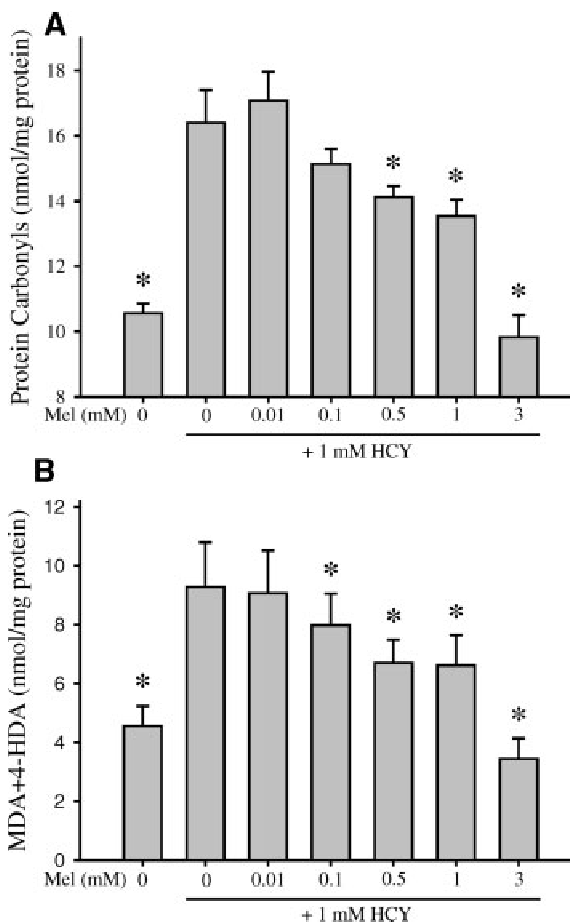

Fig. 3. Protective effects of melatonin (Mel) on homocysteine (HCY)-induced protein (**A**) and lipid (**B**) oxidation in rat brain homogenates. Results are means ± standard errors of six experiments. *$P \leq 0.05$ versus brain homogenates exposed to HCY alone.

the carbonylation of proteins induced by 1 mM HCY by a 50%, that is, IC_{50}, was 1.19 mM.

With regard to the beneficial effects of melatonin in protecting lipids from oxidative damage, concentrations of 0.1 mM or greater significantly reduced the levels of MDA+4-HDA in brain homogenates below the level of those treated with 1 mM HCY alone. Melatonin (3 mM) also reduced lipid peroxidation even below the control samples (neither HCY nor melatonin). The melatonin IC_{50} that prevented MDA+4-HDA formation was 0.40 mM.

DISCUSSION

It has been proposed that hyperhomocysteinemia promotes cerebral and cardiovascular diseases through endothelial and platelet dysfunction, elevated susceptibility of low density lipoproteins to oxidation, and generation of large numbers of free radicals [Blundell et al., 1996; Halvorsen et al., 1996; Durand et al., 1997; Mahfouz and Kummerow, 2004]. The thiol group of HCY makes it an autoxidizable compound in the presence of oxygen and transition metals such as iron and copper. As a consequence of this oxidation, $^{\bullet}OH$, H_2O_2, and superoxide anion radicals ($O_2^{\bullet-}$) are generated [Starkebaum and Harlan, 1986; Loscalzo, 1996; Perna et al., 2003]. Our results show that melatonin effectively prevents oxidative damage due to HCY exposure in rat brain homogenates. These findings are consistent with some reports, which have shown that melatonin prevents both in vivo and in vitro HCY-induced lipid peroxidation in the brain [Osuna et al., 2002; Baydas et al., 2003, 2006].

Whereas numerous reports have documented the protective actions of melatonin on lipids and DNA in the brain tissue [Reiter, 1998], few have focused on its effectiveness in limiting oxidative damage to proteins. Two early reports showed that melatonin reduces the oxidation of the bovine serum albumin due to $^{\bullet}OH$ generated by metal catalyzed oxidation induced, for example, Cu^{2+}/H_2O_2 and ascorbate/Fe^{3+}/EDTA systems, and the alkylperoxyl radicals formed by the azo initiator 2,2′-azino(2-amidinopropane) hydrochloride [Kim et al., 2000; Mayo et al., 2003]. In addition, melatonin reduced in vivo the severity of protein oxidation induced by

gentamicin, as indicated by a decrease in the carbonyl content in proteins of rats treated with gentamicin or acetaminophen and subjected to ischemia-reperfusion [Sener et al., 2002a,b, 2003]. The current investigation shows for the first time that melatonin also preserves brain proteins from the oxidative toxicity of HCY.

Acute hyperhomocysteinemia, after an oral methionine load, impairs flow-mediated endothelium-dependent vasodilatation in healthy humans [Bellany et al., 1998; Chambers et al., 1998]. Okatani et al. [2000] showed that melatonin counteracts the vasoconstrictive effect of HCY in the human umbilical artery and they suggested that this activity of melatonin may be related to its free radical scavenging and antioxidant actions. It was found that the vasoconstrictor properties of HCY are due to a reduction in the release of nitric oxide from endothelial cells. This effect depends first on the uncoupling of the endothelial nitric oxide synthase due to the reduction of intracellular tetrahydrobiopterin availability without affecting the expression of this enzyme [Zhang et al., 2000; Rodrigo et al., 2003; Topal et al., 2004], and second, through the formation of the highly reactive peroxynitrite anion by the combination of $O_2^{\bullet-}$, generated during HCY oxidation, and nitric oxide [Pryor and Squadrito, 1995; Rodrigo et al., 2003]. Zhang et al. [1998, 1999] demonstrated that melatonin also scavenges peroxynitrite.

Another explanation regarding the mechanism by which HCY induces oxidative damage is via inhibition of several key antioxidant enzymes. Hyperhomocysteinemia decreases the expression of the cellular isoform of glutathione peroxidase [Upchurch et al., 1997; Outinen et al., 1999; Handy et al., 2005]. In addition to its ability to scavenge directly free radicals, melatonin also stimulates several antioxidant enzymes. A number of reports have shown that melatonin promotes the activities and gene expression of glutathione peroxidase and reductase, CuZn and Mn superoxide dismutases, and catalase [Pablos et al., 1995; Kotler et al., 1998; Naidu et al., 2003; Rodríguez et al., 2004]. Recently, Baydas et al. [2003] documented that intracerebroventricular injection of HCY reduced the activity of the glutathione peroxidase in the cerebellum, cortex, and hippocampus in Wistar rats. Conversely, co-treatment with melatonin resulted in a significant increase in the activity of the glutathione peroxidase in these brain regions as compared with the animals that received HCY alone.

Since hyperhomocysteinemia induces oxidative stress, it seems appropriate to propose the use of antioxidants as therapeutic agents, which may attenuate this oxidative injury, especially if the antioxidant molecule would also reduce the concentration of HCY in the blood. An earlier report [Bremner et al., 2000] demonstrated that in the human, serum total HCY levels exhibit a circadian rhythm with the highest concentrations around 22:00 h and gradually decreasing thereafter to lowest levels around 10:00 h. In addition, Baydas et al. [2002a,b] demonstrated in rats that pinealectomy increases plasma HCY levels compared with those in intact animals. Melatonin treatment reverses these changes. The implications of these findings are that HCY levels are inversely related to the concentrations of melatonin in the blood.

In addition to melatonin's potential beneficial actions in hyperhomocysteinemia, melatonin use as a preventive agent may offer other additional advantages; thus, it is ubiquitously distributed in organisms and it has very low toxicity [Reiter, 1998, 2004; Jahnke et al., 1999]. On the contrary, melatonin has been proposed as an antioxidant that limits tissue damage induced by numerous drugs whose toxicity is a consequence of free radical generation during their metabolism [Brzozowski et al., 1997; García et al., 1998; López-González et al., 2000; Sener et al., 2002b, 2003].

In conclusion, the data present here provides a new mechanism by which melatonin reduces oxidative damage induced by HCY; thus, melatonin protects cerebral proteins from oxidative damage. Considering the effects reported herein and previous results relative to the antioxidant activity of melatonin and its effects on HCY metabolism, we suggest that melatonin might be an interesting potential preventive strategy for reducing cardiovascular and cerebrovascular complications of hyperhomocysteinemia.

ACKNOWLEDGMENTS

This work was financed by the Gobierno de Aragón, Spain (B40) and Instituto de Salud Carlos III (G03/137). JJG was supported by the Ibercaja—University of Zaragoza Program (Spain).

REFERENCES

Baydas G, Gursu MF, Cikim G, Canatan H. 2002a. Homocysteine levels are increased due to lack of melatonin in pinealectomized rats: Is there a link between melatonin and homocysteine? J Pineal Res 32: 63–64.

Baydas G, Gursu MF, Cikim G, Canpolat S, Yasar A, Canatan H, Kelestimur H. 2002b. Effects of pinealectomy on the levels and the circadian rhythm of plasma homocysteine in rats. J Pineal Res 33:151–155.

Baydas G, Kutlu S, Naziroglu M, Canpolat S, Sandal S, Ozcan M, Kelestimur H. 2003. Inhibitory effects of melatonin on neural lipid peroxidation induced by intracerebroventricularly administered homocysteine. J Pineal Res 34:36–39.

Baydas G, Ozer M, Yasar A, Koz ST, Tuzcu M. 2006. Melatonin prevents oxidative stress and inhibits reactive gliosis induced by hyperhomocysteinemia in rats. Biochemistry (Mosc) 71 Suppl 1:S91–S95.

Beard JL, Connor JR, Jones BC. 1993. Iron in the brain. Nutr Rev 51:157–170.

Bellany MF, McDowell IFW, Ramsey MW, Brownlee M, Bones C, Newcombe RG, Lewis MJ. 1998. Hyperhomocysteinemia after an oral methionine load acutely impairs endothelial function in healthy adults. Circulation 98:1848–1852.

Blundell G, Jones BG, Rose FA, Tudball N. 1996. Homocysteine mediated endothelial cell toxicity and its amelioration. Atherosclerosis 122:163–172.

Bradford MM. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.

Bremner WF, Holmes EW, Kanabrocki EL, Hermida RC, Ayala D, Garbincius J, Third JLHC, Ryan MD, Johnson M, Foley S, Shirazi P, Nemchausky BA, Scheving LE. 2000. Circadian rhythm of serum total homocysteine in men. Am J Cardiol 86:1153–1156.

Brzozowski T, Konturek PC, Konturek SJ, Pajdo R, Bielanski W, Brzozowska I, Stachura J, Hahn EG. 1997. The role of melatonin and L-tryptophan in prevention of acute gastric lesions induced by stress ethanol ischemia and aspirin. J Pineal Res 23:79–89.

Cabrera J, Reiter RJ, Tan DX, Qi W, Sáinz RM, Mayo JC, García JJ, Kim SJ, El-Sokkary G. 2000. Melatonin reduces oxidative neurotoxicity due to quinolinic acid: In vitro and in vivo findings. Neuropharmacology 39: 507–514.

Chambers JC, McGregor A, Jean-Marie J, Kooner JS. 1998. Acute hyperhomocysteinaemia and endothelial dysfunction. Lancet 351:36–37.

Clarke R, Daly L, Robinson K, Naughten E, Cahalane S, Fowler B, Graham I. 1991. Hyperhomocysteinemia: An independent risk factor for vascular disease. New Engl J Med 324:1149–1155.

Durand P, Lussier-Cacan S, Blache D. 1997. Acute methionine load-induced hyperhomocysteinemia enhances platelet aggregation thromboxane biosynthesis and macrophage-derived tissue factor activity in rats. FASEB J 11:1157–1168.

Faraci FM, Lentz SR. 2004. Hyperhomocysteinemia oxidative stress and cerebral vascular dysfunction. Stroke 35:345–347.

García JJ, Reiter RJ, Ortiz GG, Oh CS, Tang L, Yu BP, Escames G. 1998. Melatonin enhances tamoxifen's ability to prevent the reduction in microsomal membrane fluidity induced by lipid peroxidation. J Membrane Biol 162:59–65.

Halvorsen B, Brude I, Drevon CA, Nysom J, Ose L, Christiansen EN, Nenseter MS. 1996. Effect of homocysteine on copper ion-catalyzed azo compound-initiated and mononuclear cell-mediated oxidative modification of low density lipoprotein. J Lipid Res 37:1591–1600.

Handy DE, Zhang Y, Loscalzo J. 2005. Homocysteine down-regulates cellular glutathione peroxidase (GPx1) by decreasing translation. J Biol Chem 22:15518–15525.

Jahnke G, Marr M, Myers C, Wilson R, Travlos G, Price C. 1999. Maternal and developmental toxicity evaluation of melatonin administered orally to pregnant Sprague-Dawley rats. Toxicol Sci 50:271–279.

Janero DR. 1990. Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. Free Radical Bio Med 9:515–540.

Kang SS, Wong PWK, Malinow MR. 1992. Hyperhomocyst(e)inemia as a risk factor for occlusive vascular disease. Ann Rev Nutr 12:279–298.

Kim SJ, Reiter RJ, Qi W, Tan DX, Cabrera J. 2000. Melatonin prevents oxidative damage to protein and lipid induced by ascorbate-Fe(3+)-EDTA: Comparison with glutathione and alpha-tocopherol. Neuroendocrinol Lett 21:269–276.

Kotler ML, Rodríguez C, Sáinz RM, Antolín I, Menéndez-Peláez A. 1998. Melatonin increases gene expression for antioxidant enzymes in rat brain cortex. J Pineal Res 24:83–89.

Lapin IP, Mirzaev SM, Ryzov IV, Oxenkrug GF. 1998. Anticonvulsant activity of melatonin against seizures induced by quinolinate kainate glutamate NMDA and pentylenetetrazole in mice. J Pineal Res 24:215–218.

Lee EJ, Lee MY, Chen HY, Hsu YS, Wu TS, Chen ST, Chang GL. 2005. Melatonin attenuates gray and white matter damage in a mouse model of transient focal cerebral ischemia. J Pineal Res 38:42–52.

Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz AG, Ahn BW, Shaltiel S, Stadtman ER. 1990. Determination of carbonyl content in oxidatively modified proteins. Method Enzymol 186:464–478.

López-González MA, Guerrero JM, Rojas F, Delgado F. 2000. Ototoxicity caused by cisplatin is ameliorated by melatonin and other antioxidants. J Pineal Res 28: 73–80.

Loscalzo J. 1996. The oxidant stress of hyperhomocyst(e)inemia. J Clin Invest 98:5–7.

Mahfouz MM, Kummerow FA. 2004. Vitamin C or vitamin B_6 supplementation prevents the oxidative stress and decrease of prostacyclin generation in homocysteinemic rats. Int J Biochem Cell B 36:1919–1932.

Mayo JC, Tan DX, Sáinz RM, Natarajan M, López-Burillo S, Reiter RJ. 2003. Protection against oxidative protein damage induced by metal-catalyzed reaction or alkylperoxyl radicals: Comparative effects of melatonin and other antioxidants. Biochimica et Biophysica Acta 1620:139–150.

Moselhy SS, Demerdash SH. 2003, 2004. Plasma homocysteine and oxidative stress in cardiovascular disease. Dis Markers 19:27–31.

Naidu PS, Sinhg A, Kaur P, Sandhir R, Kulkarni SK. 2003. Possible mechanism of action in melatonin attenuation of haloperidol-induced orofacial dyskinesia. Pharmacol Biochem Be 74:641–648.

Okatani Y, Wakatsuki A, Reiter RJ. 2000. Protective effect of melatonin against homocysteine-induced vasoconstriction of human umbilical artery. Biochem Bioph Res Co 277:470–475.

Ortega-Gutiérrez S, García JJ, Martínez-Ballarín E, Reiter RJ, Millán-Plano S, Robinson M, Acuña-Castroviejo D. 2001. Melatonin improves deferoxamine antioxidant activity in protecting against lipid peroxidation caused by hydrogen peroxide in rat brain homogenates. Neurosci Lett 323:55–59.

Osuna C, Reiter RJ, García JJ, Karbownik M, Tan DX, Calvo JR, Manchester LC. 2002. Inhibitory effect of melatonin on homocysteine-induced lipid peroxidation in rat brain homogenates. Pharmacol Toxicol 90:32–37.

Outinen PA, Sood SK, Pfeifer SI, Pamidi S, Podor TJ, Li J, Weitz JI, Austin RC. 1999. Homocysteine-induced endoplasmic reticulum stress and growth arrest leads to specific changes in gene expression in human vascular endothelial cells. Blood 94:959–967.

Pablos MI, Agapito MT, Gutiérrez R, Recio JM, Reiter RJ, Barlow-Walden L, Acuña-Castroviejo D, Menéndez-Peláez A. 1995. Melatonin stimulates the activity of the detoxifying enzyme glutathione peroxidase in several tissues of chicks. J Pineal Res 19:111–115.

Perez-de-Arce K, Foncea R, Leighton F. 2005. Reactive oxygen species mediates homocysteine-induced mitochondrial biogenesis in human endothelial cells: Modulation by antioxidants. Biochem Bioph Res Co 338:1103–1109.

Perna AF, Ingrosso D, De Santo NG. 2003. Homocysteine and oxidative stress. Amino Acids 25:409–417.

Pryor WA, Squadrito GL. 1995. The chemistry of peroxynitrite: A product from the reaction of nitric oxide with superoxide. Am J Physiol 268: VIS (5 Pt 1):L699–L722.

Reiter RJ. 1998. Oxidative damage in the central nervous system: Protection by melatonin. Progress Neurobiol 56:359–384.

Reiter RJ. 2004. Mechanisms of cancer inhibition by melatonin. J Pineal Res 37:213–214.

Rodrigo R, Passalacqua W, Araya J, Orellana M, Rivera G. 2003. Homocysteine and essential hypertension. J Clin Pharmacol 43:1299–1306.

Rodríguez C, Mayo JC, Sáinz RM, Antolín I, Herrera F, Martín V, Reiter RJ. 2004. Regulation of antioxidant enzymes: A significant role for melatonin. J Pineal Res 36:1–9.

Sener G, Sehirli AÖ, Keyer-Uysal M, Arbak S, Ersoy Y, Yegen BÇ. 2002a. The protective effect of melatonin on renal ischemia-reperfusion injury in the rat. J Pineal Res 32:120–126.

Sener G, Sehirli AÖ, Altunbas HZ, Ersoy Y, Paskaloglu K, Arbak S, Ayanoglu-Dulger G. 2002b. Melatonin protects against gentamicin-induced nephrotoxicity in rats. J Pineal Res 32:231–236.

Sener G, Sehirli AÖ, Ayanoglu-Dülger G. 2003. Protective effects of melatonin vitamin E and N-acetylcysteine against acetaminophen toxicity in mice: A comparative study. J Pineal Res 35:61–68.

Sinet PM, Heikkila RE, Cohen G. 1980. Hydrogen peroxide production by rat brain *in vivo*. J Neurochem 34:1421–1428.

Stampfer MJ, Malinow MR, Willett WC, Newcomer LM, Upson B, Ullmann D, Tishler PV, Hennekens CH. 1992. A prospective study of plasma homocyst(e)ine and risk of myocardial infarction in US physicians. J Am Med Assoc 268:877–881.

Starkebaum G, Harlan JM. 1986. Endothelial cell injury due to copper-catalyzed hydrogen peroxide generation from homocysteine. J Clin Invest 77:1370–1376.

Tan DX, Manchester LC, Reiter RJ, Qi W, Hanes MA, Farley NJ. 1999. High physiological levels of melatonin in the bile of mammals. Life Sci 65:2523–2529.

Topal G, Brunet A, Millanvoye E, Boucher JL, Rendu F, Devynck MA, David-Dufilho M. 2004. Homocysteine induces oxidative stress by uncoupling of NO synthase activity through reduction of tetrahydrobiopterin. Free Radical Bio Med 36:1532–1541.

Upchurch GR, Welch GN, Fabian AJ, Freedman JE, Johnson JL, Keaney JF, Loscalzo J. 1997. Homocyst(e)ine decreases bioavailable nitric oxide by a mechanism involving glutathione peroxidase. J Biol Chem 272: 17012–17017.

Wakatsuki A, Okatani Y, Izumiya C, Ikenoue N. 1999. Melatonin protects against ischemia and reperfusion-induced oxidative lipid and DNA damage in fetal rat brain. J Pineal Res 26:147–152.

Zhang H, Squadrito GL, Pryor WA. 1998. The reaction of melatonin with peroxynitrite: Formation of melatonin radical cation and absence of stable nitrated products. Biochem Bioph Res Co 251:83–87.

Zhang H, Squadrito GL, Uppu R, Pryor WA. 1999. Reaction of peroxynitrite with melatonin: A mechanistic study. Chem Res Toxicol 12:526–534.

Zhang X, Li H, Jin H, Ebin Z, Brodsky S, Goligorsky MS. 2000. Effects of homocysteine on endothelial nitric oxide production. Am J Physiol 279:F671–F678.