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Age-related differences in the response of the brain to dietary melatonin

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Abstract The aged brain is prone to excessive levels of immune activity, not initiated by an acute response to an extrinsic agent. While dietary melatonin is reported to attenuate the extent of expression of proinflammatory genes, little is known about the extent to which these changes can be translated into altered levels of corresponding proteins. The baseline levels of the proinflammatory cytokines, tumor necrosis factor alpha (TNF-α) and interleukin-1 alpha, were greater in older (~29 months old) compared to younger (~7 months old) mouse brains. Acute (3 h) exposure to lipopolysaccharide (LPS) induced activation of nuclear factor kappa B (NF-κB), but not inflammatory cytokines in the brain. The serum level of TNF-α was increased after LPS injection, indicating a systemic immune response to the bacterial cell wall component. Dietary melatonin (40 ppm for 9.3 weeks) did not prevent LPS-induced changes in younger animals but caused an increased systemic TNF-α response in older mice. Melatonin did reduce markers of carbonyl formation in brain proteins of young animals and nitrosylative damage to peptide-bound amino acid residues, in the brains of older animals. Acute LPS challenge did not significantly affect these oxidative markers. Thus, despite lack of clear evidence of attenuation of the NF-κB–cytokine inflammatory trajectory within the CNS by melatonin, this agent did show a protective effect against free radical-initiated injury to amino acid residues within proteins. The results illustrate that previously reported changes in gene expression following melatonin treatment need not be closely paralleled by corresponding changes in protein content.

Keywords Melatonin · Aging · Inflammation · Cytokines · NF-κB · Oxidative stress

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Introduction

Melatonin can act as a modulator of immune responses. The expression of inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-1 (IL-1) is elevated with age and extended melatonin treatment can reverse this (Sharman et al. 2007). Overall, melatonin treatment of older animals is able to restore a more youthful profile of gene expression (Sharman et al. 2007). Melatonin levels go down with aging, and this may result in chronic elevations of expression of inflammatory genes (Lahiri et al. 2004;

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Bondy and Sharman 2010). Such elevated levels of expression, unprovoked by exogenous factors, may reflect the prior history of infections of an organism. Immune responses to such events may persist for an extended period after the disappearance of the original precipitating inflammatory stimulus, especially in the nervous system (Qin et al. 2007). There is a growing body of evidence that oxidative stress and inflammation increase with aging and that chronic treatment with melatonin is able to reduce these parameters (Tresguerres et al. 2012). Melatonin seems able to suppress several of the components of the inflammatory cascade initiated by lipopolysaccharide (LPS) (Tyagi et al. 2010; Shi et al. 2012). These reports suggest that levels of endogenous melatonin are decreased with age and that dietary supplementation with melatonin may reverse some of the adverse effects of senescence in cerebral tissue. We previously found that melatonin treatment of aged mice shifted the gene expression profile of immune-related mRNAs, in a direction reminiscent of the parallel profile in younger animals (Bondy and Sharman 2010). We have now focused on the effects of melatonin treatment of older animals on some of the corresponding proteins that may be consequent to such changes in gene expression, such as proinflammatory cytokines. Activity of a transcription factor (nuclear factor kappa B, NF-κB) relevant to initiation of the inflammatory cascade that may precede altered expression of genes relating to immune activation has been quantitated. Since inflammatory events generally involve a series of free radical-mediated processes, oxidative and nitrosylative damage to proteins was also examined. The hypothesis tested was that melatonin modulates protein levels of inflammatory and oxidative markers in a manner parallel to that which we observed in the genetic profile of young and old mice.

Materials and methods

Animal treatment

Male CB6F1 mice, a hybrid between C57BL/6JM and BALB/CJF from Harlan Labs (Indianapolis, IN), aged 4.5 months (young group) and 26.5 months (old group), were housed two to four per cage and were maintained on a 12-h light/dark cycle in a temperature-controlled (22± 1 °C) room. The CB6F1 hybrid was used in order to take advantage of both the genetic and phenotypic uniformity and the vigor (increased disease resistance, better survival under stress, and greater natural longevity) typical of hybrids while maintaining genetic similarity to the C57BL/6 mouse. Food and water were provided ad libitum. Control animals were fed with a pelleted minimal basal diet (AIN-93M, Dyets #100900, Dyets Inc., Bethlehem, PA) consisting of 10 % sucrose and 14 % casein (*w*/*w*) as well as a minimal salt and vitamin mix, for 2 months. The basal diet of melatonin-treated animals was supplemented with 40 ppm (*w*/*w*) melatonin (Sigma, St. Louis, MO) for 9.3 weeks. All experiments were approved by the Institutional Animal Care and Use Committee at the University of California, Irvine and conformed to the National Institute of Health guide for the care and use of laboratory animals. The final number of animals in each group was as follows: young control (n=12), young melatonin (n=13), old control (n=11), and old melatonin (n=13). Three hours prior to sacrifice, a cohort of animals of each group of mice was injected intraperitoneally with 100 μl of 300 μg/ml of *E. coli* LPS (Sigma L4005). This is approximately 1 mg/kg body weight. The control animals received 100 μl of 0.9 % saline.

Preparation of samples

Cytoplasmic and nuclear fractions were prepared using a previously published method (Lahiri and Ge 2000). The brain tissue from each animal was weighed and homogenized in 2 ml of an ice-cold buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, and 0.5 % NP-40). The samples were incubated for 10 min and centrifuged (1,500×*g*) at 4 °C for 1 min. The supernatant containing the cytoplasmic constituents was collected, and a protease inhibitor cocktail was added. The samples were aliquoted and stored at −80 °C. The nuclear pellet was resuspended in 200 μl of a buffer composed of 20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM DTT, 1 mM EDTA, 1 mM EGTA, and 1 mM PMSF. The samples were then centrifuged at 11,000×*g* for 5 min at 4 °C. The supernatant (nuclear extract) was aliquoted and stored at −80 °C. Blood was collected and centrifuged at 5,000 rpm for 10 min.

Electrophoretic mobility shift assay

The extent of NF-κB activation was determined in the nuclear fraction of brain tissue using a protocol developed by Promega (Madison, WI). The amount of protein in 5 μl of the nuclear extract was determined by the BCA

protein assay method (Pierce, Rockford, IL) and 50 μg of each sample, incubated with ^{32}P-labeled oligonucleotides containing the NF-κB consensus sequence, was loaded onto a gel. A negative control containing no cell extract, as well as competitor reactions, was included. The specific competitor contained unlabeled NF-κB consensus nucleotide while the nonspecific competitor contained unlabeled SP-1 consensus oligonucleotide. The competitor reactions also contained 50 μg of nuclear fraction derived from control mouse brains. X-ray films were manually developed and the intensity of each band was measured and quantitated using an image analyzer.

Competitive enzyme immunoassay

Brain levels of TNF-α and IL-1 alpha (IL-1α) were determined using competitive enzyme immunoassay kits from Neogen Corp. (Lexington, KY); 100 μl of the brain sample was used. Antibody was added to plates precoated with goat anti-rabbit antibody and incubated at room temperature for 3 h. After the addition of murine cytokine conjugate, the plate was incubated further for 30 min. The plate was washed and incubated for 30 min with streptavidin–alkaline phosphatase. After another wash, 200 μl of a color reagent solution was added to the plate and the color generated was determined with a spectrophotometric plate reader at 492 nm.

ELISA

The blood level of TNF-α was determined in the serum using a sandwich enzyme immunoassay kit (Neogen Corp., Lexington, KY), following the protocol provided by the manufacturer.

Protein nitrotyrosine residues

The nitrotyrosine enzyme immunoassay from OxisResearch (Portland, OR) was used to determine protein nitrotyrosine levels in the cytoplasmic fractions. This kit utilizes a monoclonal antibody specific for nitrotyrosine residues and a biotinylated goat polyclonal anti-nitrotyrosine antibody. Streptavidin peroxidase conjugate binds to the biotinylated antibody, and tetramethylbenzidine substrate is added to produce a yellow product that is measured at 450 nm.

Protein carbonyl levels

Carbonyl residues within proteins in the cytoplasmic fractions were assayed using an enzyme immunoassay kit from ZenTech Corp. (Dunedin, New Zealand). Samples were reacted with dinitrophenylhydrazine (DNP) and then adsorbed to an ELISA plate. The levels of protein carbonyl were determined using a biotinylated anti-DNP antibody followed by streptavidin peroxidase conjugate. Absorbance was measured at 450 nm.

Statistical analysis

ANOVA statistical analysis was used to assess the significance among the animal groups. The P values that are indicated on the graphs are derived from the Student's t test: two-sample assuming equal variances. $P \leq 0.05$ was accepted as significant.

Results

NF-κB activation

The activation of NF-κB was enhanced 3 h after an i.p. injection of LPS in older but not in young animals. Pretreatment for the preceding 2 months with dietary melatonin did not alter this response of older animals to LPS. However, in younger animals, melatonin treatment did lead to establishment of LPS-induced NF-κB activation in the brain (Fig. 1).

Proinflammatory cytokines

The baseline cortical content of two cytokines associated with inflammatory events, TNF-α and IL-1α, was higher in older than in young animals and melatonin treatment had no significant effect on this increase. In younger animals, melatonin treatment increased the basal level of IL-1α (Fig. 2). In the serum, LPS treatment caused an increase in the levels of TNF-α and the effect was more pronounced in old animals treated with melatonin (Fig. 3).

End-products of oxidative damage to proteins

Two parameters of oxidative damage to cortical proteins were evaluated, namely, the extent of protein carbonylation and of nitrosylation of tyrosine residues

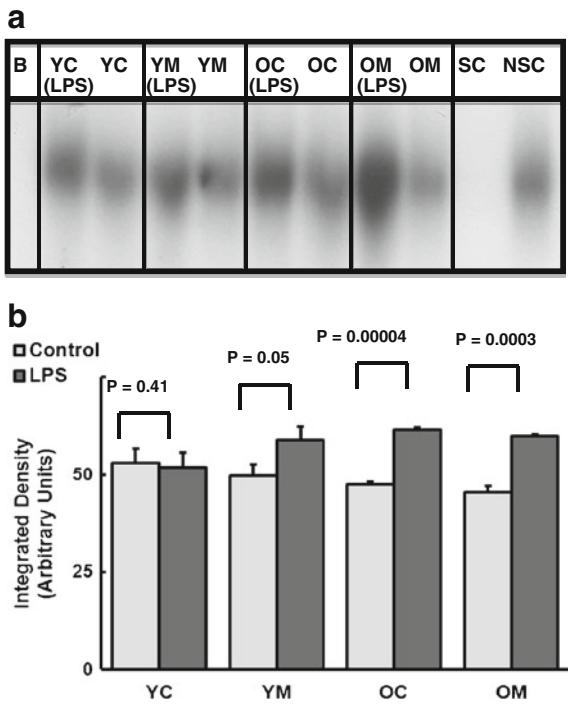

Fig. 1 Activation levels of transcription factor NF-κB in the brain. **a** A typical gel shift showing levels of activated NF-κB. **b** Integrated density of the shifted band for NF-κB (n=3). *B* blank, *YC* young control, *YM* young melatonin, *OC* old control, *OM* old melatonin, *SC* specific competitor present, *NSC* nonspecific competitor present

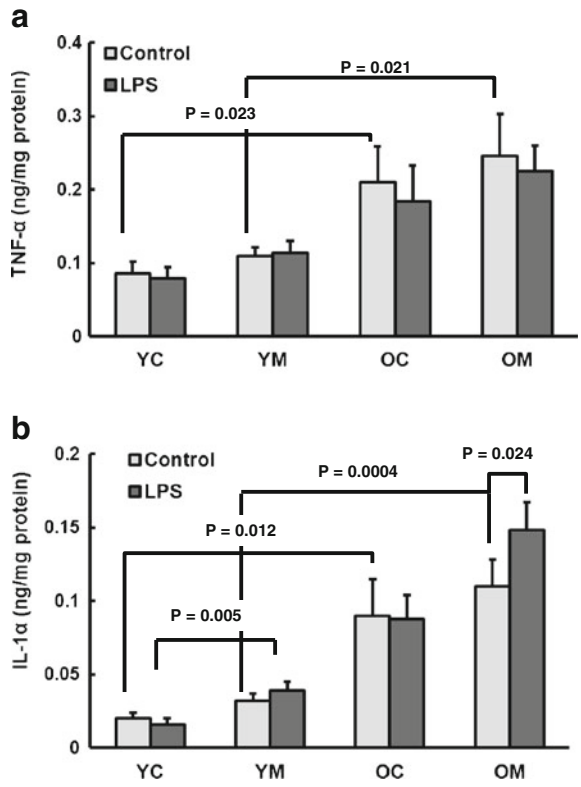

Fig. 2 Brain levels of **a** TNF-α and **b** IL-1α after saline or LPS injection. *Bars* represent mean of five to seven individual determinations±SE. *YC* young control, *YM* young melatonin, *OC* old control, *OM* old melatonin

within peptide chains. No significant age-related changes could be found in either one of the indices assessed. Melatonin pretreatment significantly diminished protein carbonyl levels in young but not in old animals. Conversely, protein nitrotyrosine levels were only decreased in old melatonin-treated animals (Fig. 4).

Discussion

While the plasma and brain content of melatonin was not assayed in this group of animals, we have previously performed such an analysis on a set of mice of the same strain and of parallel ages, receiving an identical dietary protocol. In untreated mice, basal levels of melatonin were 0.9±0.1 pg/g tissue in 6 month-old mice and reduced to 0.3±0.05 pg/g tissue in 27 month-old animals. Treatment with melatonin increased the levels of melatonin to 5.2±1.8 and 3.2±1.1 pg/g in younger and older animals, respectively (Lahiri et al. 2004). The serum concentrations of melatonin in these animals were much higher in the younger than the older group of mice (76.6±9.5 pg/ml serum for the younger group vs 16.7±1.5 pg/ml for the older group). Melatonin treatment elevated these values to 666±137 and 318±10 pg/ml serum, respectively. Thus, a significant fraction of dietary melatonin was able to traverse out of the digestive tract and into the serum while a lesser amount crossed directly into the brain. The ELISA method used for the assay allowed unequivocal determination that this melatonin was in an intact and unconjugated form.

A response of NF-κB activation to LPS was strong in aged mice but was not detected in younger mice not treated with melatonin. This may be because older animals are more sensitive to inflammatory stimuli (Escames et al. 2006) or because the kinetics of NF-κB activation is more rapid in older animals (Brink et al. 2009). Prolonged treatment with melatonin did change the responsiveness of younger animals to the LPS challenge, and this may be due to the

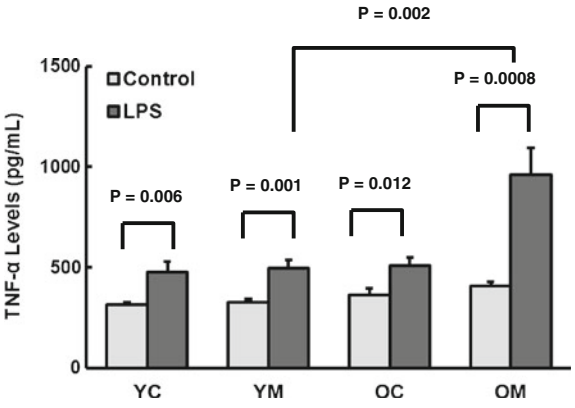

Fig. 3 Levels of TNF-α in the serum of animals injected with saline or LPS. *Bars* represent mean of five to seven individual determinations±SE. *YC* young control, *YM* young melatonin, *OC* old control, *OM* old melatonin

immune modulatory effect of the molecule (Hardeland et al. 2011). The immune modulatory role of melatonin is further supported by the observation that in young animals, IL-1α was increased after LPS challenge only in the brain of the melatonin-treated group. Furthermore, in older animals, IL-1α levels were increased after LPS challenge only if animals had been pretreated with melatonin (Fig. 2b).

The finding that TNF-α and IL-1α were elevated in older animals parallels a similar increase in the mRNA levels of these two proinflammatory cytokines. However, the absence of an attenuation of this age-related increase in melatonin-treated animals is in contrast to their reduced expression following melatonin administration (Sharman et al. 2002). This discrepancy between gene expression and content of the corresponding protein reflects the complex factors determining the rate of translation.

The failure of brain cytokine proteins to increase 3 h after LPS injection, a time when their mRNAs in young animals are greatly enhanced (Sharman et al. 2002), could be due to temporal factors, whereby detectable elevations in proteins may take longer than the peaking of mRNA levels. In the peripheral circulation, we did note an increase in the levels of TNF-α production after LPS injection, but the systemic increase did not parallel a similar response in the brain.

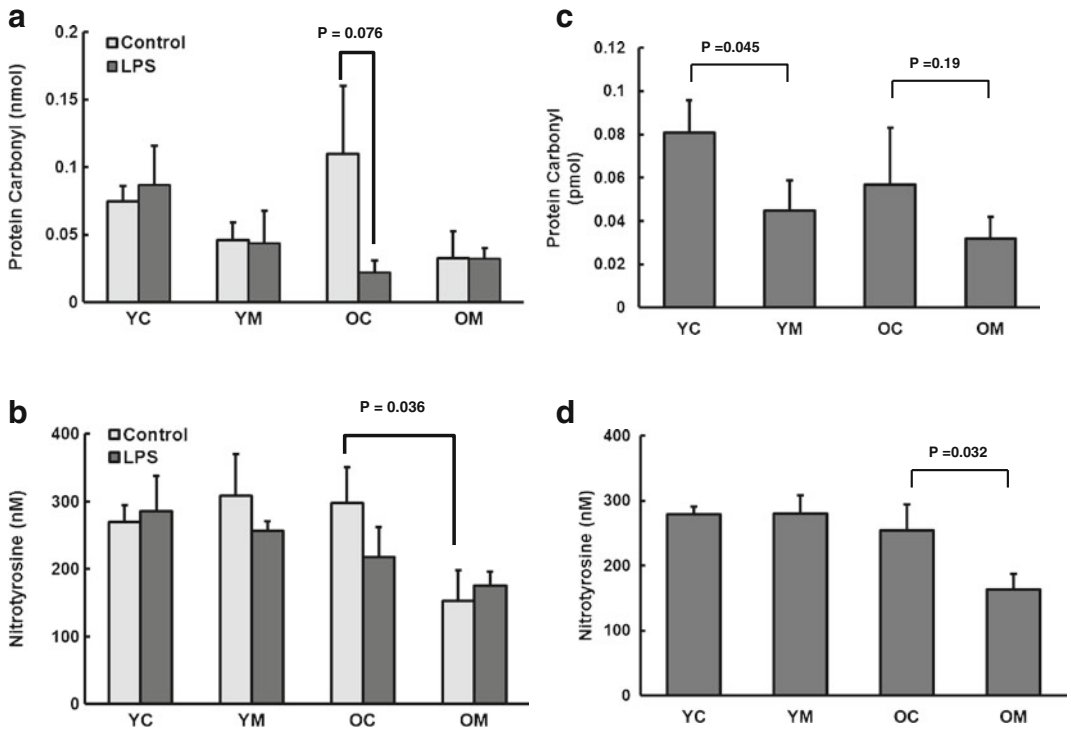

Fig. 4 Measure of oxidative stress in the brain of mice exposed to melatonin. Levels of **a** protein carbonyl and **b** nitrotyrosine were determined in saline-treated or LPS-treated animals (n=4–6). Levels of **c** protein carbonyl and **d** nitrotyrosine are shown after the saline- and LPS-treated animals are grouped together to better illustrate the age-related changes (n=9–11). *YC* young control, *YM* young melatonin, *OC* old control, *OM* old melatonin

This is in contrast to the finding of Qin et al. (2007), which found an increase in the brain and systemic levels of TNF-α as early as 1 h postintraperitoneal injection of LPS. The difference in finding may be due to the dose of LPS used, since these authors used 5 mg/kg body weight while we used only 1 mg/kg. We also found a significant increase in the levels of plasma TNF-α after LPS challenge in older melatonin-treated animals. Melatonin was not solely responsible for this effect since the younger animals treated with the methoxyindole did not show an altered systemic increase in the proinflammatory cytokine. The combination of aging and melatonin treatment was necessary for this observation. Reports have consistently shown an attenuation of TNF-α levels in melatonin-treated animals (Sacco et al. 1998; Baykal et al. 2000; Xu et al. 2007). However, in Wistar rats acutely infected with a parasite, melatonin induced upregulation of the cytokine (Santello et al. 2008). Thus, the age, health status, species of the animal model, concentration of LPS used, and the duration of melatonin treatment may all be factors determining the direction of cytokine modulation in the blood. Whether the increase in TNF-α in the older melatonin-treated animals is protective or harmful is currently unknown and requires further investigation.

Melatonin appeared to be protective of carbonyl formation in younger animals and nitrosylative damage to proteins in older animals. This finding is in agreement with the published literature (Akbulut et al. 2008; Garcia-Macia et al. 2010; Dkhar and Sharma 2011). This is unlikely to be due to a direct antioxidant property as intracellular content of melatonin is very low compared to glutathione or α-tocopherol (Lahiri et al. 2004; Kornbrust and Mavis 1980). More likely, the protective effect reflects modulation of genetic regulatory processes (Bondy and Sharman 2010) or indirect antioxidant effects (Reiter et al. 2001). LPS treatment 3 h before sacrifice did not affect the indices of oxidative or nitrosylative damage to proteins. However, we did observe a nonsignificant decrease in protein carbonyl levels after LPS challenge in the old control group (Fig. 4a), which may be a consequence of age-related alterations in the immune/oxidative pathway cross talk. Melatonin treatment in either the young or older animals did not significantly alter oxidative markers after the LPS challenge. This is not surprising since these measures indicate progressive overall damage to proteins, which take an extended period to develop. Thus, these indices would not be expected to be rapidly responsive to acute inflammation.

Melatonin was clearly protective against extended age-related accumulation of nitrosylative stress-induced damage to proteins. There was however no evidence of the ability of melatonin to suppress acute inflammatory events. Melatonin has been found to improve survival in mice treated with an acute lethal dose of LPS (Wu et al. 2001), and the molecule can block many of the rapidly induced inflammatory effects of LPS (Nava et al. 1997; Raghavendra et al. 2000). Much of the literature reporting a protective effect of melatonin on inflammatory responses has relied on quantitative gene expression (Sharman et al. 2008; Naidu et al. 2010). There are, however, reports on melatonin-induced reduction of cytokine levels (Xu et al. 2007; Tyagi et al. 2010). In the present study, other than a systemic increase in LPS-induced TNF-α production in melatonin-treated old animals, we did not observe melatonin-induced alteration of inflammatory indices. Melatonin does not behave as a broad-spectrum immunosuppressant but rather may act as a more complex modulator of immune activity. The overall interpretation of a range of studies on melatonin is that it functions to draw the senescent brain into a response profile more closely resembling that of the younger brain that can both involve suppression or activation of components of immune function (Bondy and Sharman 2010). Thus, an intricate and not always consistent response to an inflammatory stimulus is not surprising.

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