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Melatonin Causes Gene Expression in Aged Animals to Respond to Inflammatory Stimuli in a Manner Differing from that of Young Animals

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Abstract: Groups of younger and aged mice were fed either minimal basal diet or the same diet containing 40 ppm melatonin. After 9.3 weeks half of each of these 4 groups of animals received either an intraperitoneal injection of lipopolysaccharide (LPS) or of saline. Three hours after this treatment, all animals were killed and mRNA from brains extracted. Quantitative PCR was performed on 13 selected mRNA species reflecting various aspects of the inflammatory pathway, the melatonin receptor, and a key glycolytic enzyme. An overall trend observed was that the effect of melatonin in modulating LPS-provoked immune responses differed markedly in old and young animals. Melatonin tended to enhance the reaction of younger animals to LPS but suppressed the inflammatory response of older mice. This difference with aging suggests that key immune processes are markedly altered by aging. It is likely that the ability of the immune system to mount a defense is impaired in older animals.

Keywords: Aging, brain, melatonin, gene expression.

INTRODUCTION

The expression of genes is known to be altered during senescence and, such changes may relate to the gradual decline of organ function that characterizes aging [1]. In the brain these changes are accompanied by an altered response of gene expression, to exogenous stimuli. This is true both of adverse challenges originating systemically and of the ability to react to potentially beneficial agents in the circulation [2]. We have previously reported that lipopolysaccharide provokes a greater elevation of expression of cortical genes related to immune function of old mice than in young. This difference can be prevented by extended dietary treatment of old animals with melatonin [3, 4, 5]. The current study further examines the responses of a wider range of genes to an inflammatory stimulus. The effects of dietary melatonin (40 ppm for 2.1 months) on gene expression in young and old B6C3F1 male mice were compared. Expression in selected genes was assayed by quantitative reverse transcription polymerase chain reaction (qRT-PCR). The roles of selected genes are listed in Table 1.

Genes of young untreated animals exhibited an increased expression following provocation of systemic inflammation with the inflammogen lipolysaccharide (LPS). This was also generally the case with old animals. In nearly all instances studied, melatonin-induced modifications of responses to LPS were opposite in young mice (4.5 months-old) than in aged mice (26.5 months-old). This difference typically involved melatonin effecting attenuation of expression in old animals treated with LPS, while in young animals, melatonin treatment led to unchanged or enhanced responses to LPS.

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METHODS

Animal Treatment

Male B6C3F1 mice, a hybrid between C57BL/6 and C3H from Harlan Labs (Indianapolis, IN), aged 5.5 months (young group) and 23.4 months (old group), were housed two to four per cage and were maintained on a 12 hour light/dark cycle in a temperature controlled (22±1 °C) room. The B6C3F1 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 published C57BL/6 mouse genome sequence [6]. Food and water were provided *ad libitum*.

Young (YC) and old (OC) control animals were fed 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.

The basal diet of two similarly aged cohorts (YM and OM, respectively) 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. Three hours prior to sacrifice, half of each group of mice was injected intraperitoneally with 100 µl of 100µg/ml of *E. coli* lipopolysaccharide (LPS, Sigma L4005), the other half with 100 µl of 0.9% saline.

RNA Extraction and Purification

Three hours after injection with LPS or saline, all mice were killed by cervical dislocation and visually inspected for signs of disease or other pathology; brain tissues were ex-

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Table 1. Genes Selected for Detailed Analysis

Gene for	Function of Protein
TLR2	Cell surface receptors responding to exogenous inflammatory signals
TLR4	
MD2	Regulatory component of TLR4
cJun	Transcription factor activated by JNK; component of inflammatory signaling pathway
TNFα	Inflammatory cytokines
IL6	
iNOS	Inflammatory effector
GFAP	Indicator of astroglial activation
CD11b	Indicator of microglial activation
S100A8	Pro-inflammatory chemokines
Cxcl1	
PDK4	Stress-activated inhibitor of glycolytic entry enzyme pyruvate deh.
MtnR1a	Melatonin receptor

cised quickly, immediately frozen on dry ice and stored at -70 °C. Cerebellum and brain stem were removed; total RNA was extracted from the remaining tissue using the TRI Reagent kit (Molecular Research Center Inc., Cincinnati, OH), following the manufacturer's protocol. Aliquots of the total RNA were further purified on an RNeasy column (Qiagen Inc., Valencia, CA) to yield a 260 nm to 280 nm absorbance ratio of ≥ 1.9. RNA concentrations were determined by absorption at 260 nm.

Quantitative Real-Time RT-PCR

Quantitative (real-time) polymerase chain reaction (qRT-PCR) analysis was used to measure the expression levels of selected mRNA transcripts and for β-actin. Reactions were carried out on a LightCycler instrument (Roche Diagnostics, Indianapolis, IN) using the QuantiTect SYBR Green RT-PCR reagent kit (Qiagen, Valencia, CA) according to the manufacturers' directions. Product fluorescence was detected at the end of the elongation cycle at 72 °C. Melting curves all exhibited a single peak at a temperature characteristic of the primer pair used and none of the primer pairs produced amplicons in the absence of sample or reverse transcriptase. All samples in each run were adjusted so that each sample contained the same mRNA concentration. Further adjustment for loading variations was counterproductive: computing expression levels relative to β-actin did not affect the shape of any treatment-associated expression pattern, but only degraded the results by introducing additional experimental error into the expression measurements. Expression levels for each sample were normalized to values of the corresponding untreated young control group, and were calculated as the average of duplicate determinations on 3 animals.

Statistical Analysis

Differences between the eight treatment groups were assessed by regression-modeling equivalent to three-way Analysis of Variance; where appropriate, this was confirmed by Gabriel's multiple comparisons procedure test as computed using the Clinstat program [7]. The data were log-transformed where appropriate to assure homoscedasticity before applying significance tests. In all cases, the acceptance level of significance was $p<0.05$ using a two-tailed distribution.

RESULTS

Both the basal levels and the magnitude of the response to LPS of many of the genes assayed, were elevated in aged, unsupplemented animals. These included S100A8, c-Jun, TLR2, MD2, PDK4, Cxcl1 and GFAP, Fig. (1). In another set of genes (CD11b and TLR4), the basal levels were elevated with age while the response to LPS was unaltered or diminished, Fig. (2). In the case of a third set of genes (iNOS, IL-6, TNFα) basal levels of expression were unaltered with age, but their response to LPS was enhanced, Fig. (3).

Melatonin treatment of aged animals depressed the response of several genes to LPS at 3 hours post-injection. The genes that exhibited such an attenuation included those directly related to inflammation such as mRNA for IL-6, iNOS and TNFα, Fig. (3).

Genes associated with the inflammation signal transduction pathway also expressed a diminished reaction to LPS in melatonin-treated aged animals. These included mRNAs for the cell surface receptor TLR-2 and for c-Jun, a component of the transcription factor AP-1 and a protein that is activated by JNK (JUN-N-terminal kinase). A similar trend was found for expression of the chemokines S100A8 and Cxcl1 (Fig. (1)). LPS-induced expression of the genes for GFAP, (associated with activation of astroglia) and for CD11b (indicative of activated microglia), were also reduced by melatonin although this was non-significant in the case of GFAP, Fig. (1).

Unexpectedly, the LPS-induced responses of most of these genes in the young animal were consistently shifted in

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Fig. (1). RT-PCR analysis of cortical levels of mRNA for S100A8, c-jun, TLR2. MD2, PDK4, GFAP, and Cxcl1 in 4.5 and 26.5 month-old mice. One subset of each group received 40 ppm dietary melatonin for the preceding 2.1 months. YC = young control, YM = Young melatonin, OC = old control, OM = old melatonin. Relative expression of genes are compared, without (open bars) or with (filled bars) LPS challenge. Each value is the mean of the fold-change ± SE of values from 3 individual mice with respect to the young control value = 100 %. Cxcl1 is expressed logarithmically in view of the wide range of expression values. Differences between means marked with different letters are statistically significant ($p<0.05$).

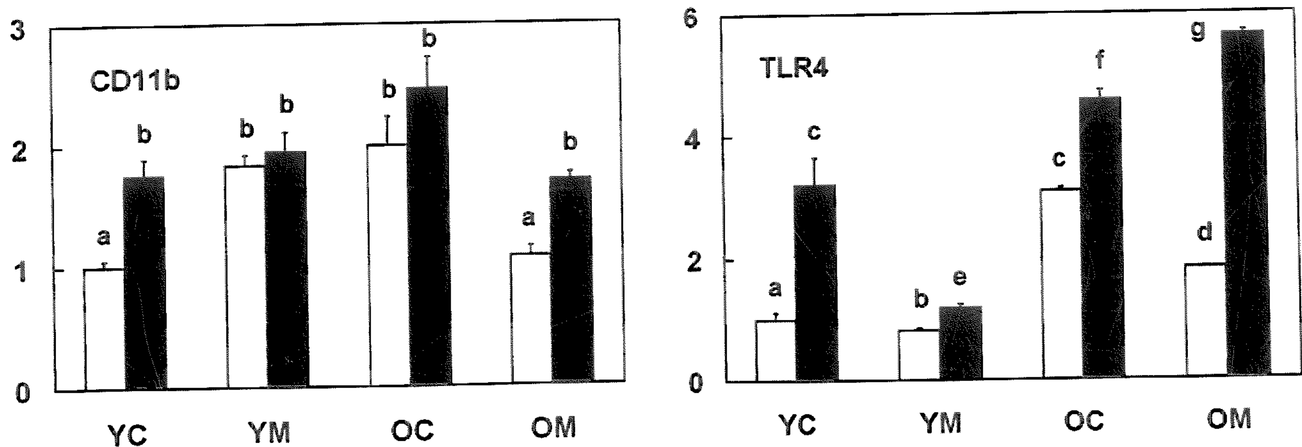

Fig. (2). RT-PCR analysis of cortical levels of mRNA for CD11b and TLR4 in 4.5 and 26.5 month-old mice. One subset of each group received 40 ppm dietary melatonin for the preceding 2.1 months. YC = young control, YM = Young melatonin, OC = old control, OM = old melatonin. Relative expression of genes are compared, without (open bars) or with (filled bars) LPS challenge. Each value is the mean of the fold-change (± SE) of values from 3 individual mice with respect to the young control value = 100 %. Differences between means marked with different letters are statistically significant ($p<0.05$).

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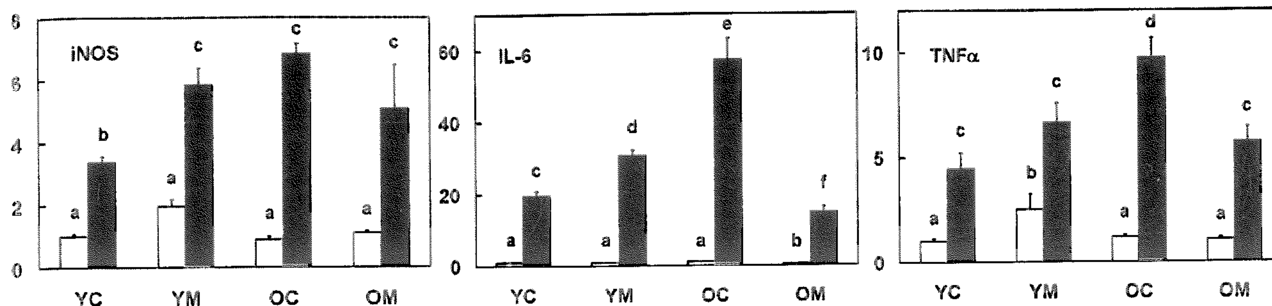

Fig. (3). RT-PCR analysis of cortical levels of mRNA for iNOS, TNFα and IL6 in 4.5 and 26.5 month-old mice. One subset of each group received 40 ppm dietary melatonin for the preceding 2.1 months. YC = young control, YM = Young melatonin, OC = old control, OM = old melatonin. Relative expression of genes are compared, without (open bars) or with (filled bars) LPS challenge. Each value is the mean of the fold-change ± SE of values from 3 individual mice with respect to the young control value = 100 %. Differences between means marked with different letters are statistically significant ($p<0.05$).

the opposite direction by prior exposure to dietary melatonin. The effect of melatonin in this case was generally to enhance the reaction to an LPS challenge. Thus there was a tendency of melatonin to diminish LPS responses in aged mice while markedly enhancing them in young mice. This was found for c-Jun, S100A8, iNOS, IL-6, and Cxcl1, while a parallel non-significant trend was exhibited by GFAP and TNFα, Figs. (1 and 3). Basal levels of expression of TLR4 (a component of the cell surface receptor initiating responses to LPS) were elevated with age and this change was depressed by melatonin treatment. The enhanced expression of TLR4 effected by LPS was diminished in young animals previously treated with melatonin while being enhanced in older animals, Fig. (2). LPS-induced augmentation of expression of MD2 (a component of the active LPS receptor site) was further increased by melatonin in animals of either age, although the increase in old animals was not significant, Fig. (1).

These changes are summarized in Table **2**.

Pyruvate dehydrogenase kinase isoenzyme 4 (PDK4) phosphorylates and inactivates pyruvate dehydrogenase and thus minimizes carbohydrate oxidation by preventing the flow of glycolytic products into the TCA cycle. In this manner it can be important in mounting effective stress responses. Basal expression of mRNA for PDK4 was higher in old than in younger mice and was reduced by melatonin in old animals, Fig. (1).

The basal level of the gene for the melatonin 1a receptor, MtnR1a, was elevated by melatonin in young mice but unchanged in old mice. LPS combined with melatonin treatment resulted in receptor down regulation in young but not old animals, Fig. (4). The large but disparate response of this gene to a short exposure to LPS suggests that the receptor exists in a dynamic state.

DISCUSSION

We have found previously that basal plasma levels of melatonin have been found to be 50±4 pg/ml for young and 10±2 pg/ml for old mice while levels resulting from this dosing regimen are 385±88 and 205±25 pg/ml for young and old

Table 2. Summary of Alterations in Gene Expression Related to Age and Melatonin Treatment

<table>
<tr><th rowspan="2">Gene</th><th colspan="2">Effect of Age on Untreated Animals</th><th rowspan="2">Effect of Melatonin Treatment on Response of Young to LPS</th><th rowspan="2">Effect of Melatonin Treatment on Response of Aged Animals to LPS*</th></tr>
<tr><th>Basal Levels</th><th>LPS Response</th></tr>
<tr><td>PDK4</td><td rowspan="7">↑Elevated</td><td>↑Elevated</td><td>Unaffected</td><td rowspan="2">Unaffected</td></tr>
<tr><td>MD2</td><td>Unaltered</td><td rowspan="5">↑Enhanced</td></tr>
<tr><td>cJun</td><td>↑Elevated</td><td rowspan="9">↓Attenuated</td></tr>
<tr><td>TLR2</td><td>↑Elevated</td></tr>
<tr><td>Cxcl1</td><td>↑Elevated</td></tr>
<tr><td>S100A8</td><td>↑Elevated</td></tr>
<tr><td>GFAP</td><td>Unaltered</td><td rowspan="2">Unaffected</td></tr>
<tr><td>iNOS</td><td rowspan="3">Unaffected</td><td rowspan="3">↑Elevated</td></tr>
<tr><td>TNFα</td><td rowspan="2">↑Enhanced</td></tr>
<tr><td>IL6</td></tr>
<tr><td>CD11b</td><td rowspan="2">↑Elevated</td><td>Unaltered</td><td rowspan="2">↓Attenuated</td></tr>
<tr><td>TLR4</td><td>↓Decreased</td><td>↑Enhanced</td></tr>
</table>

*Nonsignificant trend toward attenuation: Cxcl1, S100A8, GFAP, and iNOS.

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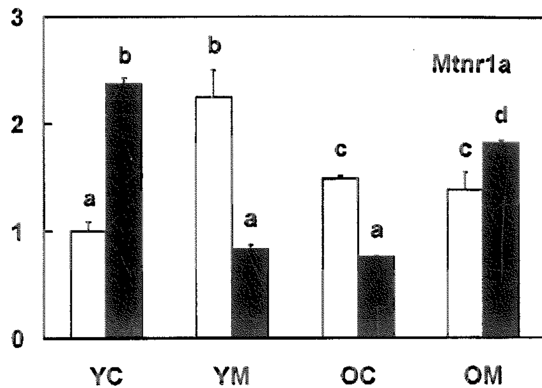

Fig. (4). RT-PCR analysis of cortical levels of mRNA of the melatonin receptor MtnR1a in 4.5 and 26.5 month-old mice. One subset of each group received 40 ppm dietary melatonin for the preceding 2.1 months. YC = young control, YM = young melatonin, OC = old control, OM = old melatonin. Relative expression is compared, without (open bars) or with (filled bars) LPS challenge. Each value is the mean of the fold-change ± SE of values from 3 individual mice with respect to the young control value = 100 %. Differences between means marked with different letters are statistically significant ($p<0.05$).

animals respectively. Cortical levels are also increased by the melatonin diet, from 1.2±0.3 to 4.1±0.8 pg/g tissue in the case of young mice and from 0.3±0.1 to 3.2±0.9 pg/g tissue in the case of old mice [8]. Thus basal melatonin levels decrease markedly with age and dietary melatonin can significantly increase brain levels of the native unconjugated compound within the brain as well as in plasma.

TLR2 plays a major role in the recognition of a broad range of cell wall components from gram positive bacteria including peptidoglycans, lipoproteins and the glucan zymosan [9, 10], as well as lipopeptide impurities commonly present in LPS derived from bacteria [11]. Such impurities may account for TLR2 being responsive to LPS [12]. TLR4 predominately recognizes materials such as lipopolysaccharides from gram negative bacteria [13]. The LPS-induced increase in expression of the mRNA for the TLR2 receptor of younger mice was modest and non-significant, but in aged mice TLR2 mRNA was greatly elevated by LPS, Fig. (**1**). Since TLR2 normally responds less than TLR4 to LPS — as might be anticipated by the relatively small amounts of lipopeptide present [14], this may represent an aberrant reaction and this could underlie the higher basal level and/or increased reaction to LPS of many components of the inflammatory cascade in aged animals. These include expression of iNOS, c-Jun, IL-6, and TNFα.

TLR4, which plays a key role in innate immunity, is known to respond strongly to LPS and its mRNA is increased over 300% in young animals, Fig. (**2**). In untreated older animals, basal TLR4 gene expression was much higher than in young animals but the response to LPS was significantly decreased, Fig. (**2**). The effect of melatonin treatment of aged mice was to increase the signal-noise ratio by depressing basal levels and elevating LPS-induced stimulation of TLR4 mRNA, Fig (**2**). However, this ratio is actually depressed in melatonin-treated young animals. Once again, the effect of melatonin on adult animals is the opposite of that on aged mice.

In the current study a melatonin-effected reduction of mRNAs for TNFα, IL-6 and iNOS in response to LPS was confined to brain of aged mice. This effect is likely to be widespread since melatonin has been found to inhibit the LPS-induced elevation of plasma levels of these proteins in mice [15].

The melatonin-induced suppression of many responses to LPS in old animals and elevation in young animals leads to the response of animals of either age to more closely resemble one another. These opposing effects imply the existence of differing mechanisms for regulation of immune processes in old as opposed to adult animals in mid-life and this is likely to represent a decline in optimal functionality.

S100A8 expression was greatly increased by LPS in aged animals; melatonin demonstrated a tendency to attenuate this increase. S100A8 and S100A9 are likely to mediate between activation of TLR and MAP kinases and NFκB thus leading to increased neuroinflammation [16]. Thus S100A8 may play a key role in the transmission of inflammatory stimuli.

PDK4 is responsible for inactivation of the pyruvate dehydrogenase complex when glucose availability is scarce in the body, and is selectively upregulated in most tissues in response to starvation and hormonal imbalances [17, 18]. This may account for both the higher basal levels of this gene in aged brain, and for the several-fold increase in expression levels of this gene effected by LPS. LPS has a parallel effect on expression of this gene in muscle tissue [19]. Overexpression of the PDK4 variant of pyruvate dehydrogenase kinase promotes a loss of metabolic flexibility that exacerbates cardiomyopathy caused by the calcineurin stress-activated pathway. This up-regulation is likely to be cytokine mediated [20].

In a preliminary attempt to understand the transcriptional regulatory mechanisms responsible for the observed LPS-related expression patterns, transcription factor binding sites unique to the promoter regions of all genes belonging to each of two contrasting expression patterns were identified. The first pattern was expressed in 5 genes displaying an increased response to LPS in old mice that was reduced in them by melatonin treatment (ID genes): IL-6, Jun, TLR2, and trends in S100A8 and TNFα. This melatonin-induced suppression of inflammatory genes responsive to LPS may relate to the protective effect of melatonin against organ failure caused by LPS administration [21]. A second contrasting pattern was identified in 2 genes that expressed a decreased response to LPS in old mice that was increased by melatonin treatment (DI genes): TLR4 and CD11b. Promoter sequences for all the above genes were identified by querying http://grid.abcc.ncifcrf.gov/promoters/promoterInfo.php.
These sequences were sorted to determine which are present in the promoter regions of all 5 ID genes, but not in either of the DI genes. There are 6 such sequences which can bind with the following transcription factors: AP-2, E2A_CS, E2A_consensus, Pur1, Sp1, and one unknown factor. Given melatonin's role in circadian processes, it may be significant

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that two of these factors, AP-2 and SP1, are associated not only with LPS-responsive genes [22], but with circadian clock-controlled genes as well [23].

The finding that the ligand for MtnR1a further induced expression of this receptor in young animals is unusual since down-regulation is a more common receptor response to elevated ligand levels. There are other examples of ligand induced up-regulation, notably that of nicotine which may account for the addictive nature of this compound [24, 25]. However, it is noteworthy that melatonin has analgesic and anxiolytic properties but appears to be devoid of addictive liability [26].

Melatonin has often been reported to improve some of the adverse events associated with brain aging [2]. However, it has also been reported to be neuroprotective in young animals [27]. This may be especially true of the immune response. The toxicity of LPS is reduced in young mice pretreated with melatonin [28]. Melatonin can even protect against LPS-induced uterine death [29]. Since both the basal levels and response to LPS are generally higher in aged animals, the general tendency of melatonin to attenuate immune activity in older animals is likely to be beneficial by enabling appropriate adjustment of the magnitude of the inflammatory response.

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