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RESEARCH PAPER

Melatonin modulates microsomal PGE synthase 1 and NF-E2-related factor-2-regulated antioxidant enzyme expression in LPS-induced murine peritoneal macrophages

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BACKGROUND AND PURPOSE

Increasing evidence demonstrates that melatonin regulates inflammatory and immune processes acting as both an activator and inhibitor of these responses. Nevertheless, the molecular mechanisms of its anti-inflammatory action remain unclear. Here we have characterized the cellular mechanisms underlying the redox modulation of LPS-stimulated inflammatory responses in murine peritoneal macrophages by melatonin to provide insight into its anti-inflammatory effects.

EXPERIMENTAL APPROACH

Murine peritoneal macrophages were isolated and treated with melatonin in the presence or absence of LPS (5 μg·mL^{-1}) for 18 h. Cell viability was determined using sulforhodamine B assay and NO production was measured using the Griess reaction. Pro-inflammatory enzymes and transcription factors were detected by Western blotting.

KEY RESULTS

Without affecting cell viability, melatonin (12.5, 25, 50 and 100 μM) reduced the level of nitrites, inducible NOS (iNOS), COX-2 and microsomal PGE synthase-1 (mPGES1) protein, and p38 MAPK phosphorylation, and prevented NF-κB translocation. Furthermore, melatonin treatment significantly increased NF-E2-related factor 2 (Nrf2) and haem oxygenase 1 (HO1) protein levels in murine macrophages exposed to LPS.

CONCLUSIONS AND IMPLICATIONS

Melatonin reduced pro-inflammatory mediators and enhanced the expression of HO1 via NF-κB, p38 MAPK and Nrf2 cascade signalling pathways in murine macrophages. Thus, melatonin might be a promising target for diseases associated with overactivation of macrophages.

Abbreviations

HO1, haem oxygenase 1; iNOS, inducible NOS; mPGES1, microsomal PGE synthase-1; Nrf2, NF-E2-related factor-2; ROS, reactive oxygen species; SOD, superoxide dismutase; SRB, sulforhodamine B

Introduction

Melatonin (N-acetyl-5-methoxytryptamine), is a neurohormone synthesized from the aromatic amino acid tryptophan mainly by the pineal gland of mammals (Reiter, 1991) and other extrapineal organs and tissues including skin (Slominski *et al.*, 2002), retina (Faillace *et al.*, 1995), harderian gland, gastrointestinal tract (Huether *et al.*, 1992; Bubenik, 2002), ovary, testes, bone marrow (Tan *et al.*, 1999), thymus, spleen (Sánchez-Hidalgo *et al.*, 2009) and in leukocytes (Carrillo-Vico *et al.*, 2004). The biological functions of melatonin have been investigated extensively. For instance, melatonin regulates seasonal reproduction and circadian rhythm (Reiter *et al.*, 2011). This indolamine also acts as a powerful and widely effective antioxidant, as it has been shown to scavenge different types of free radicals *in vitro* and *in vivo* (Allegra *et al.*, 2003; Reiter *et al.*, 2009; du Plessis *et al.*, 2010; Tamura *et al.*, 2013) and to activate antioxidant defences such as superoxide dismutase (SOD), catalase, GSH peroxidase, GSH reductase and glucose-6-phosphate dehydrogenase (De La Lastra *et al.*, 1997; Alarcón de la Lastra *et al.*, 1999; Hardeland, 2009), consequently reducing oxidative stress. Likewise, a large number of reports describe melatonin as an immunomodulatory compound acting on specific receptors in immunocompetent cells (Guerrero and Reiter, 2002). Nevertheless, it still remains unclear how melatonin regulates immunity. In this context, while some authors argue that melatonin is an immunostimulant, many other studies have described its anti-inflammatory properties (Carrillo-Vico *et al.*, 2013).

In experimental *in vivo* and *in vitro* inflammation, melatonin modulated arachidonic acid metabolism, preventing or reducing the inflammatory activation of PLA_2, lipoxygenase and COX-2 (Radogna *et al.*, 2010). According to recent studies, melatonin suppressed the production of NO and IL-6 at both gene transcription and translation levels in LPS-activated macrophages (Choi *et al.*, 2011). Moreover, melatonin might modulate Toll-like receptor 4-mediated inflammatory genes through MyD88- and TRIF-dependent signalling pathways in LPS-stimulated RAW264.7 macrophages (Xia *et al.*, 2012). Nevertheless, the intracellular molecular mechanisms involved in melatonin effects in inflammation remain, at least in part, unclear and need to be explored in depth.

Immunocompetent cells that have melatonin receptors are target cells for its immunomodulatory function (Carrillo-Vico *et al.*, 2003) cooperating during the onset, progression and resolution of inflammation (Soehnlein and Lindbom, 2010). In addition, melatonin exerts an important role in managing inflammatory responses, modulating the ability of endothelial cells to control the rolling, adhesion and transmigration of leukocytes through blockade of NF–κB-dependent mechanisms (Marçola *et al.*, 2013). In fact, the circadian rhythm of melatonin primes the ability of endothelial cells to adhere to neutrophils in the day whereas, at night, melatonin in the blood maintains endothelial cells in a low reactive state.

Macrophages play a critical role in inflammation. Resident macrophages produce cytokines and chemokines that attract other cells, including neutrophils and additional macrophages. All of these responses can be used as readouts and are useful in assessing the role of pathogenic genes or proteins (Schneider, 2013). Murine and human macrophages exhibit a particularly vigorous response to LPS, which induces a variety of inflammatory modulators (Adams and Hamilton, 1984). LPS stimulation of macrophages disrupts the balance of the intracellular redox state, which leads to oxidative stress characterized by a major shift in the cellular redox balance and is usually accompanied by damage mediated by reactive oxygen species (ROS) (Brüne *et al.*, 2013). Macrophages express enzymes such as inducible NOS (iNOS) and COX-2 that regulate inflammatory processes (Chang *et al.*, 2012) and these proteins are responsible for the overproduction of NO and PGE_2, respectively, during inflammation. Apart from these enzymes, production of another inflammatory mediator PGE_2 is triggered by activation of microsomal PGE synthase-1 (mPGES1), an efficient downstream enzyme co-localized and functionally coupled with COX-2 in macrophages activated by LPS (Lazarus *et al.*, 2002). The process of gene expression of these pro-inflammatory mediators involves several signal transduction pathways such as the MAPK and NF-κB pathways (Barton and Medzhitov, 2003; Qi and Shelhamer, 2005). Importantly, a key transcription factor, NF-E2-related factor-2 (Nrf2), is an orchestrator of the induction of several antioxidant enzymes, such as haem oxygenase 1 (HO1; nomenclature follows Alexander *et al.*, 2013) and thus regulates the cellular antioxidant response against ROS in murine macrophages, modulating acute inflammatory responses (Jung *et al.*, 2010a; Kang and Kim, 2013). These pro-inflammatory mediators and pathways are regarded as essential anti-inflammatory targets (Lawrence *et al.*, 2002). For this reason, the stimulation of macrophages with LPS constitutes an excellent model for the screening and subsequent evaluation of the effects of candidate drugs on the inflammatory pathway (Sánchez-Miranda *et al.*, 2013).

Taking this background into account, the aim of the present study was to address the intracellular mechanisms underlying the effects of melatonin on the inflammatory responses induced by LPS, in murine macrophages. In this model, redox changes, protein expression of pro-inflammatory (iNOS, mPGES1, COX-2) and anti-inflammatory (HO1) enzymes, along with the roles of MAPK, NF-κB and Nrf2 signalling pathways involved in melatonin effects after the induction of inflammation were also determined.

Methods

Animals

All animal care and experimental procedures complied with the Guidelines of the European Union regarding animal experimentation (Directive of the European Counsel 86/609/EC) and followed a protocol approved by the Animal Ethics Committee of the University of Seville. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). Thirty 8–10-week-old male Swiss mice provided by Harlan Interfauna Ibérica® (Barcelona, Spain) weighing 20–30 g, were randomly placed in cages (five mice per cage) and maintained under constant

conditions of temperature (20–25°C) and humidity (40–60%) with a 12 h light/dark cycle and fed standard rodent chow (Panlab A04, Seville, Spain) and water *ad libitum* throughout the experiment in our Animal Laboratory Center (Faculty of Pharmacy, University of Seville, Spain). After 1 week for acclimatisation, peritoneal macrophages were elicited by i. p. injection of 1 mL sterile thioglycollate medium (10% w/v; Scharlau®, Barcelona, Spain), from five mice. Following the thioglycollate injection, mouse behaviour, water and food consumption, loss of body weight and survival were monitored daily until killing, 3 days later (see below).

Isolation and culture of murine peritoneal macrophages

Mice were injected intraperitoneally with 1 mL of sterile thioglycollate medium (10% w/v). After 3 days, mice were killed by CO_2 inhalation, and peritoneal exudate cells were harvested by washing the peritoneal cavity with sterile ice-cold PBS (Alleva *et al.*, 2002). After centrifugation, cells were resuspended in RPMI 1640 medium (PAA®, Pasching, Austria) supplemented with 10% heat-inactivated fetal calf serum (FCS; PAA), l-glutamine (2 mM), glucose (4.5 $g \cdot L^{-1}$) and HEPES buffer (10 mM), in the presence of 100 $mg \cdot mL^{-1}$ streptomycin and 100 $U \cdot mL^{-1}$ penicillin (PAA) and then seeded in culture plates (1×10^6 $cells \cdot mL^{-1}$) for 2 h at 37°C in a 5% CO_2-humidified atmosphere. After 2 h, non-adherent cells were removed by washing with PBS and fresh RPMI 1640 medium supplemented with 5% FCS containing the treatment was added. After 30 min, murine peritoneal macrophages (1×10^6 $cells \cdot mL^{-1}$) were treated with 5 $\mu g \cdot mL^{-1}$ of LPS from *Escherichia coli* (Sigma-Aldrich®, St Louis, MO, US) in absence or presence of melatonin (12.5, 25, 50 or 100 μM) for 18 h. In each experiment, viability was always ≥95%.

Cell viability

Cells seeded in 96-well plates (1×10^5 cells per well) were incubated in presence or absence of melatonin for 18 h. At the end of the exposure time, the effect of melatonin on cell growth/viability was analysed by sulforhodamine B (SRB) assay (Sigma-Aldrich; Skehan *et al.*, 1990). After incubation time, adherent cell cultures were fixed *in situ* by adding 50 μL of 50% (w/v) cold of trichloroacetic acid (Sigma-Aldrich) and incubated for 60 min at 4°C. The supernatant was discarded and plates were washed five times with deionized water and dried. Fifty microlitres of SRB solution (0.4% w/v) in 1% acetic acid (Panreac) was added to each well and incubated for 30 min at room temperature. Plates containing SRB solution were washed five times with 1% acetic acid. Then, plates were air dried and 100 μL per well of 10 $mmol \cdot L^{-1}$ Tris base pH 10.5 (Sigma-Aldrich) were added and the absorbance of each well was read on an ELISA reader at 510 nm (BioTek®, Bad Friedrichshall, Germany). Finally, cell survival was measured as the percentage of absorbance compared with that obtained in control cells (non-treated cells).

Measurement of nitrite production

Cells in 24-well plates were untreated or treated with different concentrations of melatonin (12.5, 25, 50 or 100 μM), and 30 min later stimulated with LPS for 18 h. The culture supernatants (100 μL) were transferred to a 96-well assay plate mixed with Griess reagent (Sigma) and incubated for 15 min at room temperature. The amount of nitrite, as an index of NO generation, was determined by a spectrophotometric method using the Griess reaction (Moorcroft *et al.*, 2001) and obtained by extrapolation from a standard curve with sodium nitrite. The absorbance at 540 nm was measured by an ELISA reader. Results were expressed as nitrite production, relative to LPS control cells (stimulated but untreated cells = 100%). Dexamethasone (1μM; Sigma) was used as positive control (data not shown).

Isolation of cytoplasmic and nuclear proteins and immunoblotting detection

Cells (1×10^6 $cells \cdot mL^{-1}$) were untreated or treated with melatonin and stimulated with LPS for 18 h. After incubation, cells were rinsed, scraped off and collected in ice-cold PBS containing a cocktail of protease and phosphatase inhibitors and processed as described by Sánchez-Hidalgo *et al.*, (2005) in order to isolate cytoplasmatic or nuclear proteins. Protein concentration was measured for each sample using a protein assay reagent (Bio-Rad®, München, Germany) according to the Bradford's method and using ɣ-globulin as a standard (Bradford, 1976). Aliquots of supernatant containing equal amounts of protein (20 μg) were separated by SDS-PAGE on 10% acrylamide gel. In the next step, the proteins were electrophoretically transferred into a nitrocellulose membrane and incubated with specific primary antibodies: rabbit anti-COX-2 and rabbit anti-iNOS (Cayman®, Ann Arbor, MI, USA; 1:2500 and 1:1000, respectively), rabbit anti-mPGES1 (Cayman; 1:1000), rabbit anti-IκBα, (Cell Signalling®, Danvers, MA, USA; 1:1000), rabbit anti-p65, mouse anti-pJNK, rabbit anti-JNK, mouse anti-pp38, rabbit anti-p38 (Santa Cruz Biotechnology®, Santa Cruz, CA, USA; 1:1000), rabbit anti-HO1 and rabbit anti-Nrf2 (Santa Cruz Biotechnology; 1:500), overnight at 4°C. After rinsing, the membranes were incubated with a HRP-labelled secondary antibody anti-rabbit (Cayman Chemical; 1:50 000) or anti-mouse (Dako®, Atlanta, GA, USA; 1:2000) containing blocking solution for 1–2 h at room temperature. To prove equal loading, the blots were analysed for β-actin expression using an anti-β-actin antibody (Sigma Aldrich). Immunodetection was performed using enhanced chemiluminescence light-detecting kit (Pierce®, Rockford, IL, USA). The immunosignals were captured using LAS-3000 Imaging System from Fujifilm Image Reader (Stamford, USA) and densitometric data were studied following normalization to the house-keeping loading control. The signals were analysed and quantified by an image processing and analysis in Java (Image J, Softonic® Barcelona, Spain) and expressed in relation to the DMSO–LPS-treated cells.

Data analysis

All values in the Figures and text are expressed as arithmetic means ± SEM. Experiments were carried out in triplicate. Data were evaluated with Graph Pad Prism® Version 2.01 software (San Diego, CA, USA). The statistical significance of differences between groups was evaluated by one-way ANOVA, using Tukey's multiple comparisons test as *post hoc* test. *P*-values of <0.05 were considered statistically significant. In the

experiments involving densitometry, the figures shown are representative of at least three different experiments performed on different days.

Materials

Melatonin and the proteosome inhibitor MG 132 were purchased from Sigma-Aldrich® Co (Dorset, UK) and were always freshly prepared as stock solutions in DMSO (Panreac®, Barcelona, Spain) and diluted to the desired concentration in culture medium. The final concentration of DMSO in the culture medium was ≤1% in all experiments and it did not significantly influence cell responses.

Results

Effect of melatonin on cell viability

Our first aim was to evaluate the effect of melatonin treatment on the viability of murine peritoneal macrophage cells in presence of LPS. We evaluated the effect of melatonin (6.25–100 μM) on the growth of these cells by SRB assay. Our data demonstrated that incubation with melatonin for up to 18h, at concentrations up to 100 μM, had no effect on viability of murine macrophages, as determined by the SRB assay (Figure 1).

Melatonin inhibited nitrite production and suppressed the LPS-induced iNOS overexpression

LPS induces the synthesis and release of NO into murine macrophage cell medium by iNOS protein expression (Xie *et al.*, 1992). Nitrite production, as an indicator of NO synthesis, was substantially induced in cells treated with LPS, in comparison with that in untreated cells. However, melatonin treatment significantly reduced nitrite production ($P < 0.001$ vs. DMSO control) suggesting a possible down-regulation of iNOS enzyme activity (Figure 2A) that was later confirmed by measuring iNOS protein expression with Western blot. Immunoblotting analysis demonstrated a significant decrease in iNOS protein levels, after incubation for 18 h with melatonin at the concentrations assayed (25, 50 and 100 μM; $P < 0.01$ vs. DMSO control; Figure 2B).

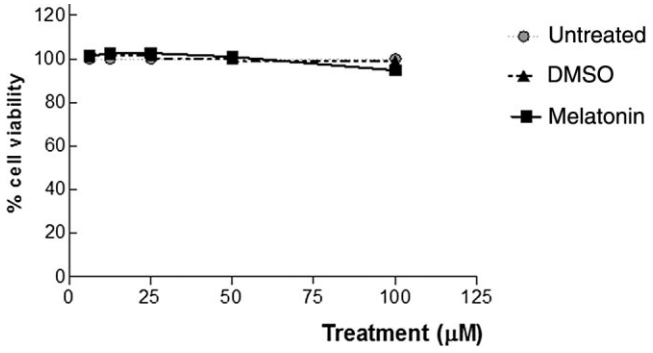

Figure 1

Effect of melatonin on cell viability. The concentrations used in this study did not affect viability of murine peritoneal macrophages. Cells were treated with melatonin (12.5, 25, 50 or 100 μM) for 24 h in presence of melatonin. Cell survival was measured as the percentage of absorbance compared with that obtained in control cells (non-treated cells).

Melatonin induced down-regulation of COX-2 and mPGES1 overexpression induced by LPS

Subsequently, we investigated the effects of melatonin on enzymes related to PGs in inflammation. COX-2 protein expression was clearly induced by LPS treatment (Figure 3A) However, a significant down-regulation of this pro-inflammatory protein was observed in those cells treated with 50 or 100 μM melatonin ($P < 0.05$ and $P < 0.001$, respectively, vs. DMSO control; Figure 3B).

It has been reported that mPGES1, one of the PGE_2 synthases, is co-localized and functionally coupled with COX-2

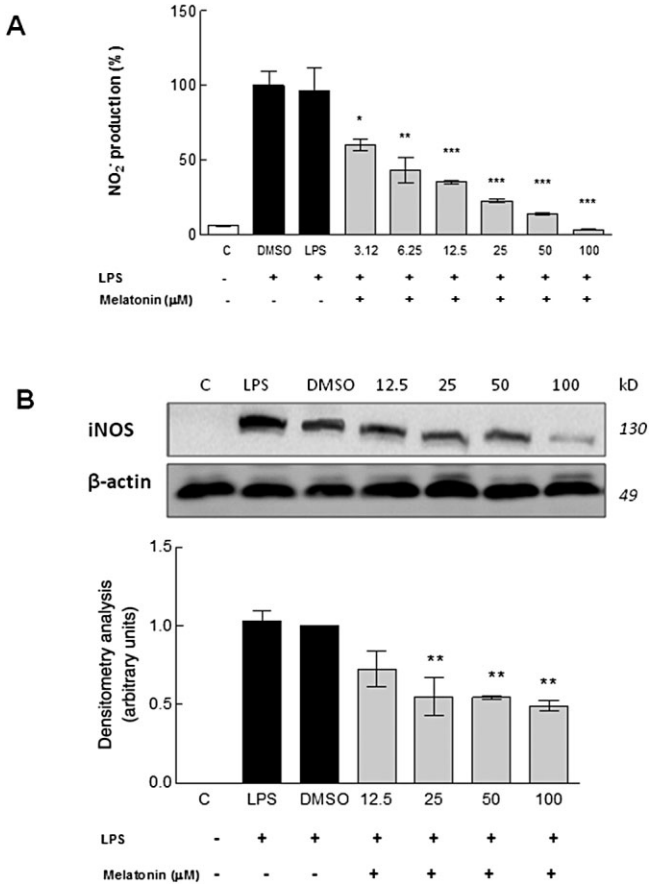

Figure 2

Effect of melatonin on LPS-induced NO production and iNOS protein expression in mouse peritoneal macrophages. Cells were incubated with melatonin and after 30 min, macrophages were treated with LPS for 18 h. (A) Nitrite generation; (B) Densitometric analysis of iNOS protein expression. The plots represent band intensity and were measured by Image J software. β-Actin served as an equal loading control for normalization. Data shown are means ± SEM for three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; significantly different from LPS–DMSO treated control cells.

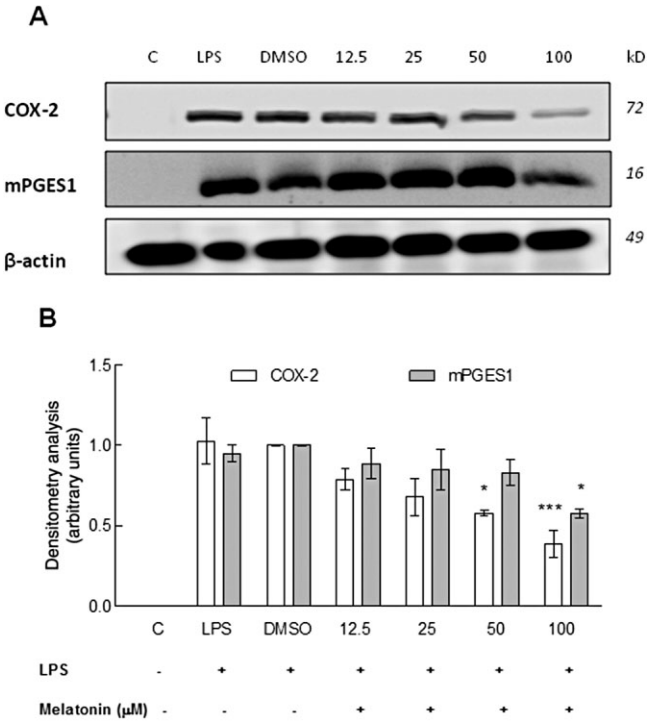

Figure 3

Melatonin inhibits COX-2 and mPGES1 protein expression in murine peritoneal isolated macrophages. Cells were untreated or treated with melatonin (12.5, 25, 50 or 100 μM) for 18 h in presence of LPS. As controls, cells were also treated with DMSO (solvent control) or left untreated in absence of LPS. The plots represent band intensity. β-Actin served as an equal loading control for normalization. Data shown are means ± SEM. *P < 0.05, ***P < 0.001; significantly different from LPS–DMSO treated control cells.

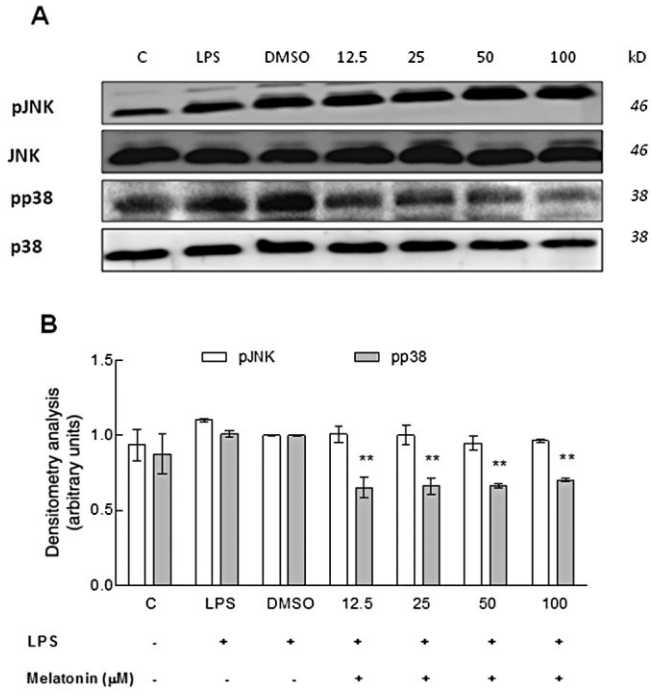

Figure 4

Effects of melatonin on pJNK and pp38 signalling pathways in murine peritoneal isolated macrophages. Melatonin treatment inhibited p38 phosphorylation but did not produce changes in JNK activation. Cells were untreated or treated with melatonin (12.5, 25, 50 or 100 μM) for 18 h in presence of LPS. As controls, cells were also treated with DMSO (solvent control) or left untreated in absence of LPS. The results are representative of three independent experiments. Densitometry was performed following normalization to the control (JNK and p38 housekeeping genes, respectively). Data shown are means ± SEM. **P < 0.01; significantly different from LPS–DMSO treated control cells.

(Murakami *et al.*, 2000). In our murine macrophages, LPS stimulation markedly increased expression of mPGES1 protein (Figure 3A). However, exposure to melatonin before LPS stimulation resulted in a significant inhibition of LPS-induced mPGES1 protein expression, in macrophages treated with the highest concentration of melatonin (P < 0.05 vs. DMSO control; Figure 3B).

Effect of melatonin on LPS-induced activation of MAPKs in murine peritoneal macrophages

The MAPK signalling pathways are involved in the expression of many inflammatory protein genes including iNOS, TNF-α and COX-2. To further explore the molecular mechanisms underlying the anti-inflammatory effects of melatonin, we also determined its role on MAPK activation by Western blot analysis, using phosphospecific MAPK antibodies (Figure 4). Cells were incubated in absence or presence of different concentrations of melatonin before LPS stimulation. LPS induced the appearance of phosphorylated (activated) JNK and p38, whereas melatonin treatment, at all doses assayed, inhibited the activation of p38 (P < 0.01 vs. DMSO control; Figure 4), but not that of JNK.

Effect of melatonin on Nrf2-mediated transcriptional activation and HO1 induction in murine peritoneal macrophages

Nrf2 is a key transcription factor that regulates the cellular antioxidant response. Upon cell stimulation, Nrf2 is translocated from the cytosol to the nucleus, and sequentially binds to a promoter sequence called the antioxidant response, element (ARE), resulting in a cytoprotective response characterized by up-regulation of antioxidant enzymes [quinone oxidoreductase-1 (NQO1), HO1 and SOD] and decreased sensitivity to oxidative stress damage (Jaiswal, 2004). Nrf2 also plays a broader role in modulating acute inflammatory responses (Owuor and Kong, 2002). To identify whether melatonin modulated the Nrf2 signalling pathway, we measured the expression of Nrf2 and HO1 protein, by Western blot. As shown in Figure 5A, LPS induced a significant down-regulation of both Nrf2 and HO1 proteins, compared with untreated macrophages. However, incubation with melatonin (100 μM) caused a marked increase in Nrf2 and HO1 expression (P < 0.001 and P < 0.05, respectively, vs. DMSO control; Figure 5B).

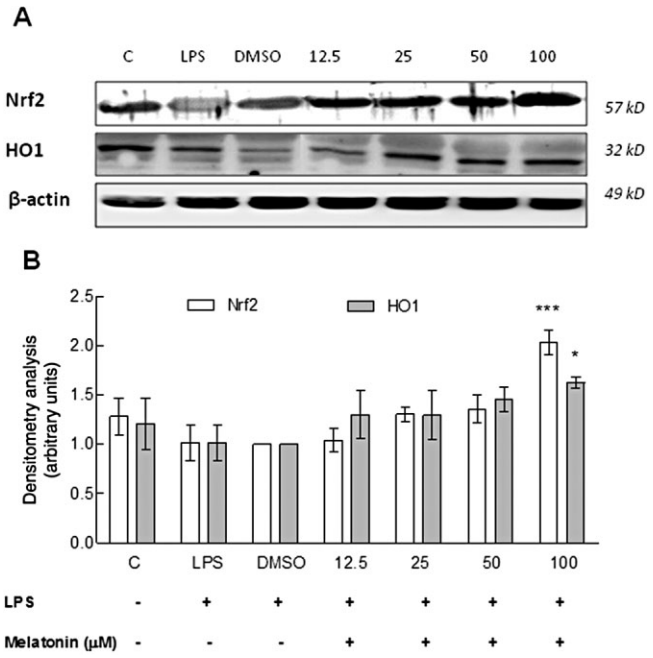

Figure 5

Melatonin treatment inhibits Nrf2 and HO1 degradation in murine peritoneal isolated macrophages. Cells were untreated or treated with melatonin (12.5, 25, 50 or100 μM) for 18 h in presence of LPS. As controls, cells were also treated with DMSO (solvent control) or left untreated in absence of LPS. The results are representative of three independent experiments. Densitometry was performed following normalization to the control (β-actin housekeeping gene). Data shown are means ± SEM. *$P < 0.05$, ***$P < 0.001$; significantly different from LPS–DMSO treated control cells.

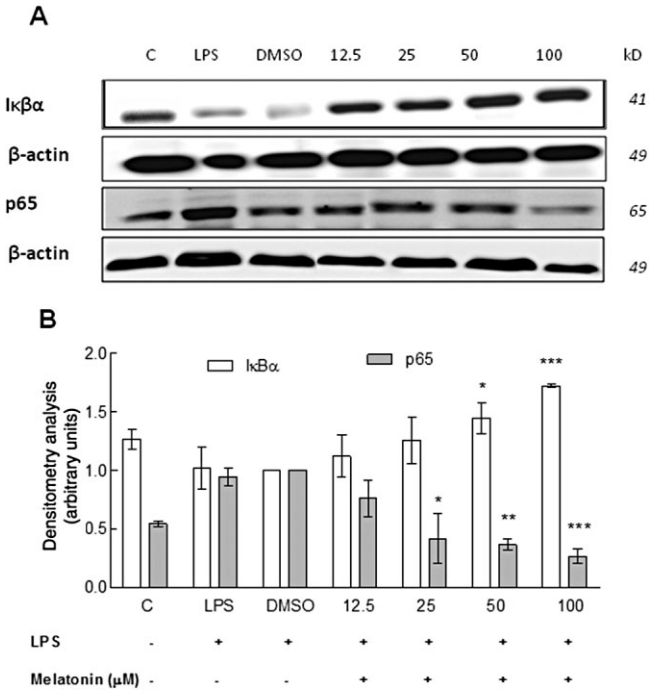

Figure 6

Melatonin treatment inhibits NF-κB-mediated transcriptional activation and prevents IκBα degradation in murine peritoneal isolated macrophages. Cells were untreated or treated with melatonin (12.5, 25, 50 or 100 μM) for 18 h in presence of LPS. As controls, cells were also treated with DMSO (solvent control) or left untreated in absence of LPS. The results are representative of three independent experiments. Densitometry was performed following normalization to the control (β-actin housekeeping gene). Data shown are means ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; significantly different from LPS–DMSO treated control cells.

Melatonin inhibited NF–κB-mediated activation of transcription and prevented degradation of IkBα in murine peritoneal macrophages

NF-κB is a pleiotropic mediator, controlling several inducible and tissue-specific genes (Lenardo and Baltimore, 1989) and is one of the key regulators of the cellular responses to oxidative stress in mammalian cells (Helenius *et al.*, 2001). Given the relevance of NF-κB to human diseases and the fact that many drugs interfere with NF-κB signalling, this signalling pathway provides a highly attractive target for anti-inflammatory therapy. The activation step allowing NF-κB to leave the cytoplasm involves the ubiquitination of IκBα by the SCF-β-TrCP ubiquitin ligase complex followed by the rapid degradation of ubiquitinated IκBα by the 26S proteasome (Scheidereit, 2006). We therefore tested the effect of melatonin on IκBα degradation and NF-κB activation in murine peritoneal macrophages. As shown in Figure 6, LPS stimulation increased IκBα degradation, which was consistent with an up-regulation of the translocation of p65 protein to the nucleus. On the contrary, pretreatment with melatonin (25, 50 and 100 μM) caused a significant parallel inhibition of NF–κB-mediated transcriptional activation, preventing IκBα degradation and the nuclear translocation of p65 protein in murine macrophages, after LPS stimulation ($P < 0.001$ vs. DMSO control).

Effects of melatonin on Nrf2 and HO1 protein expression after inhibition of the NF-κB signalling pathway in murine peritoneal macrophages

Our results revealed that melatonin blocked the activation of the NF-κB pathway, impairing the expression of genes related to the anti-inflammatory phase whereas it enhanced some anti-inflammatory signals. As a result, we addressed the causal relationship between NF-κB inhibition and Nrf2 and HO1 overexpression, mediated by melatonin in our model.

The 20S proteasome, the catalytic core of the 26S proteasome complex, is responsible for the breakdown of short-lived regulatory proteins, including Nrf2 and NF-κB (Dreger *et al.*, 2009). In the present study, macrophages were pretreated with 2 μM Z-Leu-Leu-Leu-al (MG 132), a 26S proteasome inhibitor, for 1 h, incubated in presence or absence of LPS (5 $\mu g \cdot mL^{-1}$) for 30 min and then treated with melatonin (12.5, 25, 50 and 100 μM). As expected, the nuclear level of p65 protein did not significantly alter after 18 h LPS stimulation in the presence of MG 132, with or without melatonin, in comparison with untreated cells (Figures 6 and 7). Nevertheless, Nrf2 protein expression was unaltered in presence or absence of MG 132 (Figures 5 and 7). Finally, no changes in

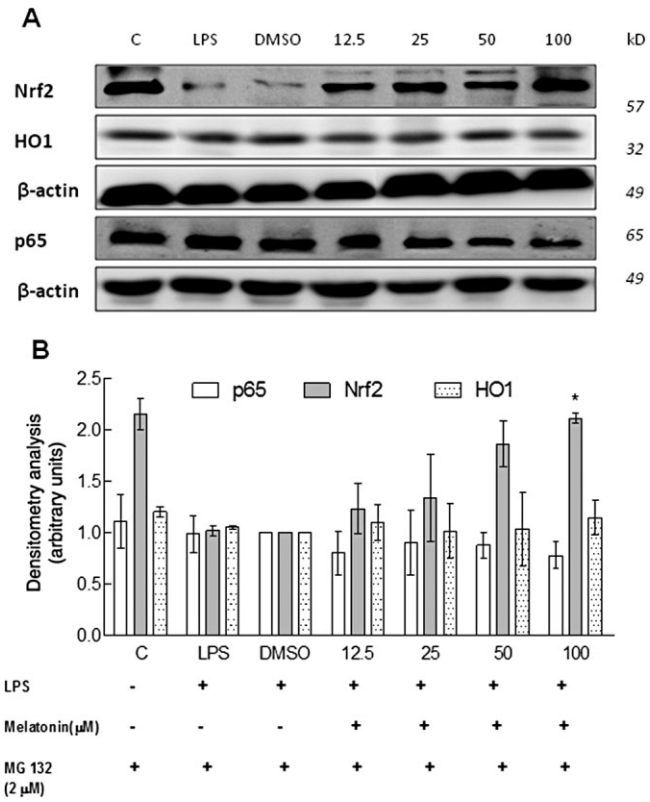

Figure 7

Effects of melatonin treatment in HO1 and Nrf2 protein expression after NF-κB signalling pathway inhibition. Cells were treated with 2 μM MG 132, a proteosome inhibitor, for 30 min, treated in presence or absence of melatonin (12.5, 25, 50 or 100 μM) for 18 h in presence of LPS. As controls, cells were also treated with DMSO (solvent control) or left untreated in absence of LPS. The results are representative of three independent experiments. Densitometry was performed following normalization to the control (β-actin housekeeping gene). Data shown are means ± SEM. *$P < 0.05$; significantly different from LPS–DMSO treated control cells.

the expression of the anti-inflammatory HO1 enzyme were detected in LPS-treated cells after incubation with MG 132 in presence or absence of melatonin when compared with untreated cells (Figures 5 and 7).

Discussion

LPS-stimulated macrophages disrupt the balance of the intracellular redox state, which leads to oxidative stress characterized by a major shift in the cellular redox balance and usually accompanied by ROS-mediated damage (Kang and Lee, 2012). Stimulation of macrophages induces transcription of the iNOS gene and large amounts of NO are generated. NO acts as an intracellular messenger, which modulates the formation of endogenous ROS including hydrogen peroxide, peroxynitrite and other potential oxidants, that orchestrate the inflammatory response (Li *et al.*, 2012). ROS are capable of eliciting a variety of pathological changes, including the peroxidation of lipids, proteins and DNA. Therefore, modulators of ROS production and ROS-induced signalling pathways, especially in macrophages, could represent potential targets for anti-inflammatory intervention (Kim *et al.*, 2012). In the present study, we found that exposure of peritoneal macrophages to LPS resulted in a significant increase in nitrite levels, as an indicator of NO production, and an up-regulation of iNOS expression. However, melatonin inhibited these effects in a concentration-dependent manner. These findings are in accordance with other studies of murine macrophages (Zhang *et al.*, 2004) and J774 and RAW 264.7 cells, stimulated with bacterial LPS (Mayo *et al.*, 2005; Deng *et al.*, 2006). mPGES1 is an efficient downstream enzyme for the production of PGE_2 in macrophages activated by LPS (Lazarus *et al.*, 2002) and is co-localized and functionally coupled with COX-2 (Murakami *et al.*, 2000). COX-2, the inducible isoform of COX, is the key enzyme that catalyses the two sequential steps in the biosynthesis of PGs from arachidonic acid, and plays a critical role in the inflammatory response. A selective inhibitor of mPGES1 would be expected to inhibit PGE_2 production induced by inflammation while sparing constitutive PGE_2 production (Kudo and Murakami, 2005; Wang *et al.*, 2006). In our study, melatonin treatment before LPS stimulation resulted in a significant down-regulation of both proteins, indicating a potential dual action on both COX-2 and mPGES1 enzymes involved in PGE_2 synthesis. These results are consistent with those obtained from other studies where melatonin, at non-cytotoxic concentrations, time and concentration-dependently inhibited the induced protein levels and promoter activities of COX-2 in LPS-activated RAW264.7 cells (Mayo *et al.*, 2005; Deng *et al.*, 2006) or stimulated with fimbriae of *Porphyromonas gingivalis* (Murakami *et al.*, 2012). Furthermore, our results are in agreement with those of Niranjan *et al.* (2012). These authors, using LPS-stimulated rat astrocytoma cells (C6), found that melatonin reversed LPS-induced changes in mRNA expression of mPGES1 and phosphorylated p38 MAPK. Similarly, melatonin treatment of C6 cells for 24 h significantly decreased LPS-induced nitrosative and oxidative stress and expressions of COX-2 and iNOS. Of the several transcription factors activated by inflammatory stimuli, the NF-κB signalling pathway plays a key role in mediating inflammation and immune responses, through induction of pro-inflammatory cytokines, chemokines and other proteins. NF-κB, as a dimeric transcription factor composed of p65 (RelA), RelB, c-Rel, NF-κB1 (p50/p105) or NF-κB2 (p52/p100) exists in the cytoplasm as an inactive complex with the inhibitory protein, IkBα. When cells are challenged with pro-inflammatory stimuli, for example LPS, IkBα is phosphorylated and subsequently ubiquitinated, allowing NF-κB to translocate to the nucleus. Consequently, NF-κB binds to kB enhancer elements present in the promoter region of many pro-inflammatory genes such as iNOS and COX-2 (Tak and Firestein, 2001; Lee and Surh, 2012).

Moreover, the MAPKs are a family of serine–threonine kinase enzymes that orchestrate the recruitment of gene transcription, protein biosynthesis, cell cycle control, apoptosis, and differentiation and allow cells to respond to oxidative stress and inflammatory stimuli, from their extracellular environment (Munoz and Ammit, 2010). MAPKs include ERKs-1 and -2, JNKs and p38 MAPKs. JNKs, encoded by three genes

(JNKs 1–3) while activated by mitogens, are also vigorously stimulated by a variety of environmental stresses, including genotoxins, ischaemia-reperfusion injury, mechanical shear stress, vasoactive peptides and pro-inflammatory cytokines. The p38 MAPKs encoded by four p38 genes are preferentially activated *in situ* by environmental stresses and pro-inflammatory cytokines such as TNF-α, IL-6, IL-7 and IL-8 in many cell types (Yong *et al.*, 2009; Kyriakis and Avruch, 2012). In effect, MAPKs have been shown to play important roles in iNOS and COX-2 up-regulation induced by various stimuli in mammalian cells (Guha and Mackman, 2001).

Our data showed that treatment with melatonin before 18 h incubation with LPS, significantly prevented IκBα degradation and blocked p65 translocation into the nuclei. In addition, such pre-treatment attenuated the activation of p38 MAPK but was unable to decrease JNK phosphorylation. These data are partially in agreement with Niranjan *et al.* (2012) and Joo and Yoo (2009), who used LPS-stimulated rat astrocytoma cells or prostate cancer cells (LNCaP), respectively, and found that melatonin reversed LPS-induced changes in mRNA expression of both phosphorylated p38 and JNK MAPKs. Also, in another model (Esposito *et al.*, 2009), melatonin treatment reduced the activation of p38, JNK and ERK1/2 MAPKs, suggesting that the reduction by melatonin of spinal cord injury in mice could also be related to a inhibition of the MAPK signalling pathways.

Altogether, our data suggest that melatonin inhibits iNOS, COX-2 and mPGES1 protein expression by a common transcriptional mechanism modulating the activation of NF-κB and p38 MAPK cascade signalling pathways, suggesting that both the NF-κB transcription factor and the p38 MAPK could be involved in mediating the anti-inflammatory effects of melatonin in murine LPS-activated macrophages.

Immunocompetent cells with melatonin receptors are target cells for its immunomodulatory function (Carrillo-Vico *et al.*, 2003) cooperating during the onset, progression and resolution of inflammation (Soehnlein and Lindbom, 2010). Large amounts of melatonin are produced by all immunocompetent cells, including macrophages, acting as an intracrine, autocrine, and/or paracrine mediator. Recently, it has been suggested that during inflammatory responses, NF-κB induced endogenous synthesis of melatonin in a physiological range, i.e., in pg amounts, in RAW 264.7 macrophages by inducing the transcription of the key enzyme involved in melatonin synthesis arylalkylamine-N-acetyltransferase (AA-NAT) and that macrophage-synthesized melatonin modulated the function of these professional phagocytes in an autocrine manner (Muxel *et al.*, 2009). Our results suggest that treatment with exogenous melatonin, in a pharmacological range, i.e., μg amounts, may modulate NF-κB translocation via AA-NAT, through a negative feedback mechanism contributing to macrophage homeostasis during resolution of inflammation. Nevertheless, further investigations are necessary to substantiate prove this proposal .

Recent reports revealed that melatonin treatment caused a significant up-regulation of LPS-induced Nrf2 and HO1 protein levels. Nrf2 is a key orchestrator of the induction of several antioxidants, which regulates the cellular antioxidant response against ROS. Nrf2 belongs to the 'cap'n'collar' basic leucin zipper family of proteins. Under basal conditions, Nrf2 is sequestered in the cytoplasm by its inhibitor Keap1, then ubiquitinylated, and finally degraded by the proteasome. In the presence of oxidative stress, Keap1 releases Nrf2, which can migrate to the nucleus, bind to the antioxidant response element sequence, and induce phase II gene transcription resulting in a cytoprotective response characterized by up-regulation of antioxidant enzymes such as NADPH NQO1, SOD, GSH peroxidase and HO1, and decreased sensitivity to oxidative stress damage (Owuor and Kong, 2002). Also, it has been reported that Nrf2 plays a broader role in modulating acute inflammatory responses (Jung *et al.*, 2010b). On the other hand, HO1 is the inducible isoform of the rate-limiting enzyme of haem degradation. HO regulates the cellular content of the pro-oxidant haem and produces catabolites with physiological functions. HO1 is strongly induced by its substrate haem and by numerous stress stimuli such as UV light, heavy metals, heat shock and hyperoxia. More recently, HO1 has also been recognized to exhibit important immunomodulatory and anti-inflammatory functions (Paine *et al.*, 2010). Our results, in LPS-stimulated macrophages, showed that melatonin increased expression of Nrf2 and the antioxidant HO1 enzyme, in parallel with the decrease of inflammatory mediators such as iNOS, COX-2 and mPGES1, suggesting that melatonin may play a role as an antioxidant defense via the Nrf2/HO1 pathway. Similar results have been obtained by other authors in *in vivo* experimental models such as dimethylnitrosamine-induced liver injury (Jung *et al.*, 2010a), cisplatin-induced nephrotoxicity (Kilic *et al.*, 2013), in hepatic ischaemia-reperfusion injury (Kang and Lee, 2012), in experimental diabetic neuropathy (Negi *et al.*, 2011) and in interstitial cystitis (Zhang *et al.*, 2013). On the other hand, NF-κB appears to be directly involved in the induction of HO1 gene expression. Increased expression of the HO1 gene is considered to be an adaptive cellular response to survive exposure to environmental stresses (Paine *et al.*, 2010). In order to clarify the role of NF-κB and Nrf2 on HO1 melatonin-mediated overexpression in LPS-stimulated macrophages, we used an inhibitor of NF-κB translocation, MG 132.

As expected, the nuclear p65 protein expression did not significantly alter after 18 h LPS stimulation in the presence of MG 132, with or without melatonin, in comparison with untreated cells, whereas Nrf2 protein expression was maintained unaltered in the presence or absence of MG 132, suggesting that Nrf2 overexpression mediated by melatonin was through a mechanism independent of the NF-κB signalling pathway. Finally, no changes in the expression of the anti-inflammatory HO1 enzyme were detected in LPS-treated cells after incubation with MG 132 in presence or absence of melatonin compared with untreated cells. These results suggest that HO1 melatonin-mediated overexpression could be controlled, at least in part, by NF-κB signalling pathways contributing to the anti-inflammatory effects of melatonin. This relationship has been previously described in human renal proximal tubule cells treated with curcumin and co-incubated with an inhibitor of IKBα phosphorylation, where HO1 induction by curcumin was mediated, at least in part, via transcriptional mechanisms and involved the NF-κB signalling pathway (Hill-Kapturczak *et al.*, 2001). Our results are also in accordance with those from Naidu *et al.*, who found that HO1 gene expression was not up-regulated in phorbol myrisate acetate-activated monocytes from mice, deficient for the NF-κB subunit p65 (Naidu *et al.*, 2008).

Similarly, Li *et al.*, showed that HO1 up-regulation was mediated by iNOS and by augmenting NF-κB binding to the region of the HO1 gene promoter in transgenic mice with cardiomyocyte-restricted expression of a dominant negative mutant of IκBα. (Li *et al.*, 2009).

In conclusion, our study showed that melatonin reduced the pro-inflammatory proteins iNOS, COX-2 and mPGES1, and enhanced the expression of HO1 via NF-κB, Nrf2 and p38 MAPK cascade signalling pathways. Thus, melatonin might be a promising target for diseases associated with an overactivation of macrophages.

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Conflicts of interest

The authors state no conflict of interest.

References

Adams DO, Hamilton TA (1984). The cell biology of macrophage activation. Annu Rev Immunol 2: 283–318.

Alarcón de la Lastra C, Motilva V, Martín MJ, Nieto A, Barranco MD, Cabeza J *et al.* (1999). Protective effect of melatonin on indomethacin-induced gastric injury in rats. J Pineal Res 26: 101–107.

Alexander SPH *et al.* (2013). The Concise Guide to PHARMACOLOGY 2013/14: Overview. Br J Pharmacol 170: 1449–1867.

Allegra M, Reiter RJ, Tan DX, Gentile C, Tesoriere L, Livrea MA (2003). The chemistry of melatonin's interaction with reactive species. J Pineal Res 34: 1–10.

Alleva DG, Johnson EB, Lio FM, Boehme SA, Conlon PJ, Crowe PD (2002). Regulation of murine macrophage proinflammatory and anti-inflammatory cytokines by ligands for peroxisome proliferator-activated receptor-gamma: counter-regulatory activity by IFN-gamma. J Leukoc Biol 71: 677–685.

Barton GM, Medzhitov R (2003). Toll-like receptor signaling pathways. Science 300: 1524–1525.

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

Brüne B, Dehne N, Grossmann N, Jung M, Namgaladze D, Schmid T *et al.* (2013). Redox control of inflammation in macrophages. Antioxid Redox Signal 19: 595–637.

Bubenik GA (2002). Gastrointestinal melatonin: localization, function, and clinical relevance. Dig Dis Sci 47: 2336–2348.

Carrillo-Vico A, García-Pergañeda A, Naji L, Calvo JR, Romero MP, Guerrero JM (2003). Expression of membrane and nuclear melatonin receptor mRNA and protein in the mouse immune system. Cell Mol Life Sci 60: 2272–2278.

Carrillo-Vico A, Calvo JR, Abreu P, Lardone PJ, García-Mauriño S, Reiter RJ *et al.* (2004). Evidence of melatonin synthesis by human lymphocytes and its physiological significance: possible role as intracrine, autocrine, and/or paracrine substance. FASEB J 18: 537–539.

Carrillo-Vico A, Lardone PJ, Alvarez-Sánchez N, Rodríguez-Rodríguez A, Guerrero JM (2013). Melatonin: buffering the immune system. Int J Mol Sci 14: 8638–8683.

Chang WT, Huang WC, Liou CJ (2012). Evaluation of the anti-inflammatory effects of phloretin and phlorizin in lipopolysaccharide-stimulated mouse macrophages. Food Chem 134: 972–979.

Choi EY, Jin JY, Lee JY, Choi JI, Choi IS, Kim SJ (2011). Melatonin inhibits *Prevotella intermedia* lipopolysaccharide-induced production of nitric oxide and interleukin-6 in murine macrophages by suppressing NF-κB and STAT1 activity. J Pineal Res 50: 197–206.

De La Lastra CA, Cabeza J, Motilva V, Martin MJ (1997). Melatonin protects against gastric ischemia-reperfusion injury in rats. J Pineal Res 23: 47–52.

Deng WG, Tang ST, Tseng HP, Wu KK (2006). Melatonin suppresses macrophage cyclooxygenase-2 and inducible nitric oxide synthase expression by inhibiting p52 acetylation and binding. Blood 108: 518–524.

Dreger H, Westphal K, Weller A, Baumann G, Stangl V, Meiners S *et al.* (2009). Nrf2-dependent upregulation of antioxidative enzymes: a novel pathway for proteasome inhibitor-mediated cardioprotection. Cardiovasc Res 83: 354–361.

Esposito E, Genovese T, Caminiti R, Bramanti P, Meli R, Cuzzocrea S (2009). Melatonin reduces stress-activated/mitogen-activated protein kinases in spinal cord injury. J Pineal Res 46: 79–86.

Faillace MP, Cutrera R, Sarmiento MI, Rosenstein RE (1995). Evidence for local synthesis of melatonin in golden hamster retina. Neuroreport 6: 2093–2095.

Guerrero JM, Reiter RJ (2002). Melatonin–immune system relationships. Curr Top Med Chem 2: 167–179.

Guha M, Mackman N (2001). LPS induction of gene expression in human monocytes. Cell Signal 13: 85–94.

Hardeland R (2009). Neuroprotection by radical avoidance: search for suitable agents. Molecules 14: 5054–5102.

Helenius M, Kyrylenko S, Vehviläinen P, Salminen A (2001). Characterization of aging-associated up-regulation of constitutive nuclear factor-kappa B binding activity. Antioxid Redox Signal 3: 147–156.

Hill-Kapturczak N, Thamilselvan V, Liu F, Nick HS, Agarwal A (2001). Mechanism of heme oxygenase-1 gene induction by curcumin in human renal proximal tubule cells. Am J Physiol Renal Physiol 281: F851–F859.

Huether G, Poeggeler B, Reimer A, George A (1992). Effect of tryptophan administration on circulating melatonin levels in chicks and rats: evidence for stimulation of melatonin synthesis and release in the gastrointestinal tract. Life Sci 51: 945–953.

Jaiswal AK (2004). Nrf2 signaling in coordinated activation of antioxidant gene expression. Free Radic Biol Med 36: 1199–1207.

Joo SS, Yoo YM (2009). Melatonin induces apoptotic death in LNCaP cells via p38 and JNK pathways: therapeutic implications for prostate cancer. J Pineal Res 47: 8–14.

Jung CH, Kim JH, Park S, Kweon DH, Kim SH, Ko SG (2010a). Inhibitory effect of Agrimonia pilosa Ledeb. on inflammation by suppression of iNOS and ROS production. Immunol Invest 39: 159–170.

Jung KH, Hong SW, Zheng HM, Lee HS, Lee H, Lee DH *et al.* (2010b). Melatonin ameliorates cerulein-induced pancreatitis by the modulation of nuclear erythroid 2-related factor 2 and nuclear factor-kappaB in rats. J Pineal Res 48: 239–250.

Kang IS, Kim C (2013). Taurine chloramine administered *in vivo* increases NRF2-regulated antioxidant enzyme expression in murine peritoneal macrophages. Adv Exp Med Biol 775: 259–267.

Kang JW, Lee SM (2012). Melatonin inhibits type 1 interferon signaling of toll-like receptor 4 via heme oxygenase-1 induction in hepatic ischemia/reperfusion. J Pineal Res 53: 67–76.

Kilic U, Kilic E, Tuzcu Z, Tuzcu M, Ozercan IH, Yilmaz O *et al.* (2013). Melatonin suppresses cisplatin-induced nephrotoxicity via activation of Nrf2/HO1 pathway. Nutr Metab (Lond) 10: 7–15.

Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG (2010). Animal research: Reporting *in vivo* experiments: the ARRIVE guidelines. Br J Pharmacol 160: 1577–1579.

Kim KJ, Yoon KY, Lee BY (2012). Low molecular weight fucoidan from the sporophyll of *Undaria pinnatifida* suppresses inflammation by promoting the inhibition of mitogen-activated protein kinases and oxidative stress in RAW264.7 cells. Fitoterapia 83: 1628–1635.

Kudo I, Murakami M (2005). Prostaglandin E synthase, a terminal enzyme for prostaglandin E2 biosynthesis. J Biochem Mol Biol 38: 633–638.

Kyriakis JM, Avruch J (2012). Mammalian MAPK signal transduction pathways activated by stress and inflammation: a 10-year update. Physiol Rev 92: 689–737.

Lawrence T, Willoughby DA, Gilroy DW (2002). Anti-inflammatory lipid mediators and insights into the resolution of inflammation. Nat Rev Immunol 2: 787–795.

Lazarus M, Kubata BK, Eguchi N, Fujitani Y, Urade Y, Hayaishi O (2002). Biochemical characterization of mouse microsomal prostaglandin E synthase-1 and its colocalization with cyclooxygenase-2 in peritoneal macrophages. Arch Biochem Biophys 397: 336–341.

Lee HN, Surh YJ (2012). Therapeutic potential of resolvins in the prevention and treatment of inflammatory disorders. Biochem Pharmacol 84: 1340–1350.

Lenardo MJ, Baltimore D (1989). NF-kappa B: a pleiotropic mediator of inducible and tissue-specific gene control. Cell 58: 227–229.

Li DY, Xue MY, Geng ZR, Chen PY (2012). The suppressive effects of Bursopentine (BP5) on oxidative stress and NF-κB activation in lipopolysaccharide-activated murine peritoneal macrophages. Cell Physiol Biochem 29: 9–20.

Li Q, Guo Y, Ou Q, Cui C, Wu WJ, Tan W *et al.* (2009). Gene transfer of inducible nitricoxide synthase affords cardioprotection by upregulating heme oxygenase-1via a nuclear factor-{kappa}B-dependent pathway. Circulation 120: 12229lat.

McGrath J, Drummond G, McLachlan E, Kilkenny C, Wainwright C (2010). Guidelines for reporting experiments involving animals: the ARRIVE guidelines. Br J Pharmacol 160: 1573–1576.

Marçola M, da Silveira Cruz-Machado S, Fernandes PA, Monteiro AW, Markus RP, Tamura EK (2013). Endothelial cell adhesiveness is a function of environmental lighting and melatonin level. J Pineal Res 54: 162–169.

Mayo JC, Sainz RM, Tan DX, Hardeland R, Leon J, Rodriguez C *et al.* (2005). Anti-inflammatory actions of melatonin and its metabolites, N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK) and N1-acetyl-5-methoxykynuramine (AMK), in macrophages. J Neuroimmunol 165: 139–149.

Moorcroft MJ, Davis J, Compton RG (2001). Detection and determination of nitrate and nitrite: a review. Talanta 54: 785–803.

Munoz L, Ammit AJ (2010). Targeting p38 MAPK pathway for the treatment of Alzheimer's disease. Neuropharmacology 58: 561–568.

Murakami M, Naraba H, Tanioka T, Semmyo N, Nakatani Y, Kojima F *et al.* (2000). Regulation of prostaglandin E2 biosynthesis by inducible membrane-associated prostaglandin E2 synthase that acts in concert with cyclooxygenase-2. J Biol Chem 275: 32783–32792.

Murakami Y, Machino M, Fujisawa S (2012). *Porphyromonas gingivalis* fimbria-induced expression of inflammatory cytokines and cyclooxygenase-2 in mouse macrophages and its inhibition by the bioactive compounds fibronectin and melatonin. ISRN Dent 2012: 350859.

Muxel SM, Pires-Lapa MA, Monteiro AW, Cecon E, Tamura EK, Floeter-Winter LM *et al.* (2009). NF-κB drives the synthesis of melatonin in RAW 264.7 macrophages by inducing the transcription of the arylalkylamine-N-acetyltransferase (AA-NAT) gene. PLoS ONE 7: e52010.

Naidu S, Wijayanti N, Santoso S, Kietzmann T, Immenschuh S (2008). An atypical NFkappaB-regulated pathway mediates phorbol ester-dependent hemeoxygenase-1 gene activation in monocytes. J Immunol 181: 4113–4181.

Negi G, Kumar A, Sharma SS (2011). Melatonin modulates neuroinflammation and oxidative stress in experimental diabetic neuropathy: effects on NF-κB and Nrf2 cascades. J Pineal Res 50: 124–131.

Niranjan R, Nath C, Shukla R (2012). Melatonin attenuated mediators of neuroinflammation and alpha-7 nicotinic acetylcholine receptor mRNA expression in lipopolysaccharide (LPS) stimulated rat astrocytoma cells, C6. Free Radic Res 46: 1167–1177.

Owuor ED, Kong AN (2002). Antioxidants and oxidants regulated signal transduction pathways. Biochem Pharmacol 64: 765–770.

Paine A, Eiz-Vesper B, Blasczyk R, Immenschuh S (2010). Signaling to heme oxygenase-1 and its anti-inflammatory therapeutic potential. Biochem Pharmacol 80: 1895–1903.

du Plessis SS, Hagenaar K, Lampiao F (2010). The *in vitro* effects of melatonin on human sperm function and its scavenging activities on NO and ROS. Andrologia 42: 112–116.

Qi HY, Shelhamer JH (2005). Toll-like receptor 4 signaling regulates cytosolic phospholipase A2 activation and lipid generation in lipopolysaccharide-stimulated macrophages. J Biol Chem 280: 38969–38975.

Radogna F, Diederich M, Ghibelli L (2010). Melatonin: a pleiotropic molecule regulating inflammation. Biochem Pharmacol 80: 1844–1852.

Reiter RJ (1991). Pineal melatonin: cell biology of its synthesis and of its physiological interactions. Endocr Rev 12: 151–180.

Reiter RJ, Paredes SD, Manchester LC, Tan DX (2009). Reducing oxidative/nitrosative stress: a newly-discovered genre for melatonin. Crit Rev Biochem Mol Biol 44: 175–200.

Reiter RJ, Rosales-Corral S, Coto-Montes A, Boga JA, Tan DX, Davis JM *et al.* (2011). The photoperiod, circadian regulation and chronodisruption: the requisite interplay between the suprachiasmatic nuclei and the pineal and gut melatonin. J Physiol Pharmacol 62: 269–274.

Sánchez-Hidalgo M, Martín AR, Villegas I, Alarcón De La Lastra C (2005). Rosiglitazone, an agonist of peroxisome proliferator-activated receptor gamma, reduces chronic colonic inflammation in rats. Biochem Pharmacol 69: 1733–1744.

Sánchez-Hidalgo M, Guerrero Montávez JM, Carrascosa-Salmoral MEP, Naranjo Gutierrez MEC, Lardone PJ, de la Lastra Romero CA (2009). Decreased MT1 and MT2 melatonin receptor expression in extrapineal tissues of the rat during physiological aging. J Pineal Res 46: 29–35.

Sánchez-Miranda E, Lemus-Bautista J, Pérez S, Pérez-Ramos J (2013). Effect of kramecyne on the inflammatory response in lipopolysaccharide-stimulated peritoneal macrophages. Evid Based Complement Alternat Med 2013: 762020–762028.

Scheidereit C (2006). IkappaB kinase complexes: gateways to NF-kappaB activation and transcription. Oncogene 25: 6685–6705.

Schneider M (2013). Collecting resident or thioglycollate-elicited peritoneal macrophages. Methods Mol Biol 1031: 37–40.

Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D *et al*. (1990). New colorimetric cytotoxicity assay for anticancer-drug screening. J Natl Cancer Inst 82: 1107–1112.

Slominski A, Pisarchik A, Semak I, Sweatman T, Wortsman J, Szczesniewski A *et al*. (2002). Serotoninergic and melatoninergic systems are fully expressed in human skin. FASEB J 16: 896–898.

Soehnlein O, Lindbom L (2010). Phagocyte partnership during the onset and resolution of inflammation. Nat Rev Immunol 10: 427–439.

Tak PP, Firestein GS (2001). NF-kappaB: a key role in inflammatory diseases. J Clin Invest 107: 7–11.

Tamura H, Takasaki A, Taketani T, Tanabe M, Kizuka F, Lee L *et al*. (2013). Melatonin as a free radical scavenger in the ovarian follicle. Endocr J 60: 1–13.

Tan DX, Manchester LC, Reiter RJ, Qi WB, Zhang M, Weintraub ST *et al*. (1999). Identification of highly elevated levels of melatonin in bone marrow: its origin and significance. Biochim Biophys Acta 1472: 206–214.

Wang JY, Wen LL, Huang YN, Chen YT, Ku MC (2006). Dual effects of antioxidants in neurodegeneration: direct neuroprotection against oxidative stress and indirect protection via suppression of glia-mediated inflammation. Curr Pharm Des 12: 3521–3533.

Xia MZ, Liang YL, Wang H, Chen X, Huang YY, Zhang ZH *et al*. (2012). Melatonin modulates TLR4-mediated inflammatory genes through MyD88- and TRIF-dependent signaling pathways in lipopolysaccharide-stimulated RAW264.7 cells. J Pineal Res 53: 325–334.

Xie QW, Cho HJ, Calaycay J, Mumford RA, Swiderek KM, Lee T *et al*. (1992). Cloning and characterization of inducible nitric oxide synthase from mouse macrophage. Science 256: 225–228.

Yong HY, Koh MS, Moon A (2009). The p38 MAPK inhibitors for the treatment of inflammatory diseases and cancer. Expert Opin Investig Drugs 18: 1893–1905.

Zhang QH, Zhou ZS, Lu GS, Song B, Guo JX (2013). Melatonin improves bladder symptoms and may ameliorate bladder damage via increasing HO1 in rats. Inflammation 36: 651–657.

Zhang S, Li W, Gao Q, Wei T (2004). Effect of melatonin on the generation of nitric oxide in murine macrophages. Eur J Pharmacol 501: 25–30.