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Effect of melatonin on the generation of nitric oxide in murine macrophages

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Abstract

In the present investigation, the effects of melatonin on the generation of nitric oxide in stimulated murine macrophages were studied by electron paramagnetic resonance techniques, and the underlying mechanisms were discussed. Upon stimulation with lipopolysaccharide/interferon-γ, macrophages generated a high concentration of nitric oxide. Melatonin pretreatment significantly inhibited the generation of nitric oxide. Western blot and reverse transcription polymerase chain reaction analysis showed that melatonin decreased the expression of inducible nitric oxide synthase at either the protein or mRNA level. Further investigation showed that melatonin significantly attenuated the nitration of cytoplasmic IκB-α, inhibited the degradation of IκB-α, and blocked the translocation of p65/RelA into the nuclei. The results suggest that melatonin inhibits the expression of inducible nitric oxide synthase by modulating the nuclear factor κB pathway.
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Keywords: Melatonin; Macrophage; Nitric oxide; Inducible nitric oxide synthase; Nuclear factor κB

1. Introduction

Melatonin, or *N*-acetyl-5-methoxytryptamine, is a hormone synthesized from tryptophan mainly by the pineal gland of mammals (Reiter, 1991). However, its production is not confined exclusively to this gland, and other organs and tissues including retina, Harderian glands, gut, ovary, testes, bone marrow and lens have been reported to produce melatonin as well (Tan et al., 1999). Melatonin concentrations in tissues, cells or other body fluids are variable and range from picomolar to micromolar levels which change with the circadian rhythm. Melatonin concentrations are considerably higher in tissues than they are in serum (Reiter and Tan, 2003).

The biological functions of melatonin have been investigated extensively. Melatonin is a powerful antioxidant (Allegra1 et al., 2003; Mayo et al., 2003) and controls important physiological functions, including seasonal reproduction and circadian rhythms. Melatonin is also an effective regulator of the immune system (Guerrero and Reiter, 2002). Macrophages and T lymphocytes, which have melatonin receptors, are target cells for the immunomodulatory function of melatonin (Garcia-Perganeda et al., 1999; Carrillo-Vico et al., 2003). However, the direct effects of melatonin on immune cells have not been investigated systematically, and the results are inconsistent. An activating effect of melatonin on monocytes/macrophages has been shown in rodents (Kaur and Ling, 1999). Additionally, melatonin has been shown to synergize with lipopolysaccharide to activate monocytes (Morrey et al., 1994). In the studies mentioned above, melatonin stimulated the secretion of cytokines such as tumor necrosis factor α and interleukin 1β. Interestingly, some investigations reported that melatonin might suppress the activation of the immune system by

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down-regulating the expression of inducible nitric oxide synthase (iNOS) (Crespo et al., 1999) and decreasing the secretion of cytokines, including tumor necrosis factor α and interleukin 1β (Mei et al., 2002). In an attempt to address some of these inconsistencies, the present investigation tested the direct or indirect effects of melatonin on nitric oxide (NO) generation in activated macrophages by using electron paramagnetic resonance (EPR) spin trapping techniques. The underlying mechanisms of the redox modulation of macrophage activation are discussed.

2. Materials and methods

2.1. Materials

Adult, female BALB/c mice (body weight 20–22 g; Beijing Vital River Experimental Animal Center) were used as cell sources in the experiments. RPMI 1640 medium and cell culture supplements were obtained from Hyclone (Logen, UT, USA). Cell culture plasticware was from Corning Costar (Acton, MA, USA). Lipopolysaccharide, melatonin and interferon-γ were products of Sigma (St. Louis, MO, USA). Antibodies against iNOS, IκB-α and p65/RelA were from BD Transduction Laboratories (Lexington, KY, USA). Antibody against nitrotyrosine was from Upstate (Lake Placid, NY, USA). Reagents for the immunoprecipitation assay were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Uniq-10™ RNA purification kit was purchased from Sangon (Shanghai, China). Access Quick™ reverse transcription polymerase chain reaction (RT-PCR) kit was purchased from Promega (Shanghai, China). Other reagents were made in China.

2.2. Cell culture and melatonin treatment

Primary macrophages were isolated from BALB/c mice by peritoneal lavage using Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution, as described previously (Shafer et al., 2001). Cells were harvested by centrifugation at 200×*g* for 5 min, washed once with Hanks' balanced salt solution, and resuspended in RPMI 1640 culture medium. The viability of cells was examined by Trypan blue exclusion. Macrophages were plated at 1×10^7 cells per 25-cm^2 cell culture flask in 5 ml of RPMI 1640 media supplemented with 10 mM Hepes buffer, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin, and cultured in a humidified 5% CO_2 incubator at 37 °C.

After incubation for 2 h, nonadherent cells (less then 5% of total cells) were removed and the remaining viable macrophages (accounting for more than 95% of total cells obtained from the peritoneal lavage fluid) were treated with melatonin by changing the culture medium into fresh RPMI 1640 containing certain concentrations of melatonin. After incubation for 30 min with melatonin, macrophages were subjected to different stimuli as described below. Incubation of macrophages with lipopolysaccharide, interferon-γ or melatonin did not influence the viability of macrophages, as assessed by Trypan blue exclusion (data not shown).

2.3. Assay of nitric oxide

iNOS gene expression and the production of nitric oxide (NO) in macrophages were induced by lipopolysaccharide/interferon-γ. NO produced by activated macrophages was detected directly by EPR spin trapping (Zhou et al., 1999). Briefly, 1×10^7 macrophages cultured in 25-cm^2 cell culture flasks were incubated with certain concentrations of melatonin for 30 min, and treated with 1 μg ml^{-1} lipopolysaccharide and 100 U ml^{-1} interferon-γ in RPMI 1640 medium for 12 h. Then the spin-trapping agent containing 1 mM of $FeSO_4$, 5 mM of diethyldithiocarbamate sodium salt (DETC) and 5 mM of $Na_2S_2O_3$ was added to the activated macrophages, which were incubated at 37 °C for an additional 3 h. The paramagnetic [ON–Fe^{2+} $(DETC)_2$] complex was enriched by extraction with 200 μl of ethyl acetate and detected with a Bruker ER-200 D-SRC EPR spectrometer under the following conditions: X-band; sweep width 400 G; microwave power 20 mW; 100 kHz modulation with amplitude 3.2 G; time constant 0.128 s.

2.4. Detection of iNOS mRNA

For the analysis of iNOS gene expression, iNOS mRNA levels in macrophages were detected by reverse transcription polymerase chain reaction (RT-PCR; El-Mahmoudy et al., 2002). After melatonin treatment, macrophages were stimulated with lipopolysaccharide/interferon-γ for 3 or 6 h. Total RNA was isolated from 10^7 activated macrophages with Sangon Uniq-10™ RNA purification kit according to the manufacturer's instructions. RT-PCR was carried out using the Promega AccessQuick™ RT-PCR system, with primers specific for iNOS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the house-keeping gene. For iNOS, the primers were 5′-GTGTTCCACCAGGAGATGTTG-3′/5′-CTCCTGCCCACTGA GTTCGTC-3′; the length of the PCR product was 576 bp. For GAPDH, the primers were 5′-GAAGGGTGGGGCCAAAAG-3′/5′-GGATGCAGG-GATGATGTTCT-3′; the length of the PCR product was 295 bp. PCR products were visualized by electrophoresis on 1.5% agarose gels stained with ethidium bromide.

2.5. Immunoblot analysis

The levels of iNOS and nuclear factor κB proteins in macrophages were determined by Western blot. Following melatonin treatment and lipopolysaccharide/interferon-γ stimulation, 10^7 macrophages were detached from the cell culture flasks with a rubber policeman and lysed with 300 μl of buffer A (0.2% Nonidet P-40, 10 mM Hepes, 10 mM KCl, 100 μM EDTA, 100 μM EGTA, 2 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin

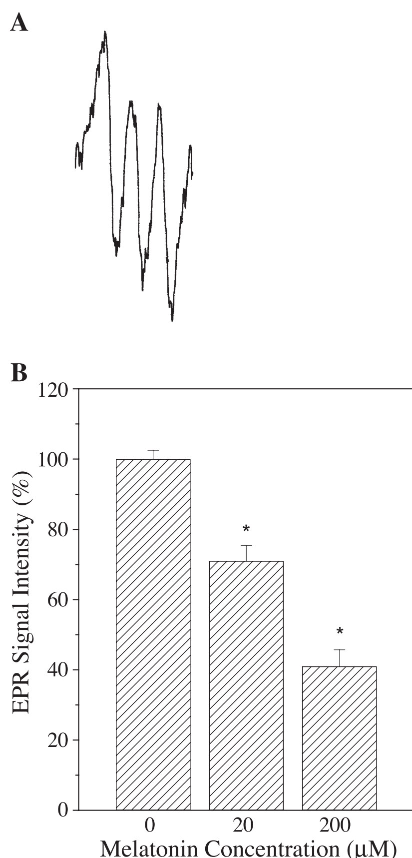

Fig. 1. (A) EPR spectrum of the [ON–Fe^{2+} $(DETC)_2$] complex. Macrophages were stimulated with 1 μg ml^{-1} lipopolysaccharide plus 100 U ml^{-1} interferon-γ for 12 h. NO was trapped with [Fe^{2+} $(DETC)_2$] and the EPR spectrum was recorded with a Bruker ER-200 D-SRC EPR spectrometer. (B) Inhibition effect of melatonin on NO production. Macrophages were pretreated with 20 or 200 μM of melatonin for 30 min and then stimulated with lipopolysaccharide/interferon-γ for 12 h. Data presented are means±SD, n=6. *: P<0.05 in comparison with control macrophages.

and 1 μg ml^{-1} leupeptin) at 4 °C for 15 min. After centrifugation at 12,000×g for 15 min, the supernatant containing cytosolic proteins was collected and used for the assay of iNOS, IκB-α and nitrated IκB-α proteins. The pellet was washed once with buffer A and then suspended in 100 μl of buffer B (20 mM Hepes, 390 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2 mM NaF, 2 mM phenylmethylsulfonyl fluoride). After centrifugation at 12,000×g for 15 min, the supernatant was collected and used as nuclear protein for the assay of p65/RelA.

For the assay of iNOS, IκB-α and p65/RelA proteins, each sample containing 60 μg of total proteins was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes. The membrane was incubated with antibodies against iNOS, IκB-α and p65/RelA proteins, respectively, and then incubated with horseradish peroxidase-conjugated second antibodies. The target proteins were detected by chemiluminescence using the Amersham Pharmacia ECL^{Plus} Western blotting detection kit and exposed to Kodak X-ray autoradiography films.

For analysis of nitrated IκB-α, immunoprecipitation was carried out (Xiong et al., 2003). The supernatants of cell lysates containing cytosolic proteins were incubated with mouse anti-IκBα monoclonal antibody at 4 °C overnight, mixed with Protein A-conjugated Sepharose beads and incubated at 4 °C for 45 min. Then the Sepharose beads were washed twice in buffer A, once in 0.25 M LiCl washing buffer, and twice in 50 mM Tris–HCl (pH 7.5) washing buffer. Proteins were eluted and analyzed by Western blot with an antibody against nitrotyrosine, as described above.

2.6. Statistical analysis

Each experiment was performed at least three times and the results are presented as means±S.D. The data were analyzed by one-way analysis of variance (ANOVA). A level of P<0.05 was considered significant.

3. Results

3.1. Inhibition of nitric oxide production by melatonin

A three-line EPR spectrum corresponding to the [ON–Fe^{2+} $(DETC)_2$] complex at g=2.035 was observed in macrophages treated with lipopolysaccharide/interferon-γ

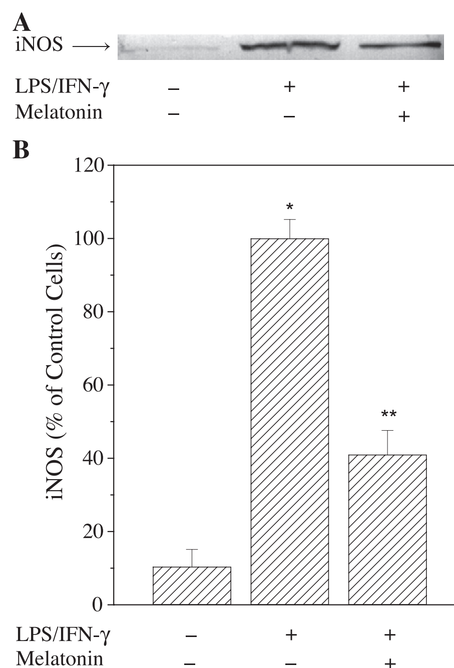

Fig. 2. (A) Typical result of Western blot analysis of iNOS gene expression. Macrophages were pretreated with 200 μM of melatonin for 30 min and then stimulated with 1 μg ml^{-1} lipopolysaccharide plus 100 U ml^{-1} interferon-γ for 12 h. iNOS protein was determined by Western blot. (B) Quantitative analysis of iNOS protein levels. Data presented are means±S.D., n=3. *: P<0.05 in comparison with untreated macrophages. **: P<0.05 in comparison with macrophages stimulated with lipopolysaccharide/interferon-γ for 12 h.

for 12 h (Fig. 1A), suggesting that stimulated macrophages do generate nitric oxide. No EPR signal was observed in macrophages without lipopolysaccharide/interferon-γ treatment. Pretreatment of macrophages with melatonin suppressed the EPR signal intensity dose dependently, as shown in Fig. 1B. In macrophages pretreated with 20 μM or 200 μM of melatonin, the EPR signal intensity of the [ON–Fe^{2+} $(DETC)_2$] complex was 71.0±6.4% and 41.5±8.4% of control macrophages, respectively.

3.2. Effects of melatonin on the expression of iNOS gene

To understand the mechanisms of the inhibition of lipopolysaccharide/interferon-γ-induced NO production by melatonin, the effect of melatonin on the expression of iNOS gene was studied. Immunoblotting with antibody against iNOS protein showed that lipopolysaccharide/interferon-γ induced the expression of iNOS gene in macrophages, as shown in Fig. 2A. Melatonin down-regulated the expression of iNOS significantly. Pretreatment of macrophages with 200 μM of melatonin for 30 min decreased the iNOS protein level to 41.0±6.6% of that of control macrophages (Fig. 2B).

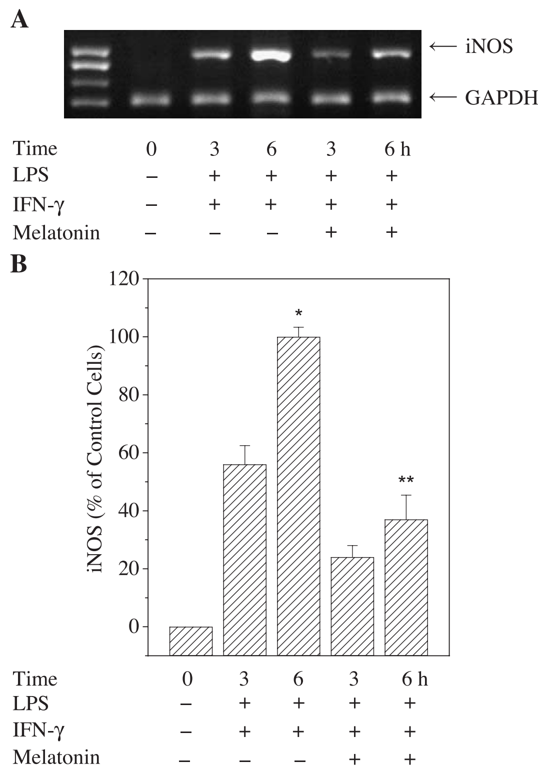

Fig. 3. (A) Typical result of RT-PCR analysis of iNOS gene expression. Macrophages were pretreated with 200 μM of melatonin for 30 min and then stimulated with 1 μg ml^{-1} lipopolysaccharide plus 100 U ml^{-1} interferon-γ for the indicated times. iNOS mRNA was determined by RT-PCR. Marker: 100-bp DNA ladder. (B) Quantitative analysis of iNOS mRNA levels. Estimates of the relative iNOS mRNA amounts were obtained by dividing the peak density of the iNOS band by the peak density of the GAPDH band. Data presented are means±S.D., n=3. *: P<0.05 in comparison with untreated macrophages. **: P<0.05 in comparison with macrophages stimulated with lipopolysaccharide/interferon-γ for 6 h.

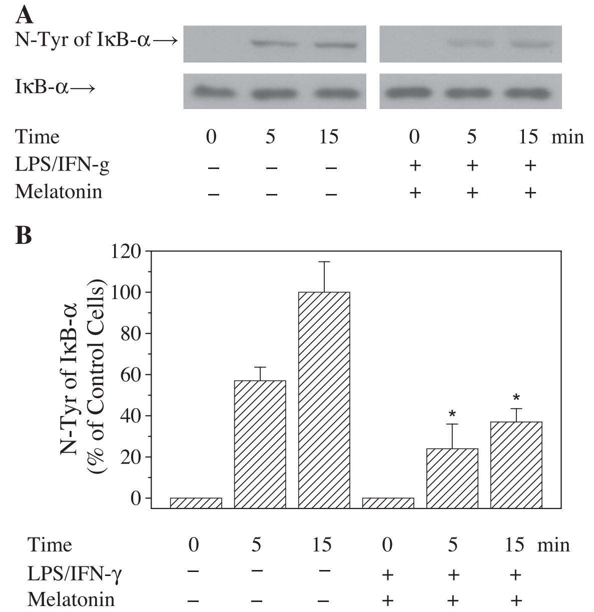

Fig. 4. (A) Rapid tyrosine nitration of IκB-α in macrophages. Macrophages were pretreated with 200 μM of melatonin for 30 min and then stimulated with 1 μg ml^{-1} lipopolysaccharide plus 100 U ml^{-1} interferon-γ for the indicated times. The formation of nitrotyrosine in cytosolic IκB-α was determined by immunoprecipitation. (B) Quantitative analysis of tyrosine nitration. Estimates of the relative nitrotyrosine amounts were obtained by dividing the peak density of the nitrotyrosine band by the peak density of the IκB-α band. Data presented are means±S. D., n=3. *: P<0.05 in comparison with macrophages stimulated with lipopolysaccharide/interferon-γ for 6 h.

Fig. 3A shows a typical result of RT-PCR analysis of iNOS mRNA in macrophages stimulated with lipopolysaccharide/interferon-γ. Melatonin significantly decreased the level of iNOS mRNA. In macrophages pretreated with 200 μM of melatonin for 30 min and then stimulated with lipopolysaccharide/interferon-γ for 6 h, the level of iNOS mRNA decreased to 37.0±8.4% of control (Fig. 3B).

3.3. Modulation of NF-κB by melatonin

The effects of melatonin on the nitration and the consequent degradation of IκB-α, as well as the translocation of p65/RelA into the nuclei, were measured. As shown in Fig. 4A, stimulation of macrophages with lipopolysaccharide/interferon-γ induced the rapid nitration of the tyrosine residues of the IκB-α, as assayed by immunoprecipitation. Then IκB-α underwent degradation. In macrophages stimulated with lipopolysaccharide/interferon-γ for 1 h, the level of IκB-α decreased to 36.0±3.4% of that of untreated cells (Fig. 5). The nitration and degradation of IκB-α were partially abolished by melatonin.

After exposure of macrophages to lipopolysaccharide/interferon-γ, p65/RelA translocated into the nucleus. One hour after stimulation, the nuclear p65/RelA level

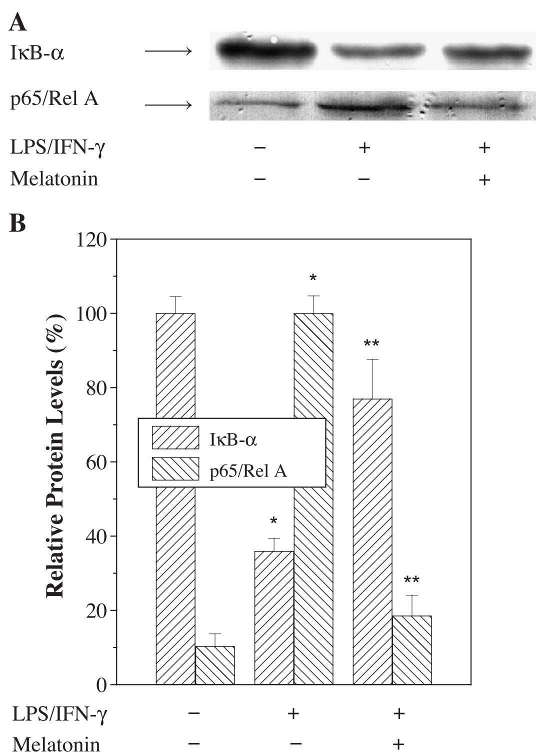

Fig. 5. (A) Degradation of IκB-α and translocation of p65/Rel A into the nuclei. Macrophages were pretreated with 200 μM of melatonin for 30 min and then stimulated with 1 μg ml^{-1} lipopolysaccharide plus 100 U ml^{-1} interferon-γ. The levels of cytosolic IκB-α and nuclear p65/Rel A were determined by Western blot. (B) Quantitative analysis of degradation of IκB-α and translocation of p65/Rel A. Data presented are means±S.D., n=3. *: P<0.05 in comparison with untreated macrophages. **: P<0.05 in comparison with macrophages stimulated with lipopolysaccharide/interferon-γ for 1 h.

increased more than 10-fold compared with that of untreated cells, as shown in Fig. 5. Pretreatment with melatonin significantly inhibited the translocation of p65/RelA into the nucleus.

4. Discussion

Macrophages are pivotal effector cells in the innate immune system. When microbial products bind to pathogen recognition receptors, macrophages are activated and release a broad array of mediators, such as cytokines, that orchestrate the inflammatory responses of the host. Upon stimulation by certain cytokines, the iNOS gene of macrophages is induced and large amounts of NO are generated. NO may cause the formation of endogenous reactive oxygen species (ROS) including hydrogen peroxide, peroxynitrite and other potentially oxidants, which are required for the microbicidal properties of macrophages. However, excessive production of NO and its downstream products has been implicated in a number of pathological processes (MacMicking et al., 1997), such as the development of neurodegenerative disorders (Minagar et al., 2002) and cancer (Xu et al., 2002). Accordingly, drugs that inhibit the generation of these mediators may have beneficial effects in the treatment of diseases due to the overactivation of macrophages.

It has been well documented that melatonin acts as an effective antioxidant (Allegra1 et al., 2003; Mayo et al., 2003) as well as an immune regulator. However, the possible relationship between its antioxidant properties and immune regulation abilities has not been discussed yet. In the present investigation, we measured the direct effect and the underlying mechanisms of melatonin on NO generation by activated macrophages.

Melatonin significantly decreased the EPR signal intensity of NO. Considering that melatonin shows no direct scavenging effects on NO (data not shown), the decreased NO concentration in melatonin-treated macrophages is due to the suppression of iNOS expression, which was confirmed by RT-PCR and Western blot.

To further understand the underlying mechanisms, the effects of melatonin on the NF-κB signaling pathway were studied. NF-κB is a sequence-specific DNA binding protein complex that regulates immune and inflammatory responses by increasing the expression of certain genes, including some cytokines and inducible enzymes such as iNOS (Baldwin, 1996). The NF-κB transcription factor family consists of five proteins: p65/Rel A, p50, c-Rel, Rel B, and p52. Each protein can dimerize with other family members to form homo- and heterodimers through the N-terminal Rel homology domain, which is also involved in translocation to the nucleus and DNA binding. NF-κB can be activated by a wide variety of stimuli including lipopolysaccharide and certain cytokines. The phosphorylation and degradation of cytoplasmic IκB-α, and the translocation of p50/p65 from the cytoplasm into the nucleus, are two key steps in NF-κB activation. In the present investigation, we found that modulation of tyrosine nitration by melatonin plays an important role in the regulation of NF-κB. Upon lipopolysaccharide/interferon-γ exposure, there was rapid tyrosine nitration of the cytoplasmic IκB-α. If the nitration of IκB-α occurs at Tyr-42, this may abolish the negative regulation of IκB-α degradation facilitated by phosphorylation of this tyrosine residue (Singh et al., 1996), resulting in accelerated IκB-α degradation. Alternatively, any nitration of other tyrosine residues may be subjected to preferential degradation (Gow et al., 1996). The nitration of IκB-α was partially abolished by melatonin pretreatment, suggesting that melatonin modulates the NF-κB signaling pathway by protein nitration-related mechanisms. This is in accordance with the report that antioxidants/pro-oxidants modulate the activation of NF-κB by tyrosine nitration-related mechanisms (Xiong et al., 2003). However, considering that the NF-κB signaling pathway is redox sensitive (Li and Karin, 1999), and that melatonin is a potent endogenous regulator of the cellular redox status, it remains to be determined whether melatonin regulates the NF-κB signaling pathway

of macrophages by other mechanisms, such as by modulating the cellular redox status.

In conclusion, melatonin is an effective modulator of macrophages. It regulates the production of NO by modulating the activation of the iNOS gene via the NF-κB signaling pathway. Melatonin might be a potential therapeutic and defense agent for diseases associated with the overactivation of macrophages.

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