# ANTI-INFLAMMATORY ACTIONS OF MELATONIN AND ITS METABOLITES, N1-ACETYL-N2-FORMYL-5-METHOXYKYNURAMINE (AFMK) AND N1-ACETYL-5-METHOXYKYNURAMINE (AMK), IN MACROPHAGES

Juan C. Mayo[1,2]; Dun-Xian Tan[3]; Rosa M. Sainz[1,2]; Rüdiger Hardeland[5]; Josefa Leon[3]; Carmen Rodriquez[1,2]; Russel J. Reiter[3].

1, Departamento de Morfología y Biología Celular, Universidad de Oviedo, Asturias, España
2, Instituto Universitario de Oncología del Principado de Asturias (IUOPA), Universidad de Oviedo
3, Department of Cellular and Structural Biology, University of Texas Health Science Center at San Antonio, San Antonio, TX, USA.
4, Institute of Zoology and Anthropology, University of Göttingen, Göttingen, Germany

**ABSTRACT:**

Inflammation is a complex phenomenon involving multiple cellular and molecular interactions which must be tightly regulated. Cyclooxygenase-2 (COX) is the key enzyme that catalyzes the two sequential steps in the biosynthesis of PGs from arachidonic acid. The inducible isoform of COX, namely COX-2, plays a critical role in the inflammatory response and its over-expression has been associated with several pathologies including neurodegenerative diseases and cancer. Melatonin is the main product of the pineal gland with well documented antioxidant and immuno-modulatory actions. Since the action of the indole on the COX-2 has not been previously described, the goal of the present report was to test the effect of melatonin on the activities of COX-2 and inducible nitric oxide synthase (iNOS), using lipopolysaccharide (LPS)-activated RAW 264.7 macrophages as a model. Melatonin and its metabolites, N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK) and N1-acetyl-5-methoxykynuramine (AMK), prevented COX-2 activation induced by LPS, without affecting COX-1 protein levels. The structurally-related compound 6-methoxy-melatonin only partially prevented the increase in COX-2 protein levels induced by the toxin. Likewise melatonin prevented iNOS activation and reduced the concentration of products from both enzymes, $PGE_2$ and nitric oxide. Another endogenous antioxidant like N-acetyl-cysteine (NAC) did not reduced COX-2 significantly. The current finding corroborates a role of melatonin as an anti-inflammatory agent and, for the first time, COX-2 and iNOS as molecular targets for either melatonin or its metabolites AFMK and AMK. These anti-inflammatory actions seem not to be exclusively mediated by the free radical scavenging properties of melatonin. As a consequence, the present work suggests these substances as a new class of potential anti-inflammatory agents without the classical side effects due to COX-1 inhibition.

## INTRODUCTION

Macrophages and their circulating counterparts, monocytes, are potent defenders of physiological integrity in vertebrates with important roles in inflammatory reactions. Inflammation is a complex and incompletely understood phenomenon involving numerous mediators of cellular and plasma origin which trigger a number of biological effects that are crucial for the host's normal defense against insults, pathogens or stress. If the inflammatory response is not tightly regulated, chronic inflammation occurs, which accounts for a variety of different pathologies, e.g., cancer and neurodegenerative diseases (1;2). Among the effectors released during the inflammatory process, prostanoids are some of the most important biological mediators implicated in many stages of the response.

Vasoactive prostaglandins (PGs) are essential components in the regulation of vascular function under normal physiological conditions, and their role in the inflammatory process is well known. PGs synthesis pathway is a major source of reactive oxygen species (ROS) during the conversion of $PGG_2$ to $PGH_2$. Cyclooxygenase (COX) or prostaglandin $H_2$ synthase is the key enzyme that catalyzes the two sequential steps in the biosynthesis of PGs from arachidonic acid (AA) (3). This enzyme exists in at least two isoforms: a constitutively expressed enzyme known as COX-1, which is highly localized in endothelial cells and platelets; and an inducible form, referred as COX-2, which is induced by a variety of stimuli associated with cell activation and inflammation. COX-1 has clear physiological functions whereas COX-2 is induced by pro-inflammatory stimuli in migratory cells and inflamed tissues (3).

Lipopolysaccharide (LPS) binds to the Toll-like receptor 4 (Tlr4) and initiates several major cellular responses which are involved in the pathogenesis of endotoxic shock, including the expression of COX-2 and the inducible 120 kD isoform of nitric oxide synthase (iNOS). Both PGs, metabolites of the COX pathway from AA, or nitric oxide (NO) formed from L-arginine by the enzymatic action of nitric oxide synthase (NOS), play important roles in inflammation, immune functions, blood vessel dilatation

and neurotransmission. Over production of PGs and NO during inflammation, often elicited by the same stimuli, is associated with local and systemic symptoms of fever, pain and edema (4;5) . Therefore, mechanisms controlling PG production are of special interest to counteract the an inflammatory process (6;7). Since both COX-2 and iNOS are inducible forms up-regulated in response to inflammatory challenge, they are traditionally associated with pathological states (3;8). To date, several cell systems have been successfully used for the study of COX-2 regulation, such as RAW 264.7 murine macrophage cells previously stimulated with LPS (9). The results of these studies are usually extrapolated to macrophages in general and/or microglial cells. Insights into this cellular model can give critical data about the regulation of the inflammatory response.

A major mechanism of action of nonsteroid anti-inflammatory drugs (NSAIDs) is the inhibition of biosynthesis of PGs (3;10). Discovery of COX-2 specific inhibitors could solve one of the main problems in therapeutics, i.e., to suppress inflammation while reducing the side effects of classical NSAIDs treatment, including gastrointestinal ulceration and bleeding, renal damage and platelet dysfunction. Results published to date support the hypothesis that the unwanted side effects of NSAIDs, such as damage to the gastric mucosa and kidneys, are due to their ability to inhibit COX-1, while their anti-inflammatory (therapeutic effects) are due to inhibition of COX-2. Thus, drugs that have high potency for COX-2 and a lesser effect on COX-1 would provide potent anti-inflammatory activity with fewer side effects. COX-2 is abundantly expressed in human colon cancer cells, and NSAIDs delay the progress of colon tumors possibly by causing apoptosis of the tumor cells. Furthermore, the risk of developing Alzheimer's disease, which may involve an inflammatory component, is reduced by chronic ingestion of NSAIDs. A new highly selective inhibitor of COX-2 could provide a means of delaying premature labor and lead to advances in cancer therapy and protection against Alzheimer's disease (3;11).

Melatonin, or N-acetyl-5-methoxytryptamine, is an indole derived from tryptophan mainly produced in the mammalian pineal gland during the dark phase. Melatonin secretion from the pineal gland exhibits a distinctive circadian rhythm (12). Thus, melatonin has been classically associated with circadian

and circanual rhythm regulation, and with adjustments of physiology of animals to seasonal environmental changes (12;13). Melatonin production, however, is not confined exclusively to the pineal gland and other organs and tissues including retina, Harderian glands, gut, ovary, testes, bone marrow and lens also have been reported to produce it (14;15). Melatonin is also synthesized in non-mammalian vertebrates, invertebrates and in other organisms including dinoflagellates, algae and bacteria (16). The presence of melatonin in such a variety of organisms suggests that this substance is phylogenetically highly conserved and plays an important role in the function and survival of organisms. In addition, during the last decade a large number of articles have shown melatonin to be a potent antioxidant and free radical scavenger, protecting against a number of radical species in both in vivo and in vitro models of oxidative stress (17;18). Melatonin directly scavenges several ROS, especially the highly reactive hydroxyl radical, and protects against oxidative stress-related processes in experimental models of ischemia/reperfusion, aging and neurodegenerative disorders among others. In addition to its roles as an adjustor to circadian rhythms and protector against ROS, its function in oncostasis (19) and as a modulator of the immune system (20) have also been widely reported. Due to the potential role of melatonin as an endogenous antioxidant and a regulator of immune system and since the pro-inflammatory enzyme COX-2 plays an important role in the immune response and seems to be subjected to redox control, the aim of the present work was to determine whether melatonin and its metabolites N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK) and N1-acetyl-5-methoxykynuramine (AMK), might regulate the expression and activity of COX-2 and iNOS in LPS-activated macrophages and, if so, whether this is related to its antioxidant activity.

## MATERIALS AND METHODS

### *Drugs and Treatments*

LPS (from *E.coli*, isotype 0111:B4) was purchased from SIGMA (SIGMA-Aldrich, St. Louis, MO, USA). Ultra pure grade melatonin was a kind gift from Helsinn Chemical (Biasca, Switzerland). All culture reagents were purchased from Invitrogen (GBCO-BRL, Rockville, MD, USA). Vitamin C, N-acetylcysteine (NAC), trolox, 3-(4,5-dimethyl-2-thiazolyl) 2,5-diphenyl-2H-tetrazolium bromide (MTT), propidium iodide, and 2',7'-dichlorofluorescein diacetate (DFCH-DA) were obtained from Sigma Chemical (St. Louis, MO, USA). Culture flasks, plates, and dishes were purchased from Falcon (Falcon BD Biosciences, Franklin Lakes, NJ, USA). RAW cells were purchased from American Type Culture Collection (ATCC, #TIB-71). Vitamin C and NAC were freshly prepared in Hanks-Balance Salt Solution (HBSS) and the pH was adjusted to 7.4 using sterile 1N NaOH. For all the experiments reported here, cells were seeded at a density of $7x10^5$ cells/ml in 60 or 100 mm Petri dishes and left to attach overnight. In the groups indicated, melatonin, other indoles or antioxidants used were added from a 500x stock solution in 100% DMSO 30 min prior to LPS addition. Vehicle (0.2% DMSO) was always added to the controls and LPS groups. LPS (1 μg/ml) was added from a 1000x stock solution in sterile water and cells were incubated for 18 h, unless otherwise indicated in the time-course experiments.

### *Synthesis of AFMK and AMK*

AFMK was synthesized according to previous reported method (21). Briefly, $H_2O_2$ was diluted to 50 mM with PBS (50 mM, pH 7.0) and melatonin was added to make a final concentration of 1 mM. The mixture was incubated at room temperature for 2 h. The majority components of this solution were then mixed with an equal volume of dichloromethane and shaken horizontally for 10 min. The water phase was discarded and the organic phase was left dried in a vacuum chamber. The residue was redissolved in a small volume of methanol and fractionated by analytical thin layer chromatography with silica gel on

polyester, fluorescent indicator, layer of 250 µm and 20 × 20 cm (TLC) using ethyl acetate as the solvent. The major spot (about 90% in all metabolites), which migrated with an Rf of 0.2 (detected with UV lamp at 254 nm) was scraped from the TLC plate and extracted with methanol. The TLC purification was repeated two additional times. The purified product was then identified by HPLC (21). AMK was synthesized according to Ressmeyer and co-workers from AFMK (22).

*Culture of RAW 264.7 cells*

RAW 264.7 murine macrophages were maintained in Dulbecco's modified Eagle's Medium (DMEM) containing 4.5 g/L glucose, 2 mM glutamine, 250 UI penicillin, 250 µg/ml streptomycin, 10% of FBS (Invitrogen GIBCO, Carlsbad, CA, USA) and 20 mM HEPES buffer under a 5% $CO_2$ atmosphere at 37º C. The medium was changed every 2 days. Cells were always subcultured before they reached confluency. For all experiments, RAW cells were collected in trypsin-free PBS with sterile rubber cell scrapers.

*Protein lysis and SDS-polyacrylamide gel electrophoresis (SDS-PAGE)*

Cells were harvested by scrapping and they were pelleted by centrifugation. After washing twice with ice-cold PBS buffer, cells were resuspended in protein lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% SDS, 1% Igepal C, 0.5% deoxycholate, 1 mM DTT and fresh added protease inhibitors, 2 µg/ml aprotinin, 1µg/ml leupeptin, 1 mM PMSF, 1µg/ml pepstatin 1mM NaF, 0.2 mM sodium orthovanadate) and incubated on ice for 30 min. Then, after centrifugation at 10,000 xg for 5 min., protein concentration was determined (Bradford protein assay kit, Bio-Rad Laboratories, Hercules, CA, USA). Samples were mixed with loading buffer (4x concentrated: 40% glycerol, 8% SDS, 0.25 mM Tris-HCl pH=6.8, 20% ß-mercaptoethanol, 0.01% bromophenol blue) and heated at 100 ºC for 5 min. Fifty µg of protein sample were then loaded in a 12.5% polyacrylamide gel and electrophoresed according to Laemmli's method using the Mini-Protean® III system (Bio-Rad Laboratories, Hercules, CA, USA).

*DAPI staining and quantification of apoptotic cells*

Cells were collected by scrapping and they were pelleted by centrifugation. After washing twice in PBS, cells were resuspendend in a small volume of PBS to avoid clusters and fixed in 4% paraformaldehyde 0.1M phosphate buffer. Cells (20 µl) were placed on a poly-L-Lysine treated slide, left to air-dry and stained with DAPI (2 µg/ml). After 5 min, cells are washed in PBS and observed under a fluorescence microscope (Leica DMIL, Leica Microsystems, Wetzlar, Germany). Cells with apoptotic morphology were considered as positive and the percentage of apoptotic *vs* total cells were counted. At least 500 cells per group were counted.

*DNA electrophoresis*

Apoptosis was evaluated by DNA fragmentation, using the method described by Saldeen and Welsh (23). For this purpose cells were cultured and harvested as described above and lysed in 400 µl of lysis buffer containing 100 mM Tris HCl pH=8.5, 200 mM NaCl, 5 mM EDTA, 0.2% SDS and fresh-added 250 µg/ml of proteinase K. After incubating samples overnight at 37 ºC, high molecular weigh DNA was precipitated with isopropanol and discarded. Then, low molecular weigh DNA was precipitated by adding 2 volumes of ethanol. DNA was recovered by centrifugation, loaded in a 2 % agarose gel. After running 2 hours, DNA was photographed using a polaroid camera.

*Western blotting*

Proteins were electrophoretically transferred to PVDF membranes (Pall Life Sciences, VWR, NJ, USA) using the Trans-blot Cell® system (Bio-Rad, Hercules, CA, USA). After blocking the membranes with 5% non-fat dry milk in TBS-T (Tris HCl 20 mM pH=7.4, 150 mM NaCl, 0.05% Tween 20) for 1h at RT, membranes were incubated overnight at 4 ºC with the appropriate primary antibody in TBS-T (1:1,000 dilution of anti COX-1 or COX-2 polyclonal antibodies, Cayman chemicals, Ann Arbor, MI, USA; 1:200 dilution of anti NOS2, # sc-651, Santa Cruz Biotechnology, Santa Cruz, CA, USA). After

washing three times with TBS-T, membranes were reacted to the appropriate secondary antibodies and developed using the ECL® chemiluminiscent reagent (Amersham Biosciences, Buckinghamshire, UK).

*$PGE_2$ determination*

Cells were cultured in phenol red-free DMEM culture medium as described above. After treatments with LPS and/or melatonin, medium was collected, centrifuged to obtain a cell-free fraction and rapidly frozen at -70ºC until assays were performed. $PGE_2$ concentration in the supernatant of culture media was determined using the $PGE_2$ EIA kit (Cayman Chemicals, Ann Arbor, MI, USA) using the manufacturer´s instructions.

*Nitrite determination*

NO was determined indirectly by assaying the nitrite concentration using the previously described method (24). Briefly, 50 µl of phenol red-free medium from the supernatant of cells cultured in the presence of the different treatments or the adequate vehicles were collected and mixed with one volume of freshly-prepared Griess reagent (0.05% of NADE, 0.5% sulphanilic acid, 2.5% of phosphoric acid) and incubated 10 minutes at RT. Absorbance was measured in a ELISA plate reader (µQant, Bio-Tek Instruments, Winooski, VT, USA) at 550 nm, using sodium nitrite as standard.

*DCF-DH fluorescence*

To determine the potential effect of antioxidants on the COX-2 protein levels, the free radical generation was determine using DCF fluorescence. Briefly cells were treated as described above with the vehicle (0.2% DMSO) or melatonin in the absence or presence of LPS (1µg/ml). Since the purpose of this experiment was to test the possible increase in ROS during the early phase of LPS treatment, we performed the assay after 1 h of LPS treatment. At this point, cells were incubated with DCF-DH (100 µM) for 10 min at 37 ºC and then washed twice with PBS and collected by scraping. Fluorescence was

measured in a 7000 Bio Assay multiwell plate reader (Perkin-Elmer, Boston, MA, USA) with an excitation/emission filter of 485/530 nm, respectively.

## RESULTS

*Melatonin inhibits COX-2 and iNOS expression in LPS-stimulated macrophages*

As shown in Figure 1A, the indole melatonin, in a dose-dependent manner, inhibited the LPS-induced increase of COX-2 protein expression in RAW 264.7 macrophages. Melatonin did not alter the expression pattern of the constitutive isoform, COX-1 (house-keeping), therefore revealing a specific effect on the inducible form. Since both enzymes, COX-2 and iNOS are usually induced under immune stimulation, we also investigated the putative role of melatonin on the inducible isoform of NOS. Melatonin also prevented the increase in iNOS induced by the LPS in these cells (Fig. 1A). However, the efficacy of melatonin in inhibiting this enzyme was lesser. Thus, this indole reduced the major biochemical consequences of immune activation, without preventing the morphological features of early macrophage activation.

*Inhibition of $PGE_2$ release*

$PGE_2$, one of the main products of the COX pathway, is usually assayed as an indirect measure of COX-2 activity (3). Thus, we studied the effects of a wide range of melatonin concentrations in RAW 264.7 cells after 18 hours of LPS treatment. As it could be deduced from the action on COX-2 protein levels, melatonin prevented the increase in $PGE_2$ induced by LPS incubation for the times indicated in macrophage cells. Figure 1B shows that melatonin, especially at 2 mM inhibited significantly $PGE_2$ release into the culture media at all times studied. While 1 mM melatonin also reduced significantly $PGE_2$ levels, especially at short times (18h), lower concentrations ($\leq$0.5 mM) did not alter the prostaglandin release induced by LPS. Likewise melatonin by itself, without LPS induction, did not change basal concentrations of $PGE_2$ in the media (data not shown), confirming the absence of action on COX-1 as deduced from western blot.

*Inhibition of nitrite levels*

As mentioned above, melatonin also reduced iNOS protein levels expressed during LPS stimulation in macrophages. NO levels in the media reflect the direct function of iNOS activity and therefore we measured it by assaying nitrite levels in the supernatant of RAW 264.7 macrophage cells after stimulation with LPS for different time intervals. As shown in figure 1C, melatonin, at pharmacological concentrations, prevented the increase of NO along the time of LPS incubation, confirming the inhibitory action on iNOS observed by western blotting. Then we studied different concentrations of melatonin after 18 hours of LPS incubation (Fig 1D). Interestingly, the effectiveness of melatonin on NO levels is greater than on the $PGE_2$ levels, since concentrations of melatonin as low as 0.1 mM significantly reduced nitrite concentration in the supernatant of LPS-treated macrophages for 18 hours (Fig. 1D). Lower concentrations of the indole (< 0.1 mM) were totally ineffective in altering nitrite levels. Nitrite levels were unaffected by melatonin itself without LPS activation and were almost undetectable as it occurs in vehicle-treated control cells.

*Comparative effect of melatonin and other NSAIDS*

To compare the effects of melatonin with other well known NSAIDS including acetyl salicylic acid (ASA) or indomethacin (IND) we also evaluated the role of these agents on COX-2 and COX-1 and their relative effect in relation to effective concentrations of melatonin. Figure 1E shows that both non-specific COX-2 inhibitors, ASA and IND, on the contrary to melatonin, did not affect either COX-1 or COX-2 protein levels, as it was expected, since these substances only affect enzyme activity but not the protein levels. As a positive control, here we show that the cAMP synthesis activator, forskolin (FOR), completely prevented the COX-2 increase associated with LPS treatment in RAW 264.7 macrophages (Fig 1F), while low concentrations of ASA or IND did not.

*Effects of other antioxidants on COX-2 levels*

Melatonin has been demonstrated to be a very good endogenous antioxidant and in some models to increase intracellular glutathione (GSH) levels. This prompted us to test the ability of other antioxidants

to block the COX-2 activation. For this purpose we used the glutathione analogue, N-acetyl-cysteine (NAC) to artificially increase the intracellular GSH levels and test the effect on COX-2. NAC did not blocked significantly LPS-induced COX-2 activation at any of the concentrations tested, either low (0.5-1 mM) or high (5-20 mM) (Fig. 2A and 2B, respectively). In addition, the DCF-DH study was performed to evaluate the possible increase in free radicals after short term of LPS incubation. We chose a short time since the response to LPS activation leads to a rapid activation of the oxidative stress responsive factors (e.g., NFκB) which drive the expression of COX-2 and iNOS. The study, showed no effect of LPS incubation on ROS generation. Furthermore, this response was not modified by melatonin (Table 1), thus demonstration no free radical formation change during the LPS treatment.

*Effect of kynuramines and other structurally-related indoles on COX-2 levels*

Since other non-antioxidant properties of melatonin might underlie the COX-2 inhibition, and given that the kynuramines AFMK and AMK are formed from melatonin via pyrrole ring cleavage (22;25), in order to determine the possible relationship between structure and anti-inflammatory function, we tested the effect of the kynuramines on COX-2 levels in RAW 264.7 macrophage cell line stimulated with LPS. As shown in figure 3A, both, AFMK and AMK, at pharmacological concentrations, prevented the increase in COX-2 induced by LPS activation for 18 hours, without a significant influence on COX-1 levels. Therefore these results pointed out the potential anti-inflammatory properties of these kynuramines which can result from oxidative metabolism of melatonin. To determine the relative efficacy, a dose-response study was performed. In the case of AFMK, concentrations ranging from 100 to 500 μM were effective whereas AMK, at concentrations under 500 μM was ineffective (Fig 3B). Additionally, the structurally related compound 6-methoxy-melatonin (6-m-Mel) was also included in this study to determine its effect on COX-2 protein levels after LPS stimulation. Figure 3C illustrates the effect of 6-m-Mel on COX-2 levels. Only the highest concentration assayed here (1mM) is effective and partially prevents the LPS-induced increase in COX-2.

*Melatonin induces apoptosis in LPS-treated macrophages*

Since other specific COX-2 inhibitors and NSAIDs induce apoptosis in LPS-stimulated RAW cells, we tested whether melatonin, due to its effects on COX-2, would induce apoptosis in RAW 264.7 macrophages previously stimulated with LPS. Results obtained regarding the induction of apoptosis by melatonin in macrophages cultured with LPS for 24h are summarized in figure 4. Apoptosis was estimated by DAPI staining and counting of shrank cells with condensed nuclei (morphological features of apoptotic cells). Figure 4A shows DAPI-stained RAW 264.7 macrophage cells cultured under different conditions. While vehicle treated cells showed a normal fibroblastic morphology (Fig. 4A, left panel), those incubated with melatonin plus LPS displayed typical features of apoptosis with condensed nuclei; apoptotic bodies were frequently observed in many cells (Fig 4A, right panel). Cells displaying apoptotic morphology were counted and percentage of apoptosis was estimated. As shown in figure 4B, melatonin pre-treatment (2-1 mM) induced an increase of apoptosis in LPS-treated cells. Although LPS alone slightly increased apoptosis after 18 hours of incubation it was greatly enhanced by the presence of melatonin (Fig 4B). These results were further confirmed by DNA electrophoresis, showing a classical DNA fragmentation, a key feature of apoptosis (Fig. 4C).

*Modulation of NO and $O_2^{\bullet-}$ do not interfere on melatonin-induced apoptosis*

It has been previously demonstrated that $NO^{\bullet}$ and/or $O_2^{\bullet-}$ concentrations might influence the degree of apoptosis in LPS treated macrophages. Low levels of $NO^{\bullet}$ activate NFκB and AP-1, therefore preventing apoptosis in RAW 264.7 macrophages (26). This prompted us to exogenously manipulate the $NO^{\bullet}$ levels by using the $NO^{\bullet}$ donor S-Nitroso-L-glutahione (GSNO) and the specific iNOS inhibitor L-NAME, to increase and decrease intracellular $NO^{\bullet}$ levels respectively. As we show here, modulation of intracellular $NO^{\bullet}$ concentrations did not influence apoptosis induced by melatonin in LPS-treated RAW 264.7 macrophages. The specific inhibition of iNOS with L-NAME did not modify the pattern of apoptosis caused by melatonin (Fig. 5a). On the other hand, elevated levels caused by the NO donor

GSNO in macrophage cells induced apoptosis by itself in this cell line. Consequently, when it was used in combination with melatonin, it further increased the levels of DNA fragmentation in cells treated with both LPS or with melatonin (data not shown). Finally DMNQ, a redox-cycling agent that induces intracellular $O_2^{\bullet-}$ formation was used. Initially we tested this substance alone in RAW 264.7 cells in order to determine the appropriate low, non-toxic, concentration to induce a controlled short-term increase in $O_2^{\bullet-}$ that turned out to be a concentration range of 5-10 μM. Combining melatonin with DMNQ, without LPS, resulted in an increase of apoptotic cell death, whereas the combination of these two drugs with LPS showed a similar degree of apoptotic DNA fragmentation than melatonin or LPS alone (Fig 5b).

## DISCUSSION

It has long been surmised that inhibition of chronic inflammatory processes might be beneficial in several pathologies including cancer and neurodegenerative diseases. COX-2 has been intensely investigated in recent years, given its role in the initial steps of activation of the inflammatory process. COX-2 is thought to be an important target in diseases such as colon cancer (27) and Alzheimer's disease (28). Furthermore, chronic use of NSAIDs in clinical studies as well as laboratory findings have proved to be beneficial for these pathologies (29). Therefore, the search for natural or endogenous products with a high degree of specificity on COX-2 inhibition is essential to reduce the side effects that come with traditional NSAIDs treatment due to the partial (or total) inactivation of COX-1 as a collateral inhibitory effect. The present work shows that the tryptophan-derived pineal indole, melatonin, prevents specifically the activation of the pro-inflammatory enzymes COX-2 and iNOS in macrophage cells without simultaneous inhibition of COX-1 enzyme, thus indicating a new anti-inflammatory action not previously reported.

Lissoni and colleagues (30) proposed years ago that melatonin is a potent anti-inflammatory agent and may contribute to the stimulation of an immune reaction against cancer by removing the immunosuppression related to the activation of the inflammatory response (30). Since free radicals are thought to be involved in the inflammatory process, melatonin is a good candidate for anti-inflammatory properties due to its antioxidant and free radical scavenging features (31). More recently, anti-inflammatory actions of melatonin have been reported in different models, e.g., zymosan induced paw inflammation (32), LPS-induced alteration of pain perception in mice (33) or pancreaticobiliary inflammation (34). Non-mammalian melatonin-immune system relationship have been also described, thus demonstrating the strong evidence supporting the immune function of the indole (35). However, none of these studies mention the hypothetical action on COX-2 protein levels and/or activity despite the clear function of this enzyme on the inflammatory response. Wu has reviewed recently the role of C/EBPbeta and NFκB on the COX-2 transcriptional activation, suggesting the use of new drugs such as melatonin as

natural anti-inflammatory agents (36), while AFMK can inhibit prostaglandin synthesis (37), but melatonin metabolites such as AFMK or AMK have never been tested as COX-2 inhibitors in vitro or in vivo. The potential anti-inflammatory actions of these kynuramines or the indole 6-m-MEL reveal a structural link between this group of substances and the ability of COX-2 inhibition that should be studied in detail. In agreement with our results, Silva and co-workers have recently shown that melatonin and kynuramines prevent interleukin-8 release from LPS-stimutaled neutrophils, but not from peripheral blood mononuclear cells (38). According to their report, on the contrary to our results, kynuramines and melatonin exert some inhibition even at lower concentrations (1μM) which do not have any effect on COX-2 or iNOS in our study. This apparent difference in efficacy might account for some cell-specific effects. Additionally, AFMK and AMK may derive also from melatonin reaction with free radicals (17). As a result, the anti-inflammatory action of melatonin might be amplified when free radical damage is present, producing an "anti-inflammatory chain reaction" under oxidative stress conditions, e.g., cancer and neurodegenerative diseases.

The effects reported herein are consistent with melatonin's ability to reduce neurodegenerative changes in experimental models of both Alzheimer's disease (AD)(39) and colon cancer (40). Numerous studies have investigated the actions of melatonin reducing neuronal degenerative changes associated with the exposure of cells to amyloid-beta (Aβ), which is believed to be a major agent that initiates cell death in the AD brain (41). Melatonin actions in reducing Aβ toxicity have usually been attributed to its multiple antioxidant and free radical scavenging actions (42). The current finding provide another action of melatonin, i.e., COX-2 inhibition, that may help it to reduce AD progression considering the proposed role of inflammatory processes in this debilitating neurodegenerative condition (43). Recently, Chung and co-workers (44) have shown that hippocampal degeneration induced by kainic acid injection is prevented by the anti-oxidant and anti-inflammatory actions of melatonin since the indole reduced also the microglial activation that usually precedes neuronal death observed in this rat model (44). Collectively these results indicate that COX-2 inhibition by melatonin in activated macrophages or microglial cells (macrophages in

the brain) may account for many protective effects including those observed in neurodegenerative diseases.

Melatonin has previously been tested for its actions in cancer inhibition (45) including as a potential treatment for colon cancer. Several mechanisms by which melatonin either reduces initiation of cancer as well as retarding tumor growth have been proposed (46). The oncostatic actions of melatonin include its antioxidant functions (47), inhibition of uptake of tumor growth factors (19), reduction of telomerase activity in cancer cells (48) and modulation of cAMP levels in prostate cancer cells (49). Given the present findings, melatonin may have an alternate action to reduce at least colon cancer via COX-2 inhibition. Evidences of inflammation being involved in cancer progression are abundant in the literature. Chronic inflammatory conditions are associated with a variety of conditions, e.g. bronchitis, gastritis, inflammatory bowel disease or hepatitis and are often etiologically implicated in lung, gastric cancer, colorectal or liver cancers (50). Persistent inflammation can oxidize DNA suficiently to promote neoplastic transformation (51). Additionally, the resultant increase in necrosis classically associated with the oxidative damage as a result of the inflammatory process leads to the release of cellular elements, which in turn promote cell growth, cancer progression and tumor-associated mast cell chemotaxis (52).

Importantly, melatonin, unlike ASA, did not alter COX-1 protein level. Reduction of COX-1 activity by aspirin is believed to be a major contributory factor to its toxicity (53). Indeed, one of the major disadvantages of using NSAIDs is their side effect on COX-1, therefore reducing basal levels of $PGE_2$ (54). This PG usually has a physiological role Due to its lack of effect on COX-1, melatonin could potentially share the benefits of NSAIDs while avoiding their side effects; this will be further tested in subsequent studies.

The high efficacy of melatonin in reducing inflammatory processes in newborn humans has already been documented. Gitto and colleagues found that melatonin treatment of premature newborn infants suffering with septic shock, where LPS is causative and inflammation prominent, significantly

aided in their recovery and markedly reduced the inflammatory parameters of this very serious condition (55). Furthermore, melatonin prevented death in septic newborns while there was a 30% mortality in the children given conventional therapy without melatonin. It seems likely that the inhibition of COX-2 in these newborns by melatonin may have been one factor in their accelerated recovery and survival.

Antioxidant properties of melatonin are usually proposed as the major mechanism underlying the reduction of NO production in several oxidative stress-related pathological conditions, specially in neurodegenerative models such as Aβ-induced apoptotic cell death in PC-12 cells or in an Alzheimer's disease mouse model (41;56), domoic acid-induced neurotoxicity in hippocampal neurons (57) or during ischemia in a rat middle cerebral artery occlusion stroke model (58). Regulation of NOS by melatonin has also been studied in detail but most of these reports have focused on the calcium-dependent constitutively expressed nNOS isoform (59;60). This inhibitory effect on nNOS is mediated by the $Ca^{2+}$-calmodulin binding properties of melatonin and other related compounds (60). Nevertheless Gilad and colleagues (61), in agreement with our data, found that melatonin do not react directly with NO but instead the indole lower the nitrite/nitrate production by reducing iNOS expression in LPS-activated macrophage through the inhibition of NFκB activation. Furthermore, it has been shown that melatonin inhibits in vivo the expression of iNOS in liver and lung of LPS-treated rats (62). It is noteworthy to point out that present findings do not rule out the possibility that antioxidant properties of melatonin and its metabolites would participate in the regulation of COX-2 and iNOS under some pro-oxidant conditions. However, as deduced from the data presented here, we did not find evidence at a short-term of oxidative stress in the LPS-stimulated macrophage cells. Therefore, regulation of iNOS –and COX-2- by melatonin might be mediated by other structure-dependent mechanisms not necessary requiring the pre-requisite of NFκB inactivation, which is a major oxidative stress responsive transcription factor. Whether this factor is critical for the anti-inflammatory actions of melatonin or if this effect is somehow related to a non-antioxidant mechanism should be further investigated. Since AFMK, AMK or 6-m-Mel show a comparable efficacy as anti-inflammatory drugs, a similar mechanism to those described for the $Ca^{2+}$-

dependent nNOS should be investigated, although participation of calmodulin in iNOS should probably ruled out.

Surprisingly, we found that COX-2 inhibition by melatonin leads to apoptosis in LPS-treated macrophage cells. Likewise, other antioxidant and anti-inflammatory substances including protein pigment c-phycocyanin also induces apoptosis in RAW 264.7 macrophages (63). The induction of apoptosis in non-cancer cells has not been reported either in vivo or in vitro. Although Wölfler et al. (64) reported pro-oxidant acitivity of melatonin in Jurkat cells, this phenomenon has never been observed elsewhere in vivo or in cell culture. Additionally, the possibility of this pro-oxidant action is discarded as deduced from the DCF-DA studies shown here. Furthermore, melatonin by itself does not induce a significant apoptosis, indicating that LPS activation is a pre-requisite for melatonin mediated programmed cell death. It is not clear from the literature why COX-2 inhibition might trigger apoptosis in RAW 264.7 macrophages, although several data indicate that this may occur in other tissues or in cancer cells (65). It has been shown however that COX-2 and p53 expression, induced by LPS and NO-releasing compounds respectively, are inversely related in RAW 264.7 macrophage cells and low levels of NO mitigates apoptosis in macrophages (66). Here we show that non-toxic low concentrations of NO not only did not prevent melatonin induced cell death but even enhanced apoptosis, indicating that restoration of NO levels after melatonin treatment did not influence melatonin's intracellular actions. cAMP levels also confer protection against NO-induced apoptosis in macrophages. Whether this would give protection to melatonin-treated cells or not deserves further study. Our group has previously observed that cAMP levels are elevated in melatonin treated LNCaP prostate cancer cells indicating that cAMP could be implicated in protection and/or differentiation rather than in inducing apoptosis (49).

Collectively the results shown here demonstrate the potential role of melatonin and its metabolites AFMK or AMK, or other structurally-related molecule such as 6-m-Mel, as modulatory agents during the inflammatory process, as well as a rationale for the possible use of melatonin as an anti-inflammatory agent due to its specific action on COX-2, thereby avoiding the undesired side effects associated with

COX-1 inhibition. These immuno-modulatory actions may have important applications in pathologies in which inflammation plays a key role, e.g., neurodegeneration and cancer. Further studies are necessary to elucidate the molecular pathways involved in COX-2 and iNOS inhibition by melatonin and other indoles such as AFMK or AMK. The physiological role of these compounds in immunomodulation should also be addressed in the future.

## ABREVIATIONS

AA: Arachidonic acid

ASA: Acetyl Salicylic acid

AFMK: N1-acetyl-N2-formyl-5-methoxykynuramine

AMK: N1-acetyl-5-methoxykynuramine

COX: Cyclooxygenase

GSH/GSSG: Reduced/Oxidized glutathione

IND. Indomethacin

LPS: Lipopolysacharide

6-m-Mel: 6-methoxy-melatonin

NAC: N-Acetyl-Cysteine

NO: Nitric oxide

NOS: Nitric oxide synthase

PG: Prostaglandin

NSAIDs: Nonsteroid anti-inflammatory drugs

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**Table 1.-** DCF-DH Fluorescence, expressed in arbitrary units (a.u.) in different groups of cells incubated with vehicle (CON) or with melatonin (MEL) in the absence of presence of LPS for 1h.

| | **DCFH-DA Fluorescence (a.u.)/mg protein** |
|---|---|
| CON | 30460 ±1241 |
| MEL | 28706 ± 1035 |
| LPS | 28114 ± 923 |
| LPS + MEL | 27645 ± 301 |

**Figure 1.** Effect of melatonin on COX-2 and iNOS protein and activity levels. A. Effect of different doses of melatonin on COX-2, iNOS and COX-1 protein levels in LPS-stimulated RAW 264.7 macrophages for 18 h. Lanes 1 to 8: vehicle-treated control (1), LPS (2) and LPS plus melatonin 2, 1, 0.5, 0.1 mM, 1 μM and 10 nM (3-8, respectively). B. Effect of different doses of melatonin on $PGE_2$ levels in the supernatant of vehicle-treated controls or LPS-stimulated RAW 264.7 macrophages for the times indicated. Symbols: ●, control; ○, LPS;▼ LPS + 2 mM melatonin, ▽ LPS + 1 mM melatonin; n, LPS + 0.1 mM melatonin. **, P<0.01 vs rest of groups. C,D. Effect of different concentrations of melatonin for the indicated times (C) or for 18 h (D) on the nitrite levels in the culture media; symbols: ●, control; ○, LPS;▼ LPS + 2 mM melatonin, ▽ LPS + 1 mM melatonin; **, P<0.01 vs rest of groups; a, P<0.01 vs CON; b, P<0.01 vs LPS group. E. Comparative effect of melatonin (MEL) with the NSAIDs, acetyl-salicylate (ASA) and indomethacin (IND) at 0.1, 0.5 and 1 mM concentration. F. Effect of the cAMP synthesis stimulator, forskolin (FOR) on COX-2 and COX-1 levels in the same cell model (NSB, Non specific binding).

**Figure 2.** A. Effect of the antioxidant NAC at low (A) or high concentrations (B) on the COX-2 protein levels in RAW 264.7 macrophage cells stimulated with LPS. Representative western blots of three different experiments are shown here. Effect of melatonin (MEL) is also shown to compare the relative effect. C. Relative DCF-fluorescence (arbitrary units) in cells treated with vehicle (CON), melatonin (1mM) alone, LPS 1μg/ml or LPS plus melatonin for 1 h. RAW 264.7 macrophage cells were cultured with vehicle (0.2% DMSO) or melatonin with or without LPS (1 μg/ml) and the DCF-DH (100 μM) was added 10 minutes before reading the fluorescence. DCF fluorescence was standardized with respect to the protein concentrations.

**Figure 3**. Effect of related molecules of melatonin on the COX-2 levels of LPS (1μg/ml) treated RAW 264.7 macrophage cells. Cells were treated with vehicle (CON) or LPS (18h) alone or plus kynuramines for 18h. A. Dose-response effect of AFMK (1-500 μM) B. Dose-response effect of AMK (1-500 μM). C. Comparative action of melatonin (MEL), AFMK or AMK, at 1 mM concentration, on COX-2 protein levels. D. Effect of 6-methoxy-melatonin (6-m-Mel) on COX-2 protein levels. Westerns blots shown here are representatives of four different experiments.

**Figure 4**. Melatonin induced apoptosis in LPS-treated RAW 264.7 macrophage cells. Cells were treated with or without melatonin for 30 min, at the doses indicated, prior to LPS treatment (1 μg/ml) and cultured additionally for 24 h. A. Cells stained with DAPI to show nuclear morphology: vehicle-treated cells (left panel), LPS (center) or LPS + melatonin (right panel); some apoptotic cells (arrows) are shown in LPS + melatonin treated cells. B. Quantification of DAPI-stained apoptotic cells, expressed as percentage of apoptotic cells *vs* total; *, P<0.01 vs LPS. C. DNA gel electrophoresis from cells cultured with vehicle (C) alone, LPS (L) or LPS plus the indicated doses of melatonin (2-0.1 mM) for 24h.

**Figure 5**. Effect of modulation of nitric oxide and superoxide anion levels during melatonin-induced apoptosis. A. RAW 264.7 macrophage cells were treated with vehicle, with or without LPS (1 μg/ml), L-NAME (10 μM) and/or melatonin at the indicated dose (2 or 1 mM) for 24h. B. Macrophages were incubated in the presence of vehicle, DMNQ (5 or 10 μM), melatonin (1 mM) and/or LPS (1 μg/ml) for 24h. Data show a representative of three different experiments.

**Table I.** Relative DCF-fluorescence (arbitrary units) in cells treated with vehicle (CON), melatonin (1mM) alone, LPS 1μg/ml or LPS plus melatonin for 1 h. RAW 264.7 macrophage cells were cultured with vehicle (0.2% DMSO) or melatonin with or without LPS (1 μg/ml) and the DCF-DH (100 μM) was added 10 minutes before reading the fluorescence. DCF fluorescence was standardized with respect to the protein concentrations.