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Protective effects of melatonin in zymosan-activated plasma-induced paw inflammation

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Abstract

The aim of the present study was to investigate the protective effect of the pineal hormone melatonin in a model of acute local inflammation (zymosan-activated plasma-induced paw oedema), in which oxyradicals, nitric oxide (NO) and peroxynitrite are known to play a crucial role in the inflammatory process. The intraplantar injection of zymosan-activated plasma elicited an inflammatory response that was characterized by a time-dependent increase in paw oedema, neutrophil infiltration and increased levels of nitrite/nitrate in the paw exudate. The maximal increase in paw volume was observed at 3 h after administration (maximal in paw volume: 1.34 ± 0.09 ml). At this time point, myeloperoxidase activity and lipid peroxidation were markedly increased in the zymosan-activated plasma-treated paw (226 ± 10.2 mU/100 mg wet tissue, 31 ± 2.1 mM/mg wet tissue, respectively). However, zymosan-activated plasma-induced paw oedema was significantly reduced in a dose-dependent manner by treatment with melatonin (given at 62.5 and 125 μg/paw) at 1, 2, 3, 4 h after injection of zymosan-activated plasma. Melatonin treatment also caused a significant reduction of the myeloperoxidase activity and lipid peroxidation and inhibited nitrite/nitrate levels in the paw exudate. The paw tissues were also examined immunohistochemically for the presence of nitrotyrosine (a marker of peroxynitrite formation). At 3 h following injection of zymosan-activated plasma, staining for nitrotyrosine was also found to be localised in the inflamed paw tissue. Treatment with melatonin (125 μg/paw) reduced the appearance of nitrotyrosine in the tissues. Our findings support the view that melatonin exerts anti-inflammatory effects.

Keywords: Melatonin; Oedema; Peroxynitrite; Nitric oxide (NO); Zymosan-activated plasma

1. Introduction

Zymosan is a wall component of the yeast *Saccharomyces cerevisiae* and produces an intense inflammatory response (Lundberg and Arfors, 1983; Teixeira et al., 1993; Cuzzocrea et al., 1997a,b). Treatment of plasma with zymosan is known to activate complement and to generate C_{5a}, which is a potent chemotactic factor for neutrophils (Fernandez et al., 1978). Zymosan can trigger the production of oxygen-derived free radicals in various cell types (Murohara et al., 1993; Cuzzocrea et al., 1997c). With this experimental model, previous studies have demonstrated the anti-inflammatory potential of various therapeutic approaches aimed at the scavenging of oxyradicals, at inhibition of nitric oxide synthase (NOS) and peroxynitrite formation; at reduction of prostaglandins formation and at inhibition of poly (ADP-Ribose) synthetase (PARS) (Cuzzocrea et al., 1997a, 1998a).

Melatonin is an indole that is synthesized in and secreted from the pineal gland during the night (Reiter, 1991). Its lipophilicity ensures that melatonin rapidly enters cells, where it may accumulate in the nucleus (Menemdez-Pelaez et al., 1994). Recently, it was demonstrated that melatonin is a free radical scavenger (Tan et al., 1993; Reiter, 1995; Reiter et al., 1995; Marshall et al., 1996), an antioxidant that protects cells against the damage induced by several oxidative agents including lipopolysaccharide (Sewerynek et al., 1995), paraquat (Melchiorri et al., 1994) and carbon tetrachloride (Daniels et al., 1996). Furthermore, melatonin is also a scavenger of peroxynitrite (Gilad et al., 1997) and it inhibits the production of NO (Pozo et al., 1994; Cuzzocrea et al., 1997d, 1998b,c).

The purpose of the present study was to demonstrate the anti-inflammatory effect of melatonin against zymosan-activated plasma-induced paw oedema in the rat.

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2. Materials and methods

2.1. Zymosan-activated plasma-induced paw oedema

Paw oedema was induced in male Wistar rats (Charles River, Italy; 200–225 g) by subplantar injection of 0.2 ml of zymosan-activated plasma. Briefly, heparinized rat plasma was incubated with zymosan (10 mg/ml; Sigma, Milan, Italy) for 60 min at 37°C in order to activate the complement system. Zymosan was then removed by centrifugation at $10,000 \times g$ for 5 min at room temperature (Lundberg and Arfors, 1983). The inflammatory agent was administered alone or in combination with melatonin (Sigma). Melatonin was dissolved in ethanol and further dissolved in saline solution (the final concentration of ethanol was 1%) and the injected volume was 0.2 ml. The paw volume was measured with a plethismometer (model 7140; Ugo Basile, Milan, Italy) immediately after the injection, as previously described (Di Rosa and Willoughby, 1971). Subsequent volume readings for the same paw were carried out at 30- or 60-min intervals and compared to the initial reading.

2.2. Determination of nitrite + nitrate concentration in the paw exudate

At specified times after the intraplantar injection of zymosan-activated plasma, rats were killed and each paw was cut at the level of the calcaneus bone. Paws were gentle centrifuged at $250 \times g$ for 20 min in order to recover a sample of the oedematous fluid. The volume of fluid that was recovered from each paw was measured. Blood was removed from the fluid sample by filtering through a 10,000 mol cut-off filter. Nitrite + nitrate (NO_x) production, an indicator of NO synthesis, was measured in exudate samples as previously described (Zingarelli et al., 1996). First, nitrate in the exudate was reduced to nitrite by incubation with nitrate reductase (670 mU/ml) and NADPH (160 μM) at room temperature for 3 h. After 3 h, nitrite concentration in the samples was measured by the Griess reaction, by adding 100 μl of Griess reagent (0.1% naphthylethylenediamide dihydrochloride in H_2O and 1% sulphanilamide in 5% concentrated H_3PO_4; vol. 1:1) to 100 μl of sample. The optical density at 550 nm (OD_{550}) was measured using a Spectramax 250 microplate reader (Molecular Devices Sunnyvale, CA). Nitrate concentrations were calculated by comparison with OD_{550} of standard solutions of sodium nitrate prepared in saline solution.

2.3. Myeloperoxidase activity

Myeloperoxidase activity, an index of polymorphonuclear leucocyte accumulation, was determined as previously described (Mullane et al., 1985). Paw tissue, collected at the specified times, was homogenized in a solution containing 0.5% hexa-decyl-trimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7) and centrifuged for 30 min at $20,000 \times g$ at 4°C. An aliquot of the supernatant was then allowed to react with a solution of tetra-methyl-benzidine (1.6 mM) and 0.1 mM H_2O_2. The rate of change in absorbance was measured by spectrophotometry at 650 nm. Myeloperoxidase activity was defined as the quantity of enzyme degrading 1 μmol of peroxide per min at 37°C and is expressed in milliunits per gram weight of wet tissue.

2.4. Malonaldehyde measurement

Levels of malonaldehyde in the paw tissue was determined as an index of lipid peroxidation, as described by Ohkawa et al. (1979). Paw tissue, collected at the specified times, was homogenized in 1.15% KCl solution. An aliquot (100 μl) of the homogenate was added to a reaction mixture containing 200 μl of 8.1% sodium dodecyl sulfate (SDS), 1500 μl of 20% acetic acid (pH 3.5), 1500 μl of 0.8% thiobarbituric acid and 700 μl distilled water. Samples was then boiled for 1 h at 95°C and centrifuged at $3000 \times g$ for 10 min. The absorbance of the supernatant was measured by spectrophotometry at 650 nm.

2.5. Immunohistochemical localization of nitrotyrosine

Tyrosine nitration, a specific indicator of peroxynitrite formation, was detected as previously described (Cuzzocrea et al., 1997c) in paw sections by immunohistochemistry. At the specified times following the injection of zymosan-activated plasma, tissues were fixed in 10% buffered formalin and 8 μm sections were cut from paraffin-embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% H_2O_2 in 60% methanol for 30 min. The sections were permeabilized with 0.1% Triton X-100 in phosphate buffered saline for 20 min. Non-specific adsorption was minimized by incubating the section in 2% normal goat serum in phosphate-buffered saline for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with avidin and biotin (biotin blocking kit, Vector Laboratories). The sections were then incubated overnight with a 1:1000 dilution of primary anti-nitrotyrosine antibody (DBA, Milan, Italy) or with control solutions. Controls included buffer alone or non specific purified rabbit immunoglobulin G (IgG). Specific labelling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin–biotin peroxidase complex (DBA).

2.6. Materials

The Biotin blocking kit, biotin-conjugated goat anti-rabbit IgG, primary anti-nitrotyrosine antibody and avidin–biotin peroxidase complex were obtained from DBA. All other reagents and compounds used were obtained from Sigma (Sigma, St. Louis, MO, USA).

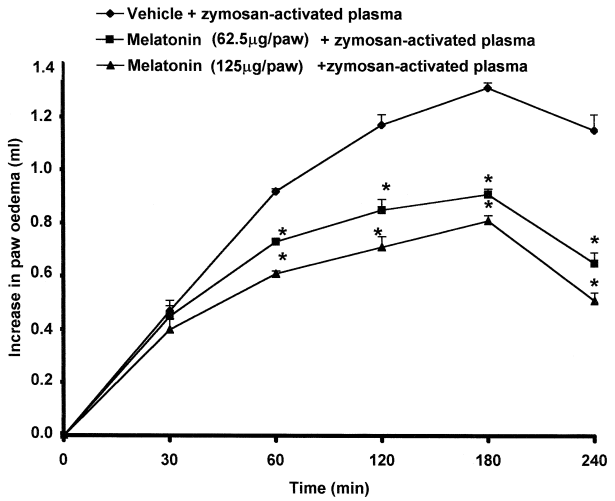

Fig. 1. Effect of melatonin (62.5 and 125 μg/paw) on paw oedema development elicited by zymosan-activated plasma in the rat. The results are expressed as means ± S.E.M. for $n = 5–6$ rats. Melatonin treatment significantly inhibited ($* P < 0.01$) in a dose-dependent manner oedema formation at the indicated time points.

2.7. Data analysis

All values in the figures and text are expressed as means ± S.E.M. *n* observations, where *n* represents the number of animals, were studied. Data sets were examined by one- and two-way analyses of variance. Individual group means were then compared by using Student's unpaired *t*-test. A *P*-value less than 0.05 was considered significant. In the experiments involving immunohistochemistry, the figures shown are representatives of at least three experiments performed on different experimental days.

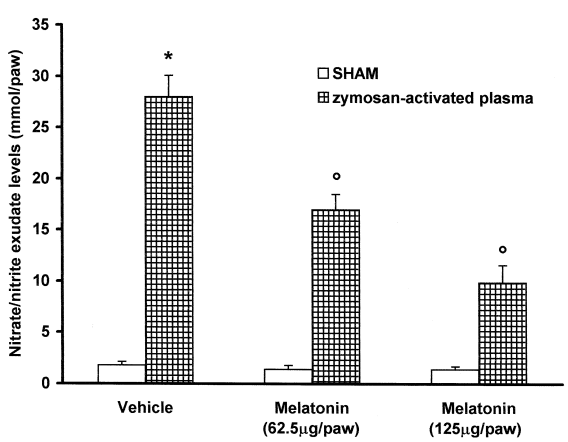

Fig. 2. Nitrite and nitrate concentrations in paw exudate. Nitrite and nitrate levels in zymosan-activated plasma-treated rats were significantly increased versus those of the sham group. Melatonin (62.5 and 125 μg/paw) treatment significantly ameliorated the zymosan-activated plasma-induced elevation of nitrite and nitrate levels. Value are means ± S.E.M. for eight rats for each group. $* P < 0.01$ versus sham. $° P < 0.01$ versus zymosan-activated plasma.

3. Results

3.1. Effects of melatonin on zymosan-activated plasma-induced paw oedema

In preliminary experiments we established that injection into the rat paw of melatonin (62.5, 125 μg/paw), diluted in heparinized plasma, did not produce any detectable oedema. Injection of zymosan-activated plasma in rat paws evoked an inflammatory reaction that resulted in oedema within 30 min and was maximal (1.34 ± 0.09 ml) at 3 h after administration (Fig. 1). However, zymosan-activated plasma-induced paw oedema was significantly reduced in a dose dependent manner by treatment with melatonin at 1, 2, 3, 4 h after injection of zymosan-activated plasma (Fig. 1).

At 3 h after injection of zymosan-activated plasma, nitrite/nitrate levels were significantly increased in the paw exudate (Fig. 2). Paw tissue was used to measure myeloperoxidase activity, as an indicator of neutrophil infiltration, and malonaldehyde, in order to estimate lipid peroxidation. As shown in Fig. 3A,B, myeloperoxidase

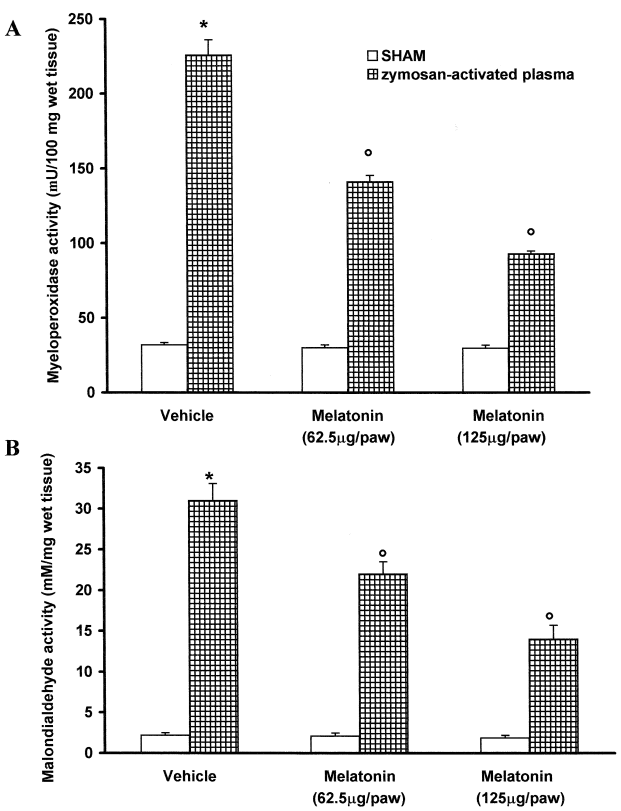

Fig. 3. Myeloperoxidase activity (A) and malondialdehyde (B) in the paw of zymosan-activated plasma-treated rats killed at 3 h. Myeloperoxidase activity and malondialdehyde levels were significantly increased in the paws of the zymosan-activated plasma-treated rats in comparison to sham rats. Melatonin treatment reduced in a dose dependent manner the zymosan-activated plasma-induced increase in myeloperoxidase activity and malondialdehyde levels. Values are means ± S.E.M. for eight rats for each group. $* P < 0.01$ vs. sham; $° P < 0.01$ vs. zymosan-activated plasma.

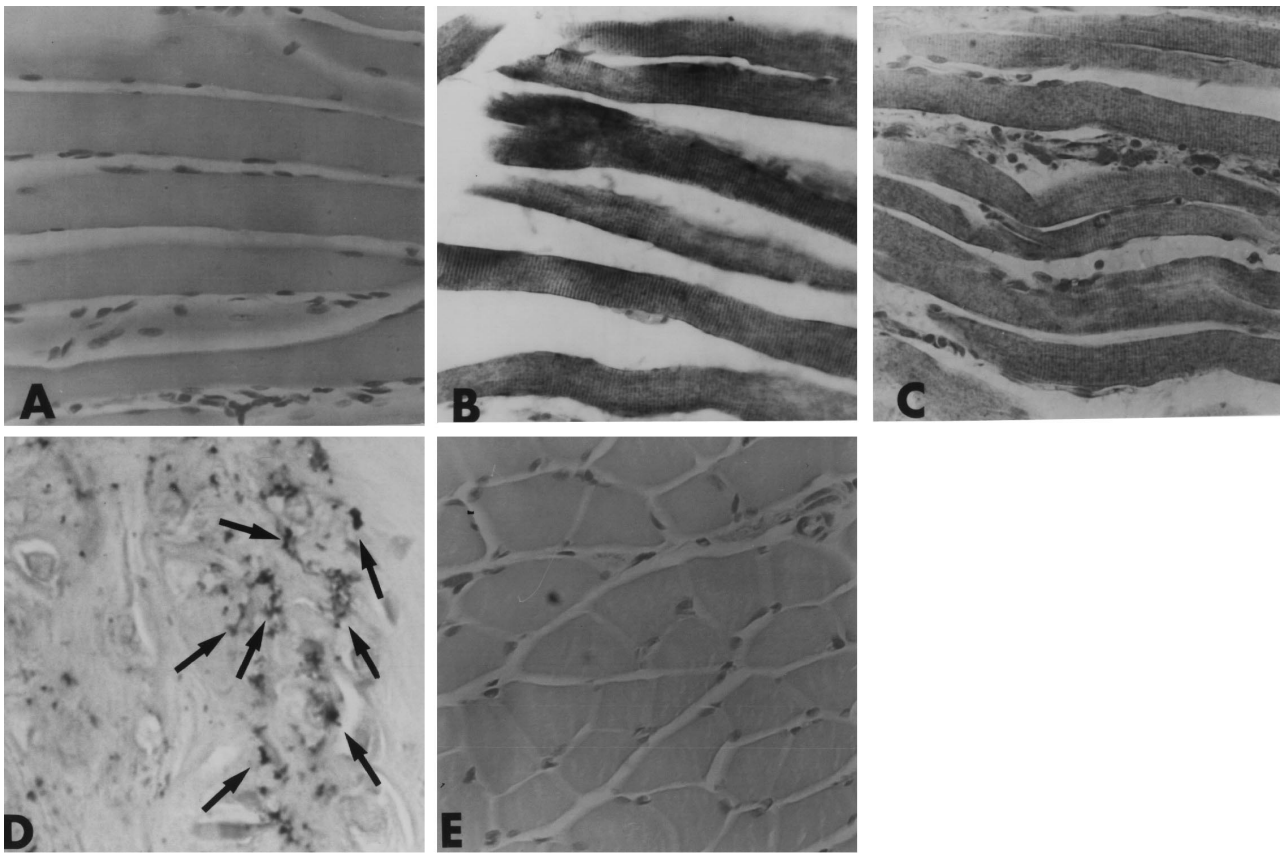

Fig. 4. Immunohistochemical localization of nitrotyrosine in the rat paw. Staining was absent in control tissue (A). Three hours following injection of zymosan-activated plasma, nitrotyrosine immunoreactivity was localised in skeletal muscle (B) and within discrete cells in the inflamed paw tissue (D). Treatment with melatonin (125 μg/paw) reduced the appearance of nitrotyrosine (C, E).

activity and malonaldehyde levels (226 ± 10.2 mU/100 mg wet tissue; 31 ± 2.1 μM/mg wet tissue respectively) were significantly ($P < 0.01$) increased in paws at 3 h after injection of zymosan-activated plasma when compared to the value for sham rats (30 ± 1.4 mU/100 mg wet tissue; 2.1 ± 0.33 mM/mg wet tissue, respectively). Nitrite/nitrate, myeloperoxidase activity and malonaldehyde levels were significantly ($P < 0.01$) reduced in a dose-dependent manner by melatonin treatment (Figs. 2 and 3). The paw tissues were also examined immunohistochemically for the presence of nitrotyrosine (a marker of peroxynitrite formation). Immunohistochemical analysis of the paw tissue of control animals showed no nitrotyrosine staining (Fig. 4A). In contrast, 3 h following injection of zymosan-activated plasma, staining for nitrotyrosine was found to be localised within discrete cells and skeletal muscle fibres in the inflamed paw tissue (Fig. 4B,D). Treatment with melatonin (125 μg/paw) reduced the appearance of nitrotyrosine in the tissues (Fig. 4C,E).

4. Discussion

Paw oedema formation is a result of a synergism between various inflammatory mediators that increase vascular permeability and/or mediators that increase blood flow (Ialenti et al., 1992). Several experimental models of paw oedema have been described. Rat paw oedema is characterised by an early phase caused by the release of histamine, 5-hydroxytryptamine and bradykinin, followed by a late phase mainly sustained by prostaglandin release (Di Rosa et al., 1971; Di Rosa and Willoughby, 1971). In recent years, the L-arginine-NO pathway has been proposed to play an important role in the inflammatory response. Pharmacological inhibitors of NO synthase have been shown to reduce the development of the inflammatory response (Tracey et al., 1995; Wei et al., 1995; Salvemini et al., 1996a, Cuzzocrea et al., 1998d). More recent studies, performed with the carrageenan-induced paw oedema model, have shown the formation of peroxynitrite in this model of inflammation (Salvemini et al., 1996b; Cuzzocrea et al., 1998d). Using nitrotyrosine immunohistochemistry, we confirmed the production of peroxynitrite in the paw of rats subjected to zymosan-activated plasma-induced paw oedema. It is well-known that acute inflammatory process, in which vascular permeability increases and leucocyte migration occurs, involve several mediators including neutrophil-derived active oxygen species and free radicals, such as hydrogen peroxide, superoxide and hydroxyl radical (Da Motta et al., 1994;

Salvemini et al., 1996a; Cuzzocrea et al., 1997a,b,d). It is well-established that zymosan activates complement and generates C_{5a}, which is a potent chemotactic factor for neutrophils (Fernandez et al., 1978). Zymosan, zymosan-activated plasma and complement can trigger the production of oxygen-derived free radicals such as hydrogen peroxide, superoxide and hydroxyl radical in various cell types (Mehta et al., 1991; Murohara et al., 1993), and it also induces peroxynitrite generation (Cuzzocrea et al., 1997c). Peroxynitrite is a strong oxidant that results from reaction between NO and superoxide (Beckman et al., 1990). Peroxynitrite plays a role in normal cellular processes, and although its actions have not been fully identified (Rubbo et al., 1994) it usually causes the oxidation of a variety of biomolecules with pathological consequences (Lancaster, 1992; Graham et al., 1993). A novel pathway of inflammation, governed by the nuclear enzyme PARS, has been proposed in relation to hydroxyl radical- and peroxynitrite-induced DNA single strand breakage (Szabó, 1996; Cuzzocrea et al., 1997c, 1998b). This pathway plays an important role in various forms of shock and reperfusion injury (Zingarelli et al., 1997; Cuzzocrea et al., 1997e; Thiemermann et al., 1997). Recently, it has been shown that melatonin, by scavenging hydroxyl radical and peroxynitrite, prevents the DNA single strand breakage and thus prevents the activation of PARS in inflammation (Cuzzocrea et al., 1998c).

Melatonin is secreted principally by the pineal gland, and levels are highest at night (Reiter, 1991). Melatonin is involved in various physiologic functions, including the regulation of seasonal reproduction, circadian rhythms, sleep, mood, performance, and the immune response, which makes it likely that the pineal hormone may be a factor in aging (Reiter, 1991; Pierpaoli and Regelson, 1994). Recently, a number of reports appeared demonstrating the protective effects of melatonin in various models of ischemia-reperfusion injury (Bertuglia et al., 1996; Sewerynek et al., 1996), inflammatory bowel disease (Pentney and Bubenik, 1995), and neuroinjury (Giusti et al., 1996). Moreover, melatonin has been shown to protect against shock induced by bacterial lipopolysaccharide (Sewerynek et al., 1995, 1996; Maestroni, 1996), to inhibit thirst and fever induced by endotoxin (Nava et al., 1997) and to protect against inflammation (Cuzzocrea et al., 1997d, 1998b,c). These studies are important in the context of the current work, inasmuch as the importance of oxyradicals, NO and peroxynitrite is well established in the pathogenesis of reperfusion injury and shock (Szabó, 1996; Cuzzocrea et al., 1997e; Zingarelli et al., 1997) and inflammation (Salvemini et al., 1996a,b; Cuzzocrea et al., 1997d, 1998d). In recent years, the sphere of influence of melatonin was further increased when the indole was found to be an effective free radical scavenger and antioxidant. Free radicals are toxic molecules, many being derived from oxygen, which are continuously produced and incessantly attack and damage molecules within cells. Collectively, the process of free radical damage to molecules is referred to as oxidative stress. Melatonin reduces oxidative stress by several means. Thus, the indole is an effective scavenger of the hydroxyl radical and the peroxyl radical (Pieri et al., 1994; Reiter, 1995), and it may stimulate some important antioxidative enzymes like superoxide dismutase, glutathione peroxidase and glutathione reductase (Reiter, 1991, 1995; Pieri et al., 1994). More recently, it has been shown that melatonin acts as a peroxynitrite scavenger and protects cultured cells against peroxynitrite-induced injury (Gilad et al., 1997). The cytotoxic effects of peroxynitrite are multiple and include protein oxidation, lipid peroxidation, inhibition of cellular metabolic pathways and signal transduction processes (Szabó, 1996; Zingarelli et al., 1996). There are a number of sites where melatonin can interfere with the inflammatory process: (A) melatonin inhibits NO production and reduces the expression of inducible NO synthase after carrageenan or zymosan exposure (Cuzzocrea et al., 1997d, 1998b,c); (B) melatonin affects the activation of the transcription factor nuclear factor kappa B (Mohan et al., 1995; Gilad et al., 1998); (C) melatonin affects the expression of inducible NO synthase at the transcriptional level (Pozo et al., 1994; Bettahi et al., 1996; Maestroni, 1996; Gilad et al., 1998); (D) melatonin affects the expression of inducible cyclooxygenase (Gilad et al., 1998); (E) melatonin inhibits neutrophil infiltration after inflammatory stimuli (Cuzzocrea et al., 1997d, 1998b).

In the present paper there are several data which support the view that melatonin prevents the inflammatory response. In fact melatonin treatment (a) inhibited paw oedema formation, (b) inhibited neutrophil infiltration and the lipid peroxidation, (c) and decreased nitrite/nitrate and peroxynitrite formation.

Taken together, the results of the present study, coupled with recent data from several groups, support the view that melatonin can exert potent anti-inflammatory effects.

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