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Chronoimmunomodulation of melatonin on bactericidal activity of human blood phagocytes

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Abstract

In this work we studied the chronoimmunomodulation effect of melatonin on blood phagocytes. This cells stimulated with melatonin and incubated with EPEC presented enhanced superoxide. Mononuclear (MN) and polymorphonuclear (PMN) phagocytes stimulated with melatonin in the presence of bacteria showed enhanced superoxide release.EPEC killing by MN phagocytes stimulated with melatonin was time dependent. The highest bactericidal index were observed over the period of 60 and 120 minutes of incubation. PMN phagocytes stimulated with melatonin, independently of time, showed increased in the bactericidal activity. Interactions between melatonin and phagocytes support the hypothesis of pineal chronomodulation microbicidal processes.

INTRODUCTION

Several works have suggested that hormones and neuropeptides act as potent immunomodulators participating in various aspects of immune system function, both in health and disease (Dardenne & Savino,1994; Blalock 1994; Lissoni et.al, 1997; Srinivasan et al., 2005).

The pineal gland, with its indole metabolism and particularly melatonin synthesis, is characterized by a series of oxidative processes involving photochemical reactions and redox reactions with the participation of free radicals or peroxides inhibitors or generators. Melatonin is an endogenous neurohormone and its actions are related to its capability to scavenge free radicals, increase the antioxidant activity of enzymes (Keplac et al, 2005; . Sudnikovich et al, 2007) mainly due to its scavenging capacity against reactive oxygen species (Keplac et al, 2005). These observations have led to the hypothesis of pineal involvement in the body oxidative processes (Ianãs, et al., 1991). Some works have reported a functional connection between the pineal gland and the immune system (Liebmann et al., 1997; Pandi-Perumal et al, 2008).

The pineal hormone melatonin due to its lipophilic nature, has access to every cell and every part of a cell in the body, suggesting that it could exert effects on blood immune cells. Probably the effects of melatonin on the immune cells, may be by indirect influence on the synthesis and release the other hormones or cytokine or by direct action on phagocytosis and phagocytic bichemical process (Liebmann et al., 1997; Rodriguez et al., 1999).

Enteropathogenic Escherichia coli (EPEC) is a bacterium that causes diarrhea in infants in developing countries (Gomes et al., 1991; Kosek et al, 2003). Studies have demonstrated that blood phagocytes present activation of oxidative metabolism and bactericidal activity when incubated with this bacteria, an activity that may be modulated by some factors present in the serum (Honorio-França et al., 1997; Honorio-França et al, 2001).

The bactericidal activity is probably due to the activation of superoxide anion-dependent killing mechanism operating on the cell surface (Asad, 1994). There is considerable evidence that phagocytes rapidly increase oxygen consumption during phagocytosis or upon stimulation of their with a variety of agents (Badwey, et al, 1983).

The products of oxygen reduction and excitation have been implicated in the destruction of bacteria, yeast, viruses and mycoplasmas by phagocytes, constituting key components in the microbicidal mechanisms of phagocytes (Babior, 1984; Badwey, et al, 1983; Honorio-França, et al, 1997).

Some studies have shown that melatonin has an antioxidant effect which includes the scavenging of highly reactive oxygen free radicals and there is evidence that melatonin may protect the effects of free radical-induced neuronal damage (Sandyk, 1990; Reiter,1993; Tan, 1994; Rodriguez

et al., 1999; Keplac et al, 2005; . Sudnikovich et al, 2007)., whereas others demonstrated a prooxidant effect (Ianãs, et al., 1991) and probably acts amplifying cellular activation reactions (Fjaerli et al., 1999).

In the present study we investigated the chronoimmunomodulation effect of melatonin on superoxide release and the bactericidal activity by blood phagocytes.

MATERIALS AND METHODS

Blood Cell Separation: Blood samples were collected from 51 volunteer donors 18 to 35 years of age whose serology for HIV, Sífilis and Hepatitis B were negative. Collections were performed only in the morning. Immediately after the donation, the donors gave informed consent to donate approximately 15 ml of blood for the study, collected into heparinized (1 U/ml) tubes. All procedures were submitted to ethical evaluation and obtained institutional approve. The samples were fractionated over a Ficoll-Paque (Pharmacia, Uppsala) density gradient (density 1.077 g/l). The mononuclear (MN) cells were separated and resuspended independently in serum-free medium 199. Polymorphonuclear (PMN) cells were obtained by the dextran (4 g/l) sedimentation hypotonic lysis method (Boyum, 1968). The cells (MN and PMN) were washed separately twice in phosphate buffered saline (PBS with 2.6 mM CaCl2 and 2.0 mM MgCl2) for the experiments of superoxide release. For the experiments of bactericidal activity, the cells were washed independently in serum-free medium 199. This procedure resulted in 95% pure mononuclear (MN) and 93% pure polymorphonuclear (PMN) preparations as analyzed by light microscopy. The resulting MN and PMN phagocyte suspensions were adjusted to 2 x 106cells/ml.

E. coli strain: Enteropathogenic Escherichia coli isolated from stools of an infant with acute diahrrea [serotype 0111:H-, LA+, eae+, EAF+, bfp+] was cultivated in Brain Heart Infusion (BHI-Difco - Detroit) for 18 hours at 37oC. Bacteria were washed twice in phosphate buffered saline (PBS) and adjusted to an approximate concentration of 2 x 107 bacteria/ml as measured by turbidimetry at 540 nm using a Coleman spectrophotometer. This bacterial concentration was previously determined by colony unit counting on Trypic Soy Agar [TSA-Difco - Detroit]

Release of superoxide anion: To measure superoxide release, cytochrome C (Sigma) reduction was determined as previously described (Pick & Mizel, 1981; Honorio-França et al, 1997). Briefly, MN and PMN phagocytes and bacteria, stimulated or not with 50 L melatonin (Sigma, final concentration of 10-7M -Paulak et al, 2005), were mixed and incubated for 30 min for phagocytosis. Cells were then resuspended in PBS containing 2.6 mM CaCl2, 2 mM $MgCl_2$, and cytochrome C (2 mg/ml). Control assays of phagocytes without bacteria stimulated or not with phorbol myristate acetate (PMA - Sigma, final concentration of 10-7M) were simultaneously carried out. The suspensions (100 L) were incubated for 60 min at 37oC in culture plates. The reaction rates were measured by absorbance at 550 nm and the results were expressed as nmol/O_2-. All the experiments were performed in duplicate or triplicate.

Bactericidal assay:Equal volumes of bacterial (2 x 107) and phagocyte (2 x 106) suspensions were mixed in duplicate in plastic tubes and shaken at 37oC for 30 min. Phagocytosis was stopped by incubation in an ice bath. In order to eliminate extracellular bacteria, the suspensions were centrifuged twice (160 x g, 10 min., 4oC) and the phagocytes were resuspended in serum-free medium 199. Bacterial killing by phagocytes from blood was determined using a microbiological plate technique (Leijh et al, 1981) and evaluated during 2 hours of incubation at 37oC under continuous shaking in the presence or not of 50 L melatonin (Sigma, final concentration of 10-7M -Paulak et al, 2005). At times 0, 30, 60 and 120 min., 500 *l of bacterial/phagocyte suspensions stimulated or not by melatonin were taken from each tube and centrifuged (160 x g, 5 min., 4oC); 95% MN and 93% PMN cells were viable throughout the assay as shown by Trypan blue exclusion . The supernatant was discarded and 600 *l of 1% Triton X100 (Sigma, St. Louis) were added in order to lyse the cells and release intracellular bacteria. In control experiments performed with bacteria incubated with 1% Triton, we did not observe killed EPEC. One hundred *l were taken from each tube and series of sixfold dilutions were then prepared in tubes containing 900 ml of Tryptic Soy Broth (TSB-Difco). One hundred *l were taken from the last series of dilution tubes and mixed with plate counting agar (TSA) on a Petri dish. After 18 hours of incubation at 37oC the number of colonies was determined. The bactericidal index was calculated as follows:

Bactericidal Index = (1 - NT/NO) x 100

NT = number of colony forming units at times 30, 60 and 120 minutes after phagocytosis

NO = number of colony forming units at time 0

Statistical analysis:Analysis of variance was used to campare superoxide release for different phagocytes and sources of treatment. When statistical significance was found ($p < 0.05$) the Tukey test was applied to determine differences between treatments. The nonparametric Friedman test ($p < 0.05$) was used to compare the bactericidal killing index at different incubation times and with different treatments (Zar, 1984).

RESULTS

MELATONIN EFFECT ON BLOOD PHAGOCYTES SUPEROXIDE REALEASE

MN or PMN blood phagocytes are able to spontaneous superoxide release. When we analyzed superoxide release by phagocytes stimulated with PMA, we observed that PMA enhanced superoxide release over the spontaneous release in both kind of cells (Table 1).

Melatonin stimulated enhance in superoxide release from either PMN or MN phagocytes. The effect was higher in PMN phagocytes, where melatonin effect was equivalent to PMA (Table 1).

PMN phagocytes stimulated with melatonin showed superoxide release at levels equivalent to those of PMA-stimulated phagocytes (Table 1).

Figure 1

TABLE 1. (MTL -107M).

Groups	Superoxide release (nmol) MN	PMN
Phagocytes	3.1 ± 0.6	4.3 ± 1.5
Phagocytes with PMA	6.9 ± 1.5*	9.8 ± 4,0*
Phagocytes with melatonin	4.3 ±1.3*	10.5 ± 4.6*
Statistical results	F=19.7	F= 19.1

*p< 0.001 comparing superoxide release the groups of phagocytes treated with the group of phagocytes without bacteria.

MELATONIN IS ABLE TO STIMULATE

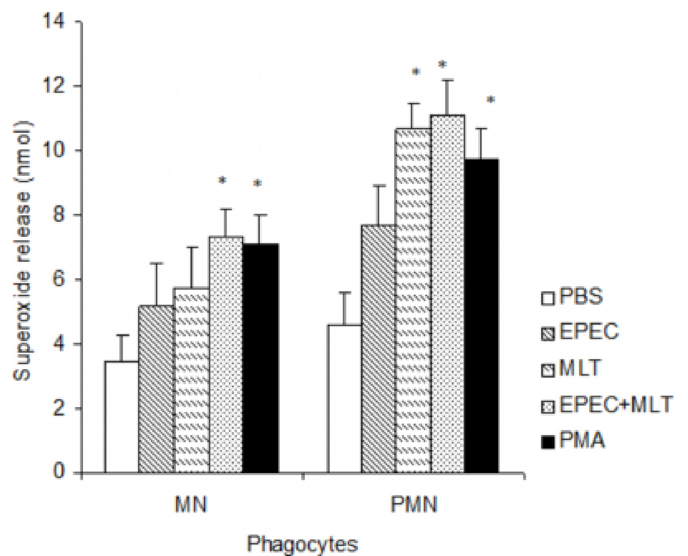
SUPEROXIDE ANION RELEASE BY BLOOD PHAGOCYTES IN THE PRESENCE OF EPEC.

MN and PMN phagocytes in the presence of bacteria increase in superoxide anion release compared with spontaneous release (Figure 1).

MN blood phagocytes stimulated with melatonin in the presence of bacteria showed enhance release superoxide anion when compared with spontaneous release and levels equivalent to those of bacteria stimulated cells (Figure 1). PMN phagocytes stimulated with melatonin in the presence of bacteria showed increase superoxide release when compared with spontaneous release and with the cells only stimulated with bacteria (Figure 1)

Figure 2

Figure 1. Effects of melatonin (MTL -107M), PBS (control) and PMA (107M - positive control) on phagocytes superoxide release (mean Â± sd) (N=10 for each treatment). *p<0.05 comparing superoxide release in cells unstimulated (PBS) with cells stimulated (EPEC, MLT, EPEC+MLT, PMA), considering a same phagocytes.



Melatonin is a Hormone Able to Enhance Bactericidal Activity by Blood Phagocytes.

Blood phagocytes showed bactericidal activity even in the absence of melatonin, although this activity was lower than observed when the cells were stimulated with melatonin (Table 2).

The bactericidal activity of MN phagocytes stimulated with melatonin was time dependent. The highest bactericidal index was observed over the period of 60 and 120 minutes of incubation. PMN phagocytes stimulated with melatonin independently of the time, showed increased in the

bactericidal activity (Table 2).

Figure 3

TABLE 2. Bactericidal activity (expressed as bactericidal index -%) of blood phagocytes against EPEC incubated or not with melatonin.. The results represent the mean and SD of six experiments with cells from different individuals

		Incubation periods (min.)		
Phagocytes	Melatonin	30	60	120
●MN	No	46.0 ± 7.4	50.4 ± 2.1†	54.7 ± 5.6†
	Yes	52.3 ± 4.0	62.8 ± .7*†	76.4 ± 4.6*†
❖PMN	No	51.7 ± 4.0	51.3 ± 2.8	58.4 ± 4.9
	Yes	66.0 ± 10.1*	73.8 ± 5.2*	81.7 ± 4.5*

● X^2 = 26.12 ❖ X2 = 25.62

*p<0.001 comparing the melatonin-treated group with the untreated group for the same phagocyte.

†p<0.001 comparing the values of each incubation time with the preceding time in the same group.

DISCUSSION

Our results demonstrate that blood phagocytes stimulated with melatonin are able to kill EPEC, supporting the idea that this hormone is able to stimulate microbicidal activity by blood phagocytes.

The modulatory role of hormones in the regulation of immunity and the pathogenesis of infection has been the focus of many studies (Stanisz et al, 1994). The evidence that melatonin is part of the intrinsic regulation of the immune cells (Liebmann et al., 1997). Studies indicate that neurohormones exert immunomodulatotory effects (Pierpaoli & Maestroni, 1987; Besedovsky & Rey, 1996) and have showed that melatonin hormone probably acts in the body oxidative processes (Ianãs, et al., 1991). Melatonin is an endogenous neurohormone produced by the pineal gland in mammals, and its beneficial action has been linked to its ability to scavenge different free radicals and increase the antioxidant activity of enzymes (Keplac et al, 2005; . Sudnikovich et al, 2007; Pandi-Perumal et al, 2008). Many studies have postulated that melatonin has a stimulatory action on the immune cells (Cutolo et al, 1999; Skwarlo-Sonta , 2003; Pawlak et al, 2005).

In the present study, melatonin showed an chronoimmunomodulatory effect on blood phagocytes in vitro. Blood PMN phagocytes stimulated with melatonin in the presence of bacteria released superoxide anion at levels equivalent to those of PMA-stimulated phagocytes. Blood MN phagocytes stimulated with melatonin in the presence of bacteria also showed enhanced superoxide release, although at a lower rate than observed in PMN phagocytes. PMN phagocytes may be more sensitive the melatonin effects.

In addition, the effects of melatonin on the immune cells, may be a direct action of on phagocytosis and the phagocytic bichemical process or an indirect acts on the synthesis and release the others hormones or cytokine (Liebmann et al., 1997, Rodriguez et al., 1999; Pandi-Perumal et al, 2008).

At this time , we do not know why melatonin induces enhanced superoxide release by PMN cells compared to MN cells. In response to infections, PMN and MN phagocytes (Babior,1984; Forman et al, 1986; Honorio-França et al, 1997) engage in the respiratory burst as a host cell-mediated immune reaction (Babior,1984).

During oxidative stress, cells generate high contents of superoxide radicals (Rodriguez et al, 2004). Free radical generation has been reported as an important mechanism for body protection in infectious processes, mainly intestinal infections (Honorio-França et al,1997; Honorio-França et al, 2001; França-Botelho et al, 2006).This microbicidal activity may be considered one of first mechanisms in host defense against bacteria and other microorganisms.

In the present study we demonstrated that increased superoxide release by blood phagocytes stimulated with melatonin has an effect on bactericidal activity.

Blood phagocytes showed bactericidal activity when stimulated with melatonin. The highest bactericidal index was observed for phagocytes that were incubated simultaneously with EPEC and melatonin. Ours results suggest that melatonin hormone acts more quickly in PMN phagocytes. The bactericidal activity these phagocytes was similar in every incubation time. By the other hand, the bactericidal activity of MN phagocytes stimulated with melatonin was dependent of time. The effects of melatonin hormone to MN phagocytes only were observed after 60 minutes the incubation. The results suggest that probably activation of microbicidal mechanism, via melatonin, to blood phagocytes may be different. These results support the

idea that superoxide is important in microbicidal mechanisms. It is still not known if melatonin activity is only linked to superoxide release. Interaction between melatonin and other free radicals may possibly be involved in microbicidal mechanisms.

This work support the idea that interactions between melatonin and blood phagocytes may induce superoxide release and increased bactericidal activity, resulting in an additional mechanism of protection against infections. In conclusion, we may state that these results support the hypothesis of pineal involvement through its main hormone melatonin as a possible chronoimmunomodulator of microbicidal processes.

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