J. Pineal Res. 2011; 51:195–206
Doi:10.1111/j.1600-079X.2011.00876.x

Journal of Pineal Research

Journal of Pineal Research
Molecular, Biological, Physiological and Clinical Aspects of Melatonin

Protective effect of melatonin against human leukocyte apoptosis induced by intracellular calcium overload: relation with its antioxidant actions

Abstract: Apoptosis or programmed cell death plays a critical role in both inflammatory and immune responses. Recent evidence demonstrates that control of leukocyte apoptosis is one of the most striking immune system-related roles of melatonin. For this reason, this study evaluated the protective effects of melatonin on human leukocyte apoptosis induced by sustained cytosolic calcium increases. Such protective effects are likely mediated by melatonin's free-radical scavenging actions. Treatments with the specific inhibitor of cytosolic calcium re-uptake, thapsigargin (TG), and/or the calcium-mobilizing agonist, *N*-formyl-methionyl-leucyl-phenylalanine (FMLP), induced intracellular reactive oxygen species (ROS) production, caspase activation as well as DNA fragmentation in human leukocytes. Also, TG- and/or FMLP-induced apoptosis was dependent on both cytosolic calcium increases and calcium uptake into mitochondria, because when cells were preincubated with the cytosolic calcium chelator, dimethyl BAPTA, and the inhibitor of mitochondrial calcium uptake, Ru360, TG- and FMLP-induced apoptosis was largely inhibited. Importantly, melatonin treatment substantially prevented intracellular ROS production, reversed caspase activation, and forestalled DNA fragmentation induced by TG and FMLP. Similar results were obtained by preincubating the cells with another well-known antioxidant, i.e., *N*-acetyl-L-cysteine. To sum up, depletion of intracellular calcium stores induced by TG and/or FMLP triggers different apoptotic events in human leukocytes that are dependent on calcium signaling. The protective effects resulting from melatonin administration on leukocyte apoptosis likely depend on melatonin's antioxidant action because we proved that this protection is melatonin receptor independent. These findings help to understand how melatonin controls apoptosis in cells of immune/inflammatory relevance.

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Key words: antioxidants, calcium signal, caspases, leukocytes, melatonin, mitochondria

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Received December 14, 2010;
Accepted February 2, 2011.

Introduction

The mode of cell death has major biological consequences. Whereas necrosis leads to plasma membrane rupture, release of proinflammatory intracellular molecules and collateral tissue damage, apoptosis removes redundant cells and maintains tissue homeostasis in a safe and nonimmunogenic manner [1]. Physiologically coordinated apoptosis is a critical goal of homeostasis, and a failure to properly control this process leads to hyper- or hypoproliferative disorders [2]. In this sense, apoptosis plays a critical role in the inflammatory and immune responses by regulating the maturation rate of B and T cells and maintaining/contrasting viability of cells engaged in the inflammatory sites [3, 4]. In addition, apoptosis precludes inflammation by confining noxious molecules within intact cell corpses marked for rapid recognition and clearance, typically by professional phagocytes such as neutrophils and macrophages [5, 6].

Although calcium is a key regulator of cell survival, the sustained and prolonged elevation of intracellular calcium plays an important role in cell death [7]. In this regard, we have previously demonstrated that both the specific inhibitor of calcium re-uptake thapsigargin (TG) and the calcium-mobilizing agonist *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) are able to induce a mitochondrial-dependent apoptosis program in human leukocytes, which is associated with the induction of mitochondrial permeability transition pore [8]. On the other hand, apoptosis also can be stimulated by oxidative stress per se, what it has been demonstrated in several cell types [7, 9].

Melatonin (*N*-acetyl-5-methoxytryptamine) is a highly conserved molecule found in organism from unicells to vertebrates [10, 11] and also has pleiotropic bioactivities that encompass numerous endocrinological and behavioral processes [10, 12, 13]. Chemically, melatonin and its metabolites can function as endogenous free-radical scav-

engers and broad-spectrum antioxidants [14, 15]. Thus, under conditions such as ischemia/reperfusion injury, neuronal excitotoxicity, and chronic inflammation, where the oxidative environment is the direct cause of cell death, melatonin was shown to counteract apoptosis by exerting its potent radical scavenging ability [16, 17]. Moreover, because melatonin easily reaches all cellular and subcellular compartments because of its small size and amphiphilic nature [18], most of the beneficial consequences resulting from melatonin administration may depend on its effect on mitochondrial physiology [19, 20].

Interestingly, emerging evidence demonstrates an unforeseen role of melatonin in the control of immune and inflammatory responses, showing that leukocytes possess all the enzymatic machinery necessary to synthesize melatonin from tryptophan [21] as well as the proper receptors MT1/MT2 [22], thereby being an autonomous compartment as far as melatonin responses are concerned. Accordingly, much evidence demonstrates that control of leukocyte apoptosis is one of the most striking immune system-related roles of melatonin [8, 23]. For this reason, it is important to clarify how melatonin controls apoptosis in cells of immune/inflammatory relevance. Additionally, melatonin was found to be at extremely high concentrations in bone marrow [24], thus possibly accounting for the intriguing observation that higher levels of melatonin are required for apoptosis control than for neuroendocrine functions.

In the present study, we demonstrated that TG- and/or FMLP-induced apoptosis in human leukocytes is likely triggered owing to intracellular calcium increases and is also dependent on mitochondrial calcium overload. Moreover, we evaluated the protective effect of melatonin on apoptosis induced by sustained intracellular calcium increases. We also proved that the beneficial consequences resulting from melatonin administration likely depend on its antioxidant effect.

Materials and methods

Reagents

Melatonin, RPMI-1640 medium, Ficoll-Histopaque separating medium, FMLP, 2-benzyl-*N*-acetyltryptamine (luzindole), polyethylene glycol-catalase (PEG-catalase), *N*-acetyl-L-cysteine (NAC), reduced glutathione (GSH), *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (AC-DEVD-AMC), nonidet-P-40 substitute (NP40), 2-(*N*-morpholino) ethanesulfonic acid hydrate (MES hydrate), PEG, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 3-[(3-chomalidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), and dithiothreitol (DTT) were obtained from Sigma Chemical Co. (Madrid, Spain). Dihydrorhodamine-123 (DHR 123), Tris–glycine gels, and TG were from Molecular Probes (Eugene, OR, USA). *N*-acetyl-Leu-Glu-His-Asp-7-amino-4-methylcoumarin (AC-LEHD-AMC) was purchased from Bachem (Bubendorf, Switzerland). Anti-caspase-9 (C9), anti-caspase-3 (8G10), and anti-β-actin (8H10D10) were from Cell Signaling (Danvers, MA, USA). In situ cell death detection kit was from Roche Diagnostics (Mannheim, Germany). Hoechst 33342 was obtained from Calbiochem (San Diego, CA, USA). All others reagents were of analytical grade.

Human leukocytes isolation

Venous blood was drawn from healthy volunteers of both genders and age between 20 and 45 yr old under informed consent according to a procedure approved by Local Ethical Committees and in accordance with the Declaration of Helsinki. Human leukocytes were separated from whole blood using Ficoll-Histopaque density centrifugation. After centrifugation at 600 g for 30 min, peripheral blood mononuclear cells were isolated from the Histopaque-1077/1119 upper interphase and maintained in RPMI-1640 medium for 1 hr to allow the adherence of monocytes so as to obtain a pure lymphocyte preparation, as previously described [8]. Similarly, peripheral blood polymorphonuclear cells were harvested from the lower interphase, and residual erythrocytes were then lysed by the short treatment of neutrophil pellet with an ice-cold isotonic NH_4Cl solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.4) so as to get a neutrophil-enriched preparation, as described elsewhere [8]. Cell purity was routinely above 95% in both cell types, after Diff-Quick staining. Once purified, both neutrophils and lymphocytes were collected and washed in phosphate-buffered saline (PBS) at 480 g for 10 min. The supernatant was then discarded, and the cell pellet was gently resuspended in RPMI-1640 medium.

Measurement of intracellular free-calcium concentration ($[Ca^{2+}]_c$)

Leukocytes were loaded with fura-2 by incubation with 4 μM fura-2 acetoxymethyl ester (Fura 2-AM) for 30 min at room temperature according to a procedure published elsewhere [8]. Once loaded, the cells were washed and used within the next 2-4 hr. Fluorescence was recorded from 2-mL aliquots of magnetically stirred cellular suspension (2×10^6 cells/mL) at 37°C by using a spectrofluorophotometer (RF-5301-PC; Shimadzu, Kyoto, Japan) with excitation wavelengths of 340 and 380 nm and emission at 505 nm. Changes in $[Ca^{2+}]_c$ were monitored by using the Fura 2-AM 340/380 nm fluorescence ratio and were calibrated according to the method of Grynkiewicz et al. [25]. In the experiments where calcium-free medium is indicated, calcium was omitted and 1 mM ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) was added.

Intracellular reactive oxygen species (ROS) measurement

DHR 123 is a nonfluorescent, noncharged dye that easily penetrates cell membrane. Once inside the cell, DHR 123 becomes fluorescent upon oxidation to yield rhodamine 123 (Rh 123), fluorescence being proportional to ROS generation. DHR 123 was found to be a nontoxic and about threefold more sensitive indicator of granulocyte respiratory burst activity than 2′,7′-dichlorofluorescin diacetate (DCFH-DA) [26, 27]. Briefly, leukocytes (1×10^6 cells/mL) were washed with serum-free RPMI-1640 medium and

incubated with 20 μM DHR 123 at 37°C for 25 min. Cells were then washed in PBS. The fluorescence intensity of Rh 123 was measured in an automatic microplate reader (Infinite M200; Tecan Austria GmbH, Groedig, Austria). Excitation was set at 488 nm and emission at 543 nm. Treatments were carried out in triplicate. The data are presented as fold-increase over the pretreatment level (experimental/control).

Assay for caspase activity

The determination of caspase-3 and caspase-9 activities was based on a method previously reported [28] with minor modifications. Stimulated or resting cells were pelleted and washed once with PBS. After centrifugation, cells were resuspended in PBS at a concentration of 1×10^7 cells/mL. Fifteen microliters of the cell suspension was added to a microplate and mixed with the appropriate peptide substrate dissolved in a standard reaction buffer that was composed of 100 mM HEPES, pH 7.25, 10% sucrose, 0.1% CHAPS, 5 mM DTT, 0.001% NP40 and 40 μM of caspase-3 substrate (AC-DEVD-AMC) or 0.1 M MES hydrate, pH 6.5, 10% PEG, 0.1% CHAPS, 5 mM DTT, 0.001% NP40, and 100 μM of caspase-9 substrate (AC-LEHD-AMC). Substrate cleavage was measured with a microplate reader (Infinite M200) with excitation wavelength of 360 nm and emission at 460 nm. Preliminary experiments reported that caspase-3 or caspase-9 substrate cleaving was not detected in the presence of the inhibitors of caspase-3 or caspase-9, DEVD-CMK or z-LEHD-FMK, respectively. The data were calculated as fluorescence units/mg protein and presented as fold-increase over the pretreatment level (experimental/control).

Western blot analysis

One-dimensional sodium dodecyl sulfate (SDS) electrophoresis was performed with a 4–12% gradient Tris–glycine, and separated proteins were then electrophoretically transferred, for 2 hr at 0.8 mA/cm^2, in a semi-dry blotter onto nitrocellulose for subsequent probing. Blots were incubated overnight with 5% (w/v) nonfat dry milk in Tris-buffered saline with 0.1% Tween 20 [Tris-buffered saline with Tween 20 (TBST)] to block residual protein-binding sites. Blocked membranes were then incubated for 3 hr with the anticaspase-3 antibody (8G10) and the anticaspase-9 antibody (C9) diluted 1:1000 in TBST. The primary antibody was removed and blots washed three times for 10 min each with TBST. To detect the primary antibodies, blots were incubated with the appropriate horseradish peroxidase-conjugated anti-IgG antibody diluted 1:5000 in TBST, washed three times in TBST, and exposed to enhanced chemiluminescence reagents for 5 min. Blots were then exposed to photographic films, and the optical density was estimated using scanning densitometry.

In situ detection of DNA fragmentation by TUNEL and Hoechst staining

At the end of a treatment protocol, human leukocytes were harvested and washed once with PBS. The cells were then fixed with 4% paraformaldehyde [in PBS, pH 7.4, at least 6 hr, room temperature (RT)] and air-dried on slides for 24 hr. Afterward, the air-dried cells were washed twice with PBS and incubated in permeabilization solution (0.1% Triton-X-100 in 0.1% sodium citrate, 15 min, RT). The permeabilization solution was then removed, and terminal deoxynucleotidyl transferase-mediated dUDP nick-end labelling (TUNEL) reaction mixture (50 μL) was added, and the cells were incubated (1 hr, 37°C) in a humidified chamber. The cells were washed again with PBS and counterstained with Hoechst 33342 (1 μg/mL) in PBS for 5 min to identify cellular nuclei. The incidence of apoptosis was assessed under an epifluorescence microscope (BX51; Olympus Spain S.A.U., Barcelona, Spain) using a FITC filter. Cells with TUNEL-positive nuclei were considered apoptotic. Hoechst staining was used to determine the total number of cells in a field. A minimum of five fields per slide was used to calculate the percentage of apoptotic cells, which was expressed as fold-increase over the pretreatment level (experimental/control).

Statistical analysis

Data are expressed as means ± S.E.M. of the numbers of determinations. To compare the different treatments, statistical significance was calculated by one-way analysis of variance followed by the Tukey's multiple comparison test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

It has been reported that a prolonged elevation in cytosolic free-calcium concentration ($[Ca^{2+}]_c$) as well as alterations in calcium homeostasis initiates the mitochondrial apoptotic pathway [8] and induces endoplasmic reticulum stress that, in turn, leads to apoptosis [29]. In the absence of extracellular calcium (calcium-free medium), fura-2-loaded human leukocytes were treated with both FMLP and TG. As shown in Fig. 1A, stimulation with 10 nM FMLP induced a typical transient increase in $[Ca^{2+}]_c$ because of calcium release from internal stores in human neutrophils. Similarly, stimulation of human neutrophils or lymphocytes with 1 μM TG caused a transient increase in $[Ca^{2+}]_c$ which reached a stable $[Ca^{2+}]_c$ plateau after 5 min of stimulation (Fig. 1B,C), thus reflecting the release of calcium from nonmitochondrial agonists-releasable pools. These increases induced by FMLP and TG were also observed in the presence of normal extracellular calcium (Fig. 1D–F), although the levels of calcium remained raised in comparison with those obtained in the absence of extracellular calcium.

To investigate the relationship between intracellular calcium overload-induced apoptosis and oxidative stress, human leukocytes were treated with the specific inhibitor of calcium reuptake, TG, or the calcium-mobilizing agonist, FMLP, and then intracellular ROS production was measured. The addition of 1 μM TG for 1 hr to human neutrophils induced a significant increase in intracellular ROS levels ($P < 0.05$, Fig. 2A). A similar result, although to a smaller extent, was obtained by incubating neutrophils

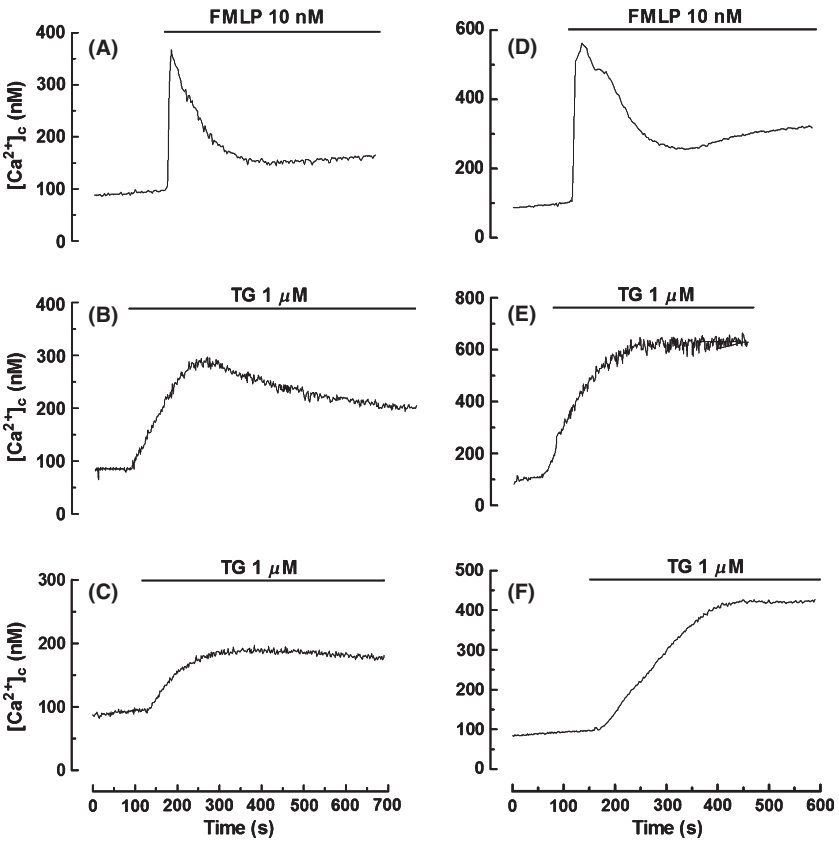

Fig. 1. Calcium mobilization in human leukocytes. Fura-2-loaded human leukocytes were stimulated with 10 nM *N*-formyl-methionyl-leucyl-phenylalanine (panels A and D) or 1 μM thapsigargin (panels B, C, E and F), as indicated, in a calcium-free medium ($[Ca^{2+}]_o$ = 0 mM + 1 mM EGTA was added; panels A, B and C) or in the presence of normal extracellular calcium ($[Ca^{2+}]_o$ = 1.2 mM; panels D, E and F). The traces shown are representative of eight separate experiments.

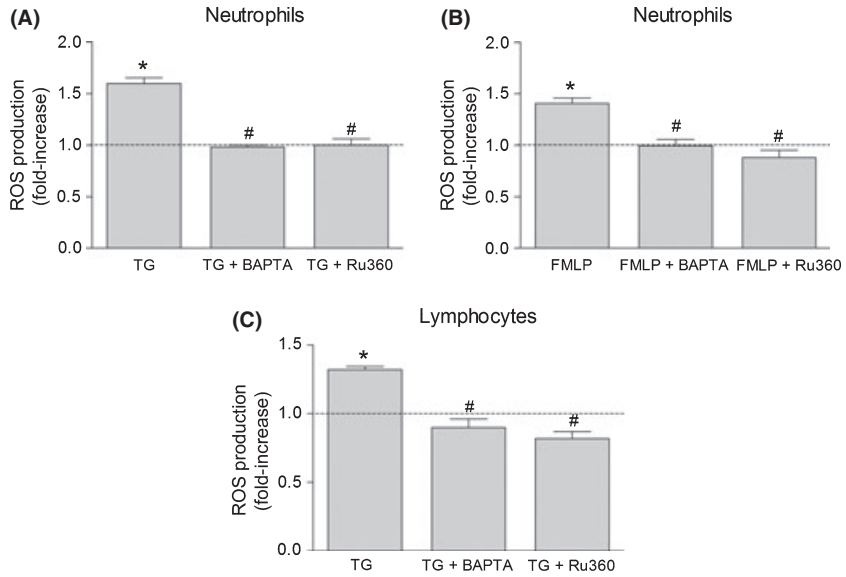

Fig. 2. Blockade of calcium signaling affects intracellular reactive oxygen species (ROS) production. Human leukocytes were preincubated with the intracellular calcium chelator dimethyl 1,2-bis(o-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) (10 μM) or the specific blocker of calcium uptake into mitochondria Ru360 (10 μM) or the vehicle for 30 min and then stimulated with 1 μM thapsigargin (TG) (A and C) or 10 nM *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) (B) for 1 hr. Changes in intracellular ROS production were estimated as described under Materials and methods section. Values are presented as means ± S.E.M. of six separate experiments and expressed as fold-increase over the pretreatment level (experimental/control). $^{*}P < 0.05$ compared to control values. $^{\#}P < 0.05$ compared to TG- or FMLP-treated cells.

with 10 nM FMLP ($P < 0.05$, Fig. 2B) for 1 hr. Moreover, the stimulation of human lymphocytes with 1 μM TG for 1 hr again provoked a marked rise in intracellular ROS levels ($P < 0.05$, Fig. 2C). Interestingly, when both neutrophils and lymphocytes were preincubated for 30 min with both the cytosolic calcium chelator, dimethyl

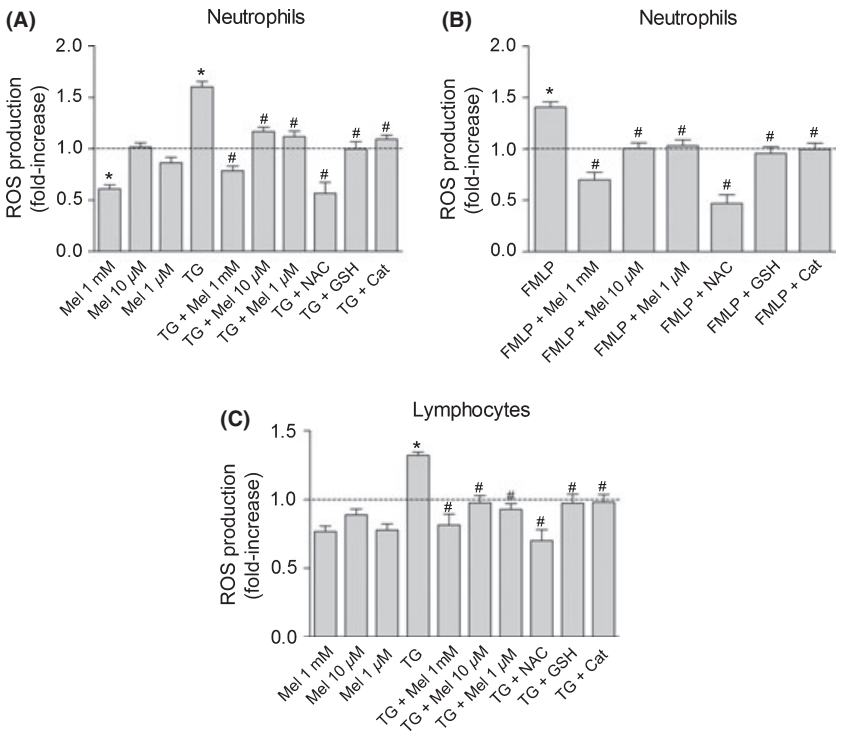

Fig. 3. Melatonin and other antioxidants antagonize intracellular reactive oxygen species (ROS) production induced by calcium signaling. Human leukocytes were alternatively preincubated with different doses of melatonin (Mel), 15 mM *N*-acetyl-L-cysteine, 5 mM reduced glutathione, 100 u/mL polyethylene glycol-catalase (Cat) or the vehicle for 1 hr and then stimulated with 1 μM thapsigargin (TG) (A and C) or 10 nM *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) (B) for 1 hr. Changes in intracellular ROS production was estimated as described under Materials and methods section. Values are presented as means ± S.E.M. of six separate experiments and expressed as fold-increase over the pretreatment level (experimental/control). $^{*}P < 0.05$ regarding control values. $^{\#}P < 0.05$ regarding TG- or FMLP-treated cells.

1,2-bis(o-aminophenoxy) ethane-*N,N,N′,N′*-tetraacetic acid (BAPTA) (10 μM), and Ru360 (10 μM), an inhibitor of mitochondrial calcium uptake to reduce mitochondrial matrix calcium, TG- and FMLP-induced ROS production was substantially abolished ($P < 0.05$, Fig. 2), thereby indicating that the above-mentioned ROS production is dependent not only on intracellular calcium increases but also on calcium uptake into mitochondria.

Both melatonin and its metabolites can function as endogenous scavengers of damaging free radicals [14, 15]. In this regard, intracellular ROS production induced by TG and/or FMLP was assessed in the absence and presence of different concentrations of melatonin. Thus, when human leukocytes were preincubated with any melatonin concentration tested (1 mM, 10 μM, and 1 μM) for 1 hr, both TG and FMLP exerted a negligible effect on ROS production ($P < 0.05$, Fig. 3), thereby confirming the well-known, antioxidant properties of melatonin. Although the strongest scavenging activity was achieved with the highest melatonin concentration used (1 mM), the antioxidant effects of melatonin do not seem to be entirely dose dependent, as both 10 and 1 μM melatonin doses displayed almost similar effects (Fig. 3). Likewise, as the lower dose of melatonin (1 μM) also proved to be effective in scavenging free radicals, such a dose was used to determine the antiapoptotic effect of melatonin in human leukocytes.

Apart from this, the effect of several well-known antioxidants on ROS production evoked by calcium signaling was also evaluated to compare their antioxidant ability with that showed by melatonin. Consequently, human leukocytes were preincubated with 15 mM NAC, 5 mM GSH or 100 u/mL cell-permeable PEG-catalase conjugate for 1 hr and then treated with 1 μM TG or 10 nM FMLP for 1 hr. In both neutrophils and lymphocytes, NAC clearly exerted the most powerful antioxidant effect ($P < 0.05$, Fig. 3). In fact, the scavenging actions revealed by NAC looked even stronger than those evidenced by 1 mM melatonin. Hence, the broad-spectrum antioxidant NAC was used in subsequent experiments so as to verify that the antiapoptotic actions of melatonin depend, at least in part, on its scavenging effects. Both GSH and PEG-catalase were also able to significantly weaken the oxidative burst induced by both calcium-mobilizing agents ($P < 0.05$, Fig. 3).

To examine the effect of melatonin on caspase-3 activation induced by calcium signaling, 1 μM TG or 10 nM FMLP was again administered to human leukocytes. Our results showed that both TG (Fig. 4A) and FMLP (Fig. 4B) succeeded in increasing the caspase-3 activity in human neutrophils ($P < 0.05$). Nevertheless, when neutrophils were preincubated with 1 μM melatonin for 1 hr, TG- or FMLP-induced caspase-3 activity was substantially lessened ($P < 0.05$, Fig. 3A,B). Similar results were obtained when human neutrophils were preincubated with 15 mM NAC for 1 hr ($P < 0.05$, Fig. 4A,B). Remarkably, blockade of both cytosolic calcium increases with 10 μM dimethyl BAPTA and mitochondrial calcium uptake with 10 μM Ru360 for 30 min also turned out effective because they managed to reverse caspase-3 activation evoked by TG and FMLP ($P < 0.05$, Fig. 4A,B), thus demonstrating that such an apoptotic feature is likely triggered owing to cytosolic calcium increases and is also dependent on mitochondrial calcium overload. Likewise, in human lymphocytes, extensive depletion of the intracellular calcium stores by using 1 μM TG for 1 hr induced caspase-3 activation similar to that observed in neutrophils ($P < 0.05$, Fig. 4C), which was almost completely forestalled by blocking cytosolic calcium increases, calcium uptake into mitochondria as well as ROS production ($P < 0.05$, Fig. 4C).

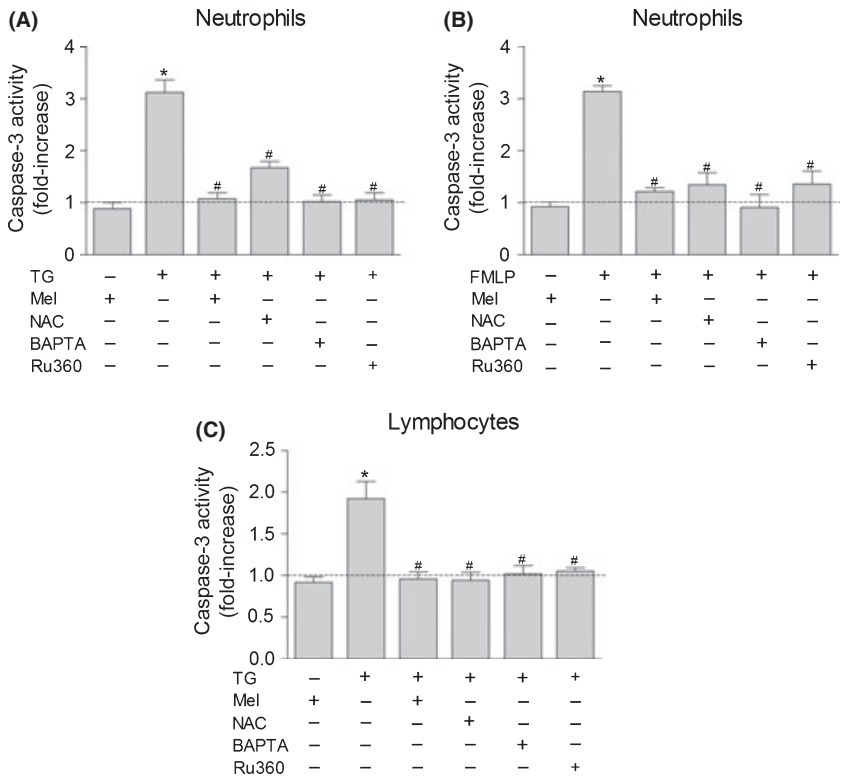

Fig. 4. Melatonin attenuates intracellular calcium overload-induced caspase-3 activation. Human leukocytes were preincubated with 1 μM melatonin (Mel) or 15 mM *N*-acetyl-L-cysteine for 1 hr, 10 μM dimethyl BAPTA or 10 μM Ru360 for 30 min, or the vehicle, and then stimulated with 1 μM thapsigargin (TG) (A and C) or 10 nM *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) (B) for 1 hr to check the caspase-3 activity. Caspase-3 activity was estimated as described under Materials and methods section. Values are presented as means ± S.E.M. of six separate experiments and expressed as fold-increase over the pretreatment level (experimental/control). $^{*}P < 0.05$ compared to control values. $^{\#}P < 0.05$ compared to TG- or FMLP-treated cells.

The results obtained by the determination of caspase activity were confirmed by western blotting. Caspase-3 activation was analyzed by using a specific monoclonal anticaspase-3 antibody, which detects the full-length inactive (procaspase) form (35 kDa) and also the active large caspase-3 fragment (17 kDa) resulting from cleavage at Asp175 [30]. Treatment of neutrophils with 1 μM TG for 1 hr resulted in a substantial activation of caspase-3 as revealed by the increase in the amount of the active form and the decrease in the inactive procaspase form of caspase-3 ($P < 0.05$, Fig. 5A). Similar results were obtained when lymphocytes were stimulated with 1 μM TG (Fig. 5B). Nonetheless, the preincubation of human leukocytes with 1 μM melatonin for 1 hr significantly prevented TG-evoked activation of caspase-3, as estimated by the content of the active form of caspase-3 ($P < 0.05$, Fig. 5A,B). Additionally, when human leukocytes were preincubated with 15 mM NAC for 1 hr, the content of caspase-3 active form was again extensively diminished ($P < 0.05$, Fig. 5A,B). Lastly, the pretreatment with both the intracellular calcium chelator, dimethyl BAPTA, and the inhibitor of mitochondrial calcium uniporter, Ru360, also succeeded in reversing the amount of active caspase-3 evoked by TG ($P < 0.05$, Fig. 5A,B).

As caspase-9 is an initiator caspase that is involved in the initial steps of mitochondrial apoptosis [31], we also checked caspase-9 activity in the presence of TG or FMLP to ascertain that intracellular calcium overload-induced oxidative stress leads to mitochondrial apoptosis. As shown in Fig. 5C,D, treatment of neutrophils with 1 μM TG or 10 nM FMLP for 1 hr obviously produced a significant rise in caspase-9 activity ($P < 0.05$). Similarly, the treatment with 1 μM TG for 1 hr caused substantial caspase-9 activation in human lymphocytes ($P < 0.05$, Fig. 5E). However, the preincubation with 1 μM melatonin for 1 hr largely prevented TG- or FMLP-induced caspase-9 activity in human leukocytes ($P < 0.05$, Fig. 5C–E), thereby suggesting that the beneficial consequences resulting from melatonin administration likely depend on its effect on mitochondrial physiology. Also, the preincubation with NAC (15 mM) for 1 hr widely counteracted caspase-9 activation produced by intracellular calcium increases ($P < 0.05$, Fig. 5C–E). Importantly, caspase-9 activation also depends on both cytosolic calcium increases and mitochondrial calcium overload as both the preincubation of leukocytes for 30 min with 10 μM dimethyl BAPTA and 10 μM Ru360 were able to decrease TG- or FMLP-induced caspase-9 activity ($P < 0.05$, Fig. 5C–E).

Caspase-9 activation was also analyzed by western blotting. In this case, a monoclonal anticaspase-9 antibody that recognizes the full-length inactive (pro-caspase) form (47 kDa) and the active large caspase-9 fragment (35 kDa) was used [32]. Once more, western blot analysis revealed that treatment of leukocytes with 1 μM TG significantly augmented the amount of the active form of caspase-9 ($P < 0.05$, Fig. 5A,B). The preincubation of leukocytes with 1 μM melatonin for 1 hr managed, however, to almost entirely prevent the stimulatory effect of TG, as inferred from the decrease in the amount of the active form and the increase in the inactive procaspase form of caspase-9 ($P < 0.05$, Fig. 5A,B). Moreover, when human leukocytes were preincubated for 1 hr with 15 mM NAC, the content of caspase-9 active form was again widely reduced ($P < 0.05$, Fig. 5A,B). Likewise, the pretreatment with both the intracellular calcium chelator, dimethyl BAPTA, and the mitochondrial calcium uniporter inhibitor, Ru360,

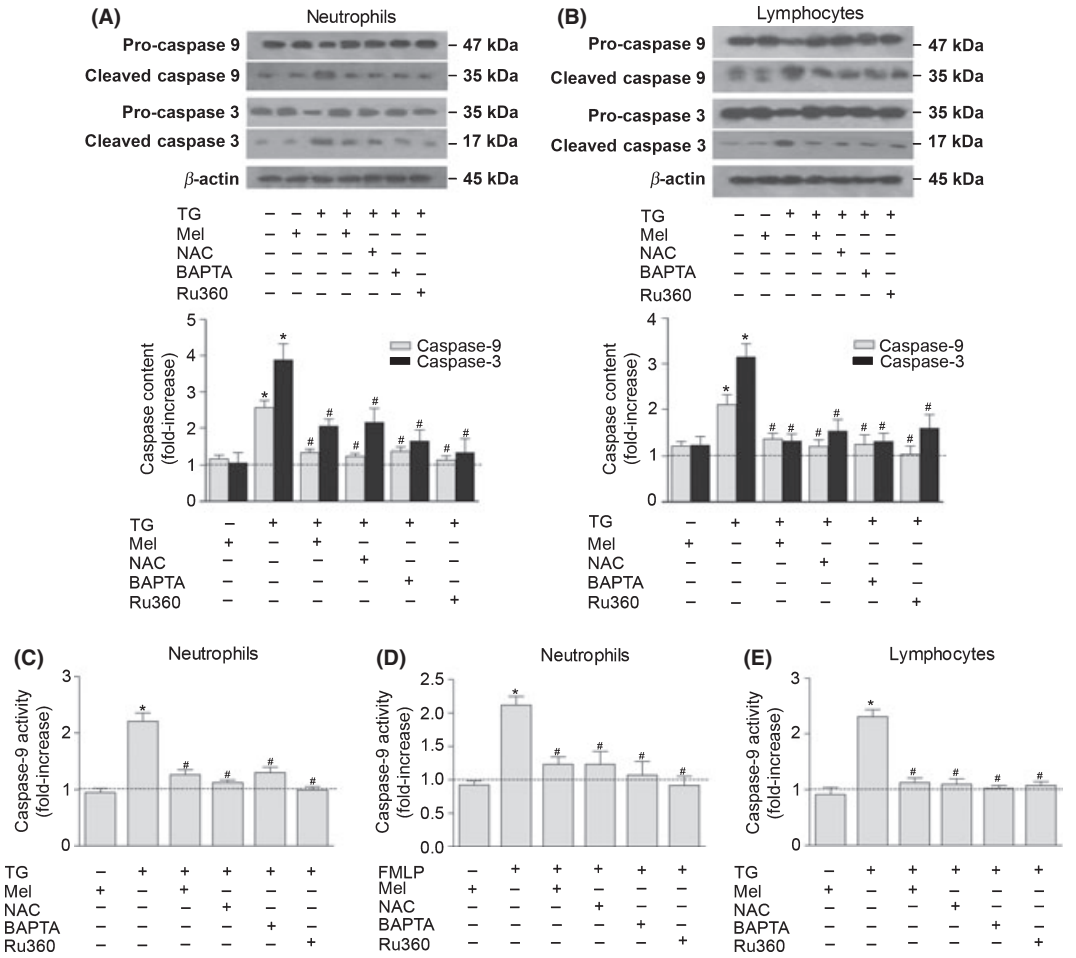

Fig. 5. Melatonin counteracts the stimulatory effect of intracellular calcium overload on the content of active caspases. To determine caspase content (A and B), human neutrophils (A) and lymphocytes (B) were preincubated as described in Fig. 4 and then stimulated with 1 μM thapsigargin (TG) for 1 hr. Samples were lysed and then subjected to gradient Tris–glycine isolation and subsequent western blotting with a specific anticaspase-3 (8G10) or anticaspase-9 (C9) antibody and reprobed with anti-β-actin (8H10D10) antibody for protein-loading control. Histograms showed in A and B represent the quantification of the 35 kDa fragment of cleaved caspase-9 (gray bars) or the 17-kDa fragment of cleaved caspase-3 (black bars) expressed as fold-increase over the pretreatment level (experimental/control). Results are presented as mean ± S.E.M. of four independent experiments. $^{*}P < 0.05$ compared to control values. $^{\#}P < 0.05$ compared to its corresponding TG-induced caspase content values. To determine caspase-9 activity (C–E), human leukocytes were again preincubated as described in Fig. 4 and then stimulated with 1 μM TG (C and E) or 10 nM *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) (D) for 1 hr to check the caspase-9 activity. Caspase-9 activity was estimated as described under Materials and methods section. Values are presented as means ± S.E.M. of six separate experiments and expressed as fold-increase over the pretreatment level (experimental/control). $^{*}P < 0.05$ regarding control values. $^{\#}P < 0.05$ regarding TG- or FMLP-treated cells.

was able to lessen the amount of active caspase-9 evoked by TG again ($P < 0.05$, Fig. 5A,B). Taken together, these findings strongly suggest that depletion of intracellular calcium pools by both TG and FMLP and subsequent mitochondrial calcium overload cause activation of caspase-3 and caspase-9 that is forestalled by melatonin likely due to its antioxidant properties.

As TUNEL assay is a well-established method for detection of DNA cleavage, a relatively late apoptotic marker [33], we assessed the amount of DNA fragmentation in the presence of TG to verify whether sustained intracellular calcium increases leads to cell death. Thus, treatment of human neutrophils with 1 μM TG for 1 hr produced a substantial increase in the proportion of cells depicting DNA fragmentation ($P < 0.05$, Fig. 6A). Similar results were found when human lymphocytes were treated for 1 hr with 1 μM TG ($P < 0.05$, Fig. 6B). Additionally, it is worth noting that the preincubation of human leukocytes with 1 μM melatonin for 1 hr significantly weakened the stimulatory effect of TG on DNA fragmentation ($P < 0.05$, Fig. 6), thus substantiating that melatonin is able to prevent intracellular calcium overload-induced cell death. On the other hand, the preincubation of leukocytes for 1 hr with the powerful antioxidant NAC (15 mM) strongly diminished the proportion of cells depicting DNA fragmentation ($P < 0.05$, Fig. 6). Curiously, both dimethyl BAPTA and Ru360 (10 μM, 30 min) also managed to significantly inhibit the stimulatory effect of TG on DNA fragmentation ($P < 0.05$, Fig. 6), thereby indicating that mitochondrial apoptosis is dependent on

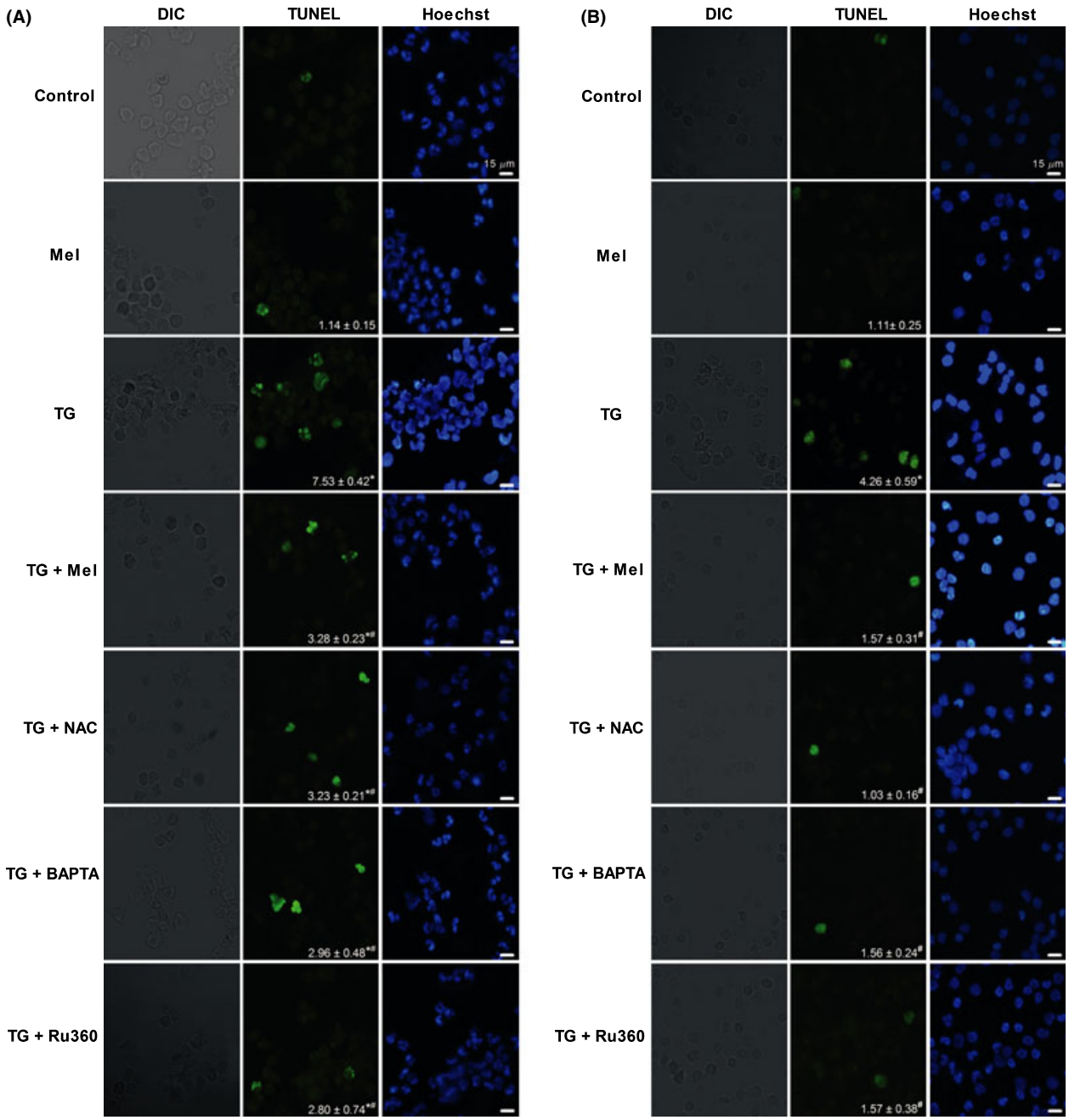

Fig. 6. Melatonin prevents cell death in human leukocytes. Neutrophils (A) and lymphocytes (B) were preincubated with 1 μM melatonin (Mel) or 15 mM *N*-acetyl-L-cysteine for 1 hr, 10 μM dimethyl BAPTA or 10 μM Ru360 for 30 min, or the vehicle, and then stimulated with 1 μM thapsigargin (TG) for 1 hr to check the proportion of cells depicting DNA fragmentation. DNA fragmentation was estimated as described under Materials and methods section. Left panel: differential interference contrast (DIC) images of cultured cells. Central and right panels: pictures of TUNEL-positive cells (green) and Hoechst 33342 nuclear stain (blue), respectively. Scale bars: 15 μm. Values inside the central panel are presented as means $\pm$ S.E.M. of three separate experiments and expressed as fold-increase over the pretreatment level (experimental/control). $^{*}P < 0.05$ regarding control values. $^{\#}P < 0.05$ regarding TG alone.

both cytosolic calcium increases and calcium uptake into mitochondria.

Finally, to further clarify whether the protective effect of melatonin on mitochondrial apoptosis actually depends on the interaction between melatonin and its cell membrane receptors, we also analyzed whether luzindole, which specifically antagonizes melatonin binding/activation of MT1/MT2 receptors, was able to counteract the inhibition of caspase-9 induced by melatonin. To this purpose, neutrophils and lymphocytes were pretreated with 50 μM luzindole, 30 min before melatonin treatments. As shown in Fig. 7, in the presence of luzindole, 1 μM melatonin was still able to forestall TG- and FMLP-induced caspase-9 activation ($P < 0.05$). These findings show that the signal transduction elicited by MT1/MT2 receptor stimulation does not seem to play a role in the protective effect of

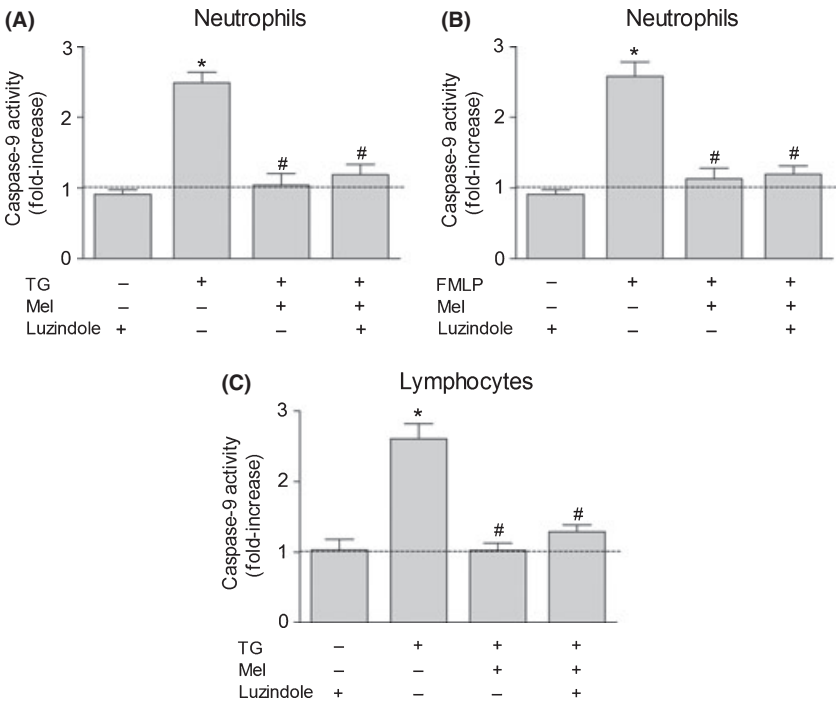

Fig. 7. The protective effect of melatonin on mitochondrial apoptosis is receptor independent. Human leukocytes were preincubated with 1 μM melatonin (Mel) for 1 hr in the absence or the presence of its MT1/MT2 receptor antagonist luzindole and then stimulated with 1 μM thapsigargin (TG) (A and C) or 10 nM *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) (B) for 1 hr to check the caspase-9 activity. Luzindole was added at the concentration of 50 μM, 30 min before melatonin treatments. Caspase-9 activity was estimated as described under Materials and methods section. Values are presented as means ± S.E.M. of six separate experiments and expressed as fold-increase over the pretreatment level (experimental/control). $^{*}P < 0.05$ compared to control values. $^{\#}P < 0.05$ compared to TG- or FMLP-treated cells.

melatonin on mitochondrial apoptosis, thus confirming that the beneficial consequences resulting from melatonin administration are melatonin receptor independent.

Discussion

The involvement of calcium in cell death has been repeatedly documented in previous studies [8, 34]. The proapoptotic effects of calcium are mediated by a diverse range of calcium-sensitive factors that are compartmentalized in various intracellular organelles including endoplasmic reticulum and mitochondria [35]. In addition, mitochondria act as calcium buffers by sequestering excess calcium from the cytosol. Excessive calcium load to the mitochondria may induce an apoptotic program by stimulating the release of apoptosis promoting factors from the mitochondria intermembrane space to cytosol and by impairing mitochondrial function [7, 36]. Apoptosis can also be stimulated by ROS in several cell types [9, 37]. It has been even shown that ROS, such as H_2O_2, increases cytosolic calcium in the absence of extracellular calcium, thus indicating that H_2O_2 mobilizes calcium from intracellular stores [38], leading cells into an apoptotic state. Our data show that intracellular calcium overload induces increases in intracellular ROS production, which is dependent not only on cytosolic calcium increases but also on mitochondrial calcium uptake.

These results are consistent with evidence showing a direct interaction between mitochondria and intracellular calcium stores and the observation of close physical contacts between both organelles [39, 40]. In fact, there is both structural and functional evidence suggesting the presence of specific and stable interactions between mitochondria and intracellular calcium stores (i.e., endoplasmic reticulum), which facilitate a rapid and nearly direct flux of calcium from endoplasmic reticulum to mitochondria [39, 41–44], and these tight endoplasmic reticulum–mitochondria couplings may also serve to modulate calcium release. In addition, it has been suggested that mitochondria co-localize in small subcellular regions where reticulum and mitochondria form close contacts [44]. This has led to the concept of 'intracellular synapse' or 'quasi-synaptic' calcium signal transmission between mitochondria and reticulum that was coined by Csordás et al. [40].

Melatonin is an indoleamine involved in many important physiological functions. In terms of immune function, the role of melatonin as an immunomodulator is widely known [45–47]. However, in recent years, interest in melatonin has markedly increased because of its influence on the apoptotic process. The exact mechanism by which melatonin influences apoptosis is not yet clear as melatonin has been reported to have both proapoptotic and antiapoptotic actions (for review, see [21]). Here, we demonstrated that melatonin is able to reduce intracellular ROS generation induced by calcium signaling in human leukocytes, in both neutrophils and lymphocytes. In addition, our results also indicate that melatonin induces a protective effect on apoptosis evoked by calcium signaling owing to the substantial reduction exerted by the indoleamine on caspase-9 and caspase-3 as well as on DNA fragmentation. Furthermore, when mitochondria were prevented from loading with calcium by preincubation of leukocytes with both dimethyl BAPTA and Ru360, then the intracellular ROS production, caspase activation, and DNA fragmentation were clearly diminished. These findings corroborate previous studies in which the blockade of cytosolic calcium increases and calcium uptake into mitochondria with BAPTA or Ru360, respectively, does inhibit apoptosis in several cell types [37, 48, 49].

Finally, to our knowledge, we have demonstrated the protective role of melatonin on calcium signaling-induced apoptosis in primary human leukocytes, instead of cell lines or cells from other species, thus avoiding the common problem of translation into humans. The inhibitory effect of melatonin on apoptosis induced by intracellular calcium overload might not be a consequence of the opposing action of melatonin in the calcium pathway because we have previously shown that melatonin is ineffective at modifying TG-induced calcium signal [8]. However, we provided evidence supporting that the inhibitory effect of melatonin on apoptosis is likely due to its antioxidant capacity and free-radical scavenging actions [14], as melatonin was able to reduce not only the intracellular ROS production but also caspase-9 and caspase-3 activation and DNA damage induced by intracellular calcium overload. This was also the case of the well-known antioxidant NAC. On the other hand, catalase almost completely inhibited the calcium overload-evoked ROS production, which might suggest that hydrogen peroxide is the major ROS produced. In this sense, despite melatonin is not a great hydrogen peroxide scavenger [50], it is worth noting that melatonin reportedly suppresses superoxide anion production, an upstream event of hydrogen peroxide production [14, 51, 52], and stimulates the activation and gene expression of several antioxidant endogenous enzymes, such as superoxide dismutase and catalase, both under physiological and under conditions of elevated oxidative stress [14, 53].

Additionally, we showed that the effects of melatonin are independent of plasma membrane MT1/MT2 receptor stimulation, as the inhibitory effect of melatonin on leukocyte apoptosis is unaffected by the MT1/MT2 antagonist luzindole. Melatonin, as an amphipathic molecule, may freely cross the plasma membrane, and so it may rapidly accumulate within cells and react with the cytosolic target [54]. Our findings are in agreement with previous reports showing that, in isolated mitochondria, melatonin prevents opening of mitochondrial permeability transition pore and release of intramitochondrial proapoptotic factors, which are cell death-associated events [8, 55], and protects mitochondria from oxidative damage [52]. However, the inhibitory effect of melatonin administration on intracellular ROS generation differs from recent, surprising findings reporting pro-oxidant ability of melatonin in both normal and tumor leukocytes [54, 56].

In conclusion, we demonstrated that depletion of intracellular calcium stores induced by TG and/or FMLP triggers different apoptotic events in human leukocytes that are dependent on cytosolic calcium increases, calcium uptake into mitochondria as well as ROS production. On the other hand, we also proved that the protective effects resulting from melatonin administration on leukocyte apoptosis likely depend on melatonin's antioxidant properties. These findings underline the potential general interest of the role of melatonin as a controller of the life/death of immune cells within organisms.

Acknowledgements

This work was supported by research grants from Junta de Extremadura (PRI07-A024) and Ministerio de Ciencia e Innovación-Fondo Social Europeo de Desarrollo Regional (BFU2007-60091 and BFU2010-15049). I. Bejarano and S.D. Paredes are beneficiaries of grants from Consejería de Economía, Comercio e Innovación – Fondo Social Europeo (Junta de Extremadura, PRE06070 and REI09009, respectively). J. Espino is beneficiary of grant from Ministerio de Educación (AP2009-0753).

Author contributions

JE carried out the experiments and drafted the manuscript. IB and SDP performed the statistical analysis and helped in drafting the manuscript. CB, ABR, and JAP designed and conceived of the study, interpreted the data, and discussed the results. All authors read and approved the final manuscript.

References

1. Kerr JFR, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 1972; **26**:239–257.
2. Thompson CB. Apoptosis in the pathogenesis and treatment of disease. Science 1995; **267**:1456–1462.
3. Wesche-Soldato DE, Swan RZ, Chung CS et al. The apoptotic pathway as a therapeutic target in sepsis. Curr Drug Targets 2007; **8**:493–500.
4. Bolitho P, Voskoboinik I, Trapani JA et al. Apoptosis induced by the lymphocyte effector molecule perforin. Curr Opin Immunol 2007; **19**:339–347.
5. Ravichandran KS, Lorenz U. Engulfment of apoptotic cells: signals for a good meal. Nat Rev Immunol 2007; **7**:964–974.
6. Schlegel RA, Williamson P. Phosphatidylserine, a death knell. Cell Death Differ 2001; **8**:551–563.
7. Brookes PS, Yoon Y, Robotham JL et al. Calcium, ATP, and ROS: a mitochondrial love-hate triangle. Am J Physiol Cell Physiol 2004; **287**:817–833.
8. Espino J, Bejarano I, Redondo PC et al. Melatonin reduces apoptosis induced by calcium signaling in human leukocytes: evidence for the involvement of mitochondria and Bax activation. J Membr Biol 2010; **233**:105–118.
9. Tan S, Sagara Y, Liu Y et al. The regulation of reactive oxygen species production during programmed cell death. J Cell Biol 1998; **141**:1423–1432.
10. Tan DX, Hardeland R, Manchester LC et al. The changing biological roles of melatonin during evolution: from an antioxidant to signals of darkness, sexual selection and fitness. Biol Rev Camb Philos Soc 2010; **85**:607–623.
11. Paredes SD, Korkmaz A, Manchester LC et al. Phytomelatonin: a review. J Exp Bot 2009; **60**:57–69.
12. Reiter RJ, Tan DX, Manchester LC et al. Melatonin and reproduction revisited. Biol Reprod 2009; **81**:445–456.
13. Rosenstein RE, Pandi-Perumal SR, Srinivasan V et al. Melatonin as a therapeutic tool in ophthalmology: implications for glaucoma and uveitis. J Pineal Res 2010; **49**:1–13.
14. Reiter RJ, Paredes SD, Manchester LC et al. Reducing oxidative/nitrosative stress: a newly-discovered genre for melatonin. Crit Rev Biochem Mol Biol 2009; **44**:175–200.
15. Hardeland R, Tan DX, Reiter RJ et al. Kynuramines metabolites of melatonin and other indoles. The resurrection of an almost forgotten class of biogenic amines. J Pineal Res 2009; **47**:109–126.

16. Juknat AA, Mendez Mdel V, Quaglino A et al. Melatonin prevents hydrogen peroxide-induced Bax expression in cultured rat astrocytes. J Pineal Res 2005; **38**:84–92.
17. Mayo JC, Sainz RM, Uria H et al. Inhibition of cell proliferation: a mechanism likely to mediate the prevention of neuronal cell death by melatonin. J Pineal Res 1998; **25**:12–18.
18. Menendez-Pelaez A, Reiter RJ. Distribution of melatonin in mammalian tissues: the relative importance of nuclear versus cytosolic localization. J Pineal Res 1993; **15**:59–69.
19. Reiter RJ, Paredes SD, Korkmaz A et al. Melatonin combats molecular terrorism at the mitochondrial level. Interdiscip Toxicol 2008; **1**:137–149.
20. Paradies G, Petrosillo G, Paradies V et al. Melatonin, cardiolipin and mitochondrial bioenergetics in health and disease. J Pineal Res 2010; **48**:297–310.
21. Sainz RM, Mayo JC, Rodríguez C et al. Melatonin and cell death: differential actions on apoptosis in normal and cancer cells. Cell Mol Life Sci 2003; **60**:1407–1426.
22. Paredes SD, Terrón MP, Marchena AM et al. Tryptophan modulates cell viability, phagocytosis and oxidative metabolism in old ringdoves. Basic Clin Pharmacol Toxicol 2007; **101**:56–62.
23. Carrillo-Vico A, Reiter RJ, Lardone PJ et al. The modulatory role of melatonin on immune responsiveness. Curr Opin Investig Drugs 2006; **7**:423–431.
24. Tan DX, Manchester LC, Reiter RJ et al. Identification of highly elevated levels of melatonin in bone marrow: its origin and significance. Biochim Biophys Acta 1999; **1472**:206–214.
25. Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 1985; **260**:3440–3450.
26. Rothe G, Oser A, Valet G. Dihydrorhodamine 123: a new flow cytometric indicator for respiratory burst activity in neutrophil granulocytes. Naturwissenschaften 1988; **75**:354–355.
27. Emmendörffer A, Hecht M, Lohmann-Matthes ML et al. A fast and easy method to determine the production of reactive oxygen intermediates by human and murine phagocytes using dihydrorhodamine 123. J Immunol Methods 1990; **131**:269–275.
28. Bejarano I, Redondo PC, Espino J et al. Melatonin induces mitochondrial-mediated apoptosis in human myeloid HL-60 cells. J Pineal Res 2009; **46**:392–400.
29. Espino J, Bejarano I, Paredes SD et al. Melatonin is able to delay endoplasmic reticulum stress-induced apoptosis in leukocytes from elderly humans. Age (Dordr) 2010 (in press). doi: 10.1007/s11357-010-9194-0.
30. Nicholson DW, Ali A, Thornberry NA et al. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature 1995; **376**:37–43.
31. Li P, Nijhawan D, Budihardjo I et al. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 1997; **91**:479–489.
32. Zou H, Li Y, Liu X et al. An APAF-1-cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. J Biol Chem 1999; **274**:11549–11556.
33. Heatwole VM. TUNEL assay for apoptotic cells. Methods Mol Biol 1999; **115**:141–148.
34. Orrenius S, Zhivotovsky B, Nicotera P. Regulation of cell death: the calcium-apoptosis link. Nat Rev Mol Cell Biol 2003; **4**:552–565.
35. Hajnóczky G, Davis E, Madesh M. Calcium and apoptosis. Biochem Biophys Res Commun 2003; **304**:445–454.
36. Hajnóczky G, Csordas G, Das S et al. Mitochondrial calcium signaling and cell death: approaches for assessing the role of mitochondrial Ca^{2+} uptake in apoptosis. Cell Calcium 2006; **40**:553–560.
37. Bejarano I, Lozano GM, Ortiz A et al. Caspase 3 activation in human spermatozoa in response to hydrogen peroxide and progesterone. Fertil Steril 2008; **90**:1340–1347.
38. Pariente JA, Camello C, Camello PJ et al. Release of calcium from mitochondrial and nonmitochondrial intracellular stores in mouse pancreatic acinar cells by hydrogen peroxide. J Membr Biol 2001; **179**:27–35.
39. Rizzuto R, Pinton P, Carrington W et al. Close contacts with endoplasmic reticulum as determinants of mitochondrial Ca^{2+} responses. Science 1998; **280**:1763–1766.
40. Csordás G, Thomas AP, Hajnóczky G. Quasi-synaptic calcium signal transmission between endoplasmic reticulum and mitochondria. EMBO J 1999; **18**:96–108.
41. Hajnóczky G, Csordás G, Madesh M et al. The machinery of local Ca^{2+} signalling between sarco-endoplasmic reticulum and mitochondria. J Physiol 2000; **529**:69–81.
42. Camello-Almaraz C, Salido GM, Pariente JA et al. Role of mithocondria in Ca^{2+} oscillations and shape of Ca^{2+} signals in pancreatic acinar cells. Biochem Pharmacol 2002; **63**:283–292.
43. Filippin L, Magalhães PJ, Di Benedetto G et al. Stable interactions between mitochondria and endoplasmic reticulum allow rapid accumulation of calcium in a subpopulation of mitochondria. J Biol Chem 2003; **278**:39224–39234.
44. Vay L, Hernández-Sanmiguel E, Santo-Domingo J et al. Modulation of Ca^{2+} release and Ca^{2+} oscillations in HeLa cells and fibroblasts by mitochondrial Ca^{2+} uniporter stimulation. J Physiol 2007; **580**:39–49.
45. Paredes SD, Marchena AM, Bejarano I et al. Melatonin and tryptophan affect the activity-rest rhythm, core and peripheral temperatures, and interleukin levels in the ringdove: changes with age. J Gerontol A Biol Sci Med Sci 2009; **64**:340–350.
46. Paredes SD, Bejarano I, Terrón MP et al. Melatonin and tryptophan counteract lipid peroxidation and modulate superoide dismutase activity in ringdove heterophils in vivo. Effect of antigen-induced activation and age. Age (Dordr) 2009; **31**:179–188.
47. Terrón MP, Delgado J, Paredes SD et al. Effect of melatonin and tryptophan on humoral immunity in young and old ringdoves (*Streptopelia risoria*). Exp Gerontol 2009; **44**:653–658.
48. Fang KM, Chang WL, Wang SM et al. Arachidonic acid induces both Na^{+} and Ca^{2+} entry resulting in apoptosis. J Neurochem 2008; **104**:1177–1189.
49. Lin H, Sue YM, Chou Y et al. Activation of a nuclear factor of activated T-lymphocyte-3 (NFAT3) by oxidative stress in carboplatin-induced apoptosis. Br J Pharmacol 2010 (in press). doi: 10.1111/j.1476-5381.2010.00989.x.
50. Tan DX, Reiter RJ, Manchester LC et al. Chemical and physical properties and potential mechanisms: melatonin as a broad spectrum antioxidant and free radical scavenger. Curr Top Med Chem 2002; **2**:181–197.
51. Rodríguez AB, Terrón MP, Durán J et al. Physiological concentrations of melatonin and corticosterone affect phagocytosis and oxidative metabolism of ring dove heterophils. J Pineal Res 2001; **31**:31–38.
52. López A, García JA, Escames G et al. Melatonin protects the mitochondria from oxidative damage reducing oxygen con-

sumption, membrane potential, and superoxide anion production. J Pineal Res 2009; **46**:188–198.
53. Rodríguez C, Mayo JC, Sainz RM et al. Regulation of antioxidant enzymes: a significant role for melatonin. J Pineal Res 2004; **36**:1–9.
54. Radogna F, Paternoster L, De Nicola M et al. Rapid and transient stimulation of intracellular reactive oxygen species by melatonin in normal and tumor leukocytes. Toxicol Appl Pharmacol 2009; **239**:37–45.
55. Petrosillo G, Moro N, Ruggiero FM et al. Melatonin inhibits cardiolipin peroxidation in mitochondria and prevents the mitochondrial permeability transition and cytochrome c release. Free Radic Biol Med 2009; **47**:969–974.
56. Bejarano I, Espino J, Barriga C et al. Pro-oxidant effect of melatonin in tumour leucocytes: relation with its cytotoxic and pro-apoptotic effects. Basic Clin Pharmacol Toxicol 2011; **108**:14–20.