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Melatonin Reduces Apoptosis Induced by Calcium Signaling in Human Leukocytes: Evidence for the Involvement of Mitochondria and Bax Activation

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Abstract We have evaluated the effect of melatonin on apoptosis evoked by increases in $[Ca^{2+}]_c$ in human leukocytes. Our results show that treatment of neutrophils with the calcium mobilizing agonist FMLP or the specific inhibitor of calcium reuptake thapsigargin induced a transient increase in $[Ca^{2+}]_c$. Our results also show that FMLP and thapsigargin increased caspase-9 and -3 activities and the active forms of both caspases. The effect of FMLP and thapsigargin on caspase activation was time-dependent. Similar results were obtained when lymphocytes were stimulated with thapsigargin. This stimulatory effect was accompanied by induction of mPTP, activation of the proapoptotic protein Bax and release of cytochrome *c*. However, when leukocytes were pretreated with melatonin, all of the apoptotic features indicated above were significantly reversed. Our results suggest that melatonin reduces caspase-9 and -3 activities induced by increases in $[Ca^{2+}]_c$ in human leukocytes, which are produced through the inhibition of both mPTP and Bax activation.

Keywords Melatonin · Caspases · Cytochrome *c* · Mitochondria · Apoptosis · Leukocytes

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Introduction

Apoptosis is a gene-regulated form of cell death that is critical for normal development and tissue homeostasis. A major component of the apoptotic machinery involves a family of aspartic acid-directed cysteine proteases, called "caspases" (cysteinyl aspartate-specific proteinases), which cleave multiple protein substrates en masse, leading to the loss of cellular structure and function and ultimately resulting in cell death (Stennicke and Salvesen 1997).

From a functional point of view, caspases involved in apoptosis act either as initiators (caspases 8, 9 and 10) or as effectors (caspases 3, 6 and 7) (Earnshaw et al. 1999). Caspase-8 was identified as the most important initiator enzyme of the Fas/CD95 pathway (Kischkel et al. 1995). Caspase-9 interacts with many other regulators and transducers, such as cytochrome *c*, in intrinsic pathways (Shi 2002). Both initiator caspases are activators of downstream caspases. Caspase-3, the most important among them, executes the final disassembly of the cell by cleaving a variety of cell structure proteins and generating DNA strand breaks (Enari et al. 1998).

Traditionally, two general apoptotic pathways have been described. The first is the extrinsic pathway, triggered by the binding of an extracellular death ligand, such as factor activating ExoS ligand (FasL), to its cell-surface death receptor, such as Fas (Ashkenazi and Dixit 1998). The second is the intrinsic pathway, which is mediated by mitochondrial alterations. In response to apoptotic stimuli, several proteins are released from the mitochondrial intermembrane space into the cytoplasm (Green and Reed 1998). Some of the well-characterized proteins include cytochrome *c*, which mediates the activation of caspase-9 (Li et al. 1997), triggering a cascade of caspase activation, including caspase-3, which promotes cellular self-destruction.

Calcium is a key regulator of cell survival since cytosolic free-calcium concentration ($[Ca^{2+}]_c$) is a major regulatory factor for a large number of cellular processes such as muscle contraction, metabolism, secretion, cell differentiation and apoptosis. However, the sustained elevation of intracellular calcium plays a role in cell death (Demaurex and Distelhorst 2003). 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 (Hajnoczky et al. 2003). Excessive calcium load to the mitochondria may induce apoptosis by both stimulating the release of apoptosis-promoting factors from the mitochondrial intermembrane space to the cytoplasm and impairing mitochondrial function (Wang 2001).

Calcium-dependent increase in mitochondrial permeability to ions and solutes with molecular masses up to 1,500 Da, matrix swelling and uncoupling of oxidative phosphorylation, which has been defined as permeability transition (Hunter and Haworth 1979), is currently ascribed to the opening of the mitochondrial permeability transition pore (mPTP) (Zoratti and Szabo 1995). The mPTP is a megachannel formed in the mitochondrial membranes which is found at the contact sites between inner and outer mitochondrial membranes. Conditions like oxidative stress, calcium overload and low ATP levels are sufficient to induce mPTP even without Bcl-2 family proteins, although under some circumstances the Bcl-2 family proteins can interact with mitochondrial pore-forming proteins (Crompton 1999; Kroemer and Reed 2000).

In the last few years, several mechanisms have been proposed for the release of cytochrome *c* into the cytosol. In many models of apoptosis, Bax-mediated permeabilization of the mitochondrial outer membrane plays a crucial role. Some studies suggest that, although mPTP is likely to be a mechanism responsible for cytochrome *c* release, it is no longer regarded as the mechanism (Marzo et al. 1998). In particular, in the absence of mPTP induction, oligomeric Bax inserts and forms a channel in the mitochondrial outer membrane large enough to allow the release of cytochrome *c* (Antonsson et al. 2000; Gogvadze et al. 2001).

The pineal gland hormone melatonin regulates seasonal and circadian rhythms of mammals and functions as a powerful free radical scavenger (Tan et al. 1993), but emerging evidence suggests that it may be involved in other important processes such as the protection of human leukocytes and other cell types against damage-induced apoptosis (Luchetti et al. 2006; Radogna et al. 2008). Recent convincing evidence suggests that the so-called intrinsic pathway might represent the main target of melatonin to antagonize apoptosis in human leukocytes (Radogna et al. 2008) and in other tumor cell lines and in vivo models (Feng and Zhang 2004; Acuña-Castroviejo et al. 2007). In fact, increasing evidence for a melatonin–mitochondria relationship includes the antiapoptotic properties of indoleamine through its interaction with mPTP (Andrabi et al. 2004; Jou et al. 2004).

Here, we focused on the role of melatonin in caspase activation induced by the specific inhibitor of calcium reuptake thapsigargin or the calcium-mobilizing agonist *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) in human leukocytes, analyzing the induction of mPTP, release of mitochondrial cytochrome *c*, caspase-9 and -3 activation and activation of the proapoptotic protein Bax.

Materials and Methods

Chemicals

Melatonin, RPMI-1640, Ficoll-Histopaque separating medium, FMLP, dithiothreitol, *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (AC-DEVD-AMC), 3-[(3-chomalidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), cyclosporin A (CsA), anti-cytochrome *c* monoclonal antibody and ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetra-acetic acid (EGTA) were from Sigma (Madrid, Spain). Fura-2 acetoxymethyl ester (fura-2/AM), calcein acetoxymethyl ester (calcein-AM), Tris–glycine gel and thapsigargin were from Invitrogen (Barcelona, Spain). *N*-Acetyl-Leu-Glu-His-Asp-7-amino-4-methylcoumarin (AC-LEHD-AMC) was from Bachem (Weil am Rhein, Germany). Anti-caspase-9 monoclonal antibody (C9) and anti-caspase-3 monoclonal antibody (8G10) were from Cell Signalling (Beverly, MA). All others reagents were of analytical grade.

Human Leukocyte Isolation

Venous blood was drawn from healthy volunteers of both genders and aged 18–75 years 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 $600\times g$ for 30 min, peripheral blood mononuclear cells were isolated from the Histopaque-1077/1119 upper interphase and maintained in RPMI-1640 medium for 1 h to allow adherence of monocytes so as to obtain a pure lymphocyte preparation, as previously described (Otton et al. 2007). Similarly, peripheral blood polymorphonuclear cells were harvested from the lower interphase, and residual erythrocytes were then lysed by short treatment of neutrophil pellet with an ice-cold isotonic NH_4Cl solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM ethylenediaminetetraacetic acid [EDTA, pH 7.4]) so as to obtain a neutrophil-enriched preparation, as described elsewhere

(Genestier et al. 2005). Cell purity was routinely above 98% and 97% in lymphocytes and neutrophils, respectively, after Diff-Quick staining. Once purified, both neutrophils and lymphocytes were collected and washed in PBS at 480×*g* for 15 min. The supernatant was then discarded and the cell pellet gently resuspended in Na-HEPES solution containing (in mM) NaCl, 140; KCl, 4.7; $CaCl_2$, 1.2; $MgCl_2$, 1.1; glucose, 10; and HEPES, 10 (pH 7.4).

Measurement of Intracellular $[Ca^{2+}]_c$

Leukocytes were loaded with fura-2 by incubation with 4 μM fura 2-AM for 30 min at room temperature according to a procedure published elsewhere (Bejarano et al. 2007). Once loaded, the cells were washed and used within the next 2–4 h. Fluorescence was recorded from 2-ml aliquots of a magnetically stirred cellular suspension (2×10^6 cells/ml) at 37°C 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 using the fura 2-AM 340/380 nm fluorescence ratio and calibrated according to the method of Grynkiewicz, Poenie and Tsien (1985). In the experiments where calcium-free medium is indicated, calcium was omitted and 1 mM EGTA was added.

Cell Viability

Cell viability was assessed using calcein, an acetoxymethyl-ester dye that accumulates in cells with leakage through the plasma membrane being related to loss of cell viability. Cells were incubated for 30 min with 5 μM calcein-AM at 37°C and centrifuged, and the pellet was resuspended in fresh Na-HEPES medium. Fluorescence was recorded from 2-ml aliquots using a spectrofluorophotometer (RF-5301-PC). Samples were excited at 494 nm, and the resulting fluorescence was measured at 535 nm.

Assessment of mPTP Opening with Calcein

We used a well-established method for detecting transient mPTP opening in the intact cell (Petronilli et al. 1999; Bejarano et al. 2009). Cells were incubated with calcein-AM (1 μM) and cobalt chloride ($CoCl_2$, 1 mM) for 15 min at 37°C, resulting in mitochondrial localization of calcein fluorescence due to the quenching of cytosolic and nuclear calcein. mPTP opening was indicated by a reduction in mitochondrial calcein signal (expressed as a percentage of the baseline value) and measured with a fluorescence spectrophotometer (RF-5301-PC) with excitation wavelength of 490 nm and emission at 510 nm. Data are expressed as fractional changes of emitted fluorescence (F/F_0). Alternatively, the decay in mitochondrial calcein fluorescence was calculated and expressed as a fold increase.

Caspase Activity Assay

To determine caspase activity, stimulated or resting leukocytes were sonicated and cell lysates were incubated with 2 ml of substrate solution (20 mM HEPES, 2 mM EDTA, 0.1% CHAPS, 5 mM dithiothreitol and 8.25 mM caspase substrate [pH 7.4]) for 1 h at 37°C, as described elsewhere (Rosado et al. 2006). Substrate cleavage was measured using a fluorescence spectrophotometer (RF-5301-PC) with excitation wavelength of 360 nm and emission at 460 nm. The activities of caspase-3 and -9 were calculated from the cleavage of the respective specific fluorogenic substrate (AC-DEVD-AMC for caspase-3 and AC-LEHD-AMC for caspase-9). Preliminary experiments reported that caspase-3 or -9 substrate cleaving was not detected in the presence of the inhibitors of caspase-3 or -9, DEVD-CMK or z-LEHD-FMK, respectively. The data were calculated as fluorescence units per milligram of protein and were presented as a fold increase over pretreatment level (untreated samples).

Subcellular Fractionation

Subcellular fractions were prepared of 5×10^6 cells by washing the cells once in ice-cold PBS before resuspension in cytosol extraction buffer (250 mM sucrose, 70 mM KCl, 250 μg/ml digitonin and complete protease inhibitor cocktail mix [PIM] in PBS) at a concentration of 100×10^6 and 40×10^6 cells/ml for neutrophils and lymphocytes, respectively. Cells were incubated for 10 min on ice, after which they were centrifuged at 1,000×*g* for 5 min at 4°C. The supernatant represented the cytosolic fraction, and the pellet, containing the mitochondria, was dissolved in mitochondrial lysis buffer (100 mM NaCl, 10 mM $MgCl_2$, 2 mM EGTA, 2 mM EDTA, 1% Triton X-100, 10% glycerol, PIM in 50 mM Tris–HCl buffer [pH 7.5]) and incubated for another 10 min on ice. After incubation, samples were centrifuged at 10,000×*g* for 10 min at 4°C. The supernatant represented the mitochondrial fraction. The cytosolic and mitochondrial fractions were collected separately and used for Western blotting.

Immunoprecipitation and Western Blotting

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 h at 0.8 mA/cm^2, in a semidry blotter onto nitrocellulose for subsequent probing. Blots were

incubated overnight with 10% (w/v) bovine serum albumin (BSA) in Tris-buffered saline with 0.1% Tween-20 (TBST) to block residual protein binding sites. Blocked membranes were then incubated with the anti-cytochrome *c* antibody diluted 1:100 in TBST for 2 h or the anti-caspase-3 antibody (8G10) and the anti-caspase-9 antibody (C9), diluted 1:1,000 in TBST for 2 and 3 h, respectively. The primary antibody was removed, and blots were washed six times for 5 min each with TBST. To detect the primary antibodies, blots were incubated with the appropriate horseradish peroxidase-conjugated anti-IgG antibody diluted 1:5,000 in TBST, washed six 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.

Bax activation was determined by immunoprecipitation as previously described (Bejarano et al. 2009). Briefly, cell suspensions were stimulated, as indicated, and lysed. Bax protein was immunoprecipitated from cell lysates by incubation with 2 μg of anti-Bax antibody (clone 6A7), which reacts only with Bax in its conformationally active state, and 15 μl of protein A-agarose overnight at 4°C on a rocking platform. Proteins were separated by 4–12% density gradient Tris–glycine and electrophoretically transferred, for 2 h at 0.8 mA/cm^2, in a semidry blotter (Hoefer Scientific, Newcastle, UK) onto nitrocellulose for subsequent Western blotting. Nonspecific protein binding sites of the nitrocellulose membranes were blocked by incubating overnight with 10% (w/v) BSA in TBST. Membranes were incubated with the anti-Bax antibody diluted 1:200 in TBST for 2 h. To detect the primary antibody, blots were incubated with the appropriate horseradish peroxidase-conjugated anti-IgG antibody diluted 1:5,000 in TBST, washed six 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.

Statistical Analysis

Data are expressed as means ± SEM of the numbers of determinations. Analysis of statistical significance was performed using Student's *t*-test. To compare the different treatments, statistical significance was calculated by one-way analysis of variance followed by Tukey's multiple comparison test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

To evaluate the effect of melatonin on cell viability, leukocytes were treated with both the calcium-mobilizing agonist FMLP and the specific inhibitor of calcium reuptake thapsigargin in the absence and presence of melatonin. As shown in Table 1, basal cell viability was higher than 93%, as assayed by calcein.Treatment of leukocytes with 10 nM FMLP or 1 μM thapsigargin for 60–120 min caused a significant reduction of cell viability in both neutrophils and lymphocytes. However, melatonin pretreatment (1 mM for 60 min) was able to reverse the cell viability reduction induced by FMLP and thapsigargin. Additionally, treatment of human leukocytes with melatonin alone had no significant effect on cell viability.

It has been reported that a prolonged elevation in $[Ca^{2+}]_c$ and alterations in calcium homeostasis initiate the mitochondrial apoptotic pathway (Demaurex and Distelhorst 2003) and induce endoplasmic reticulum stress, which in turn leads to apoptosis (Rao et al. 2004). In the absence of extracellular calcium (calcium-free medium), fura-2-loaded human neutrophils were treated with both FMLP and thapsigargin. As shown in Fig. 1a, stimulation with 10 nM FMLP induced a typical transient increase in $[Ca^{2+}]_c$ due to calcium release from internal stores in human neutrophils. Similarly, stimulation of human neutrophils with 1 μM thapsigargin caused a transient increase in $[Ca^{2+}]_c$, which reached a stable $[Ca^{2+}]_c$ plateau after 15–20 min of stimulation (Fig. 1b), reflecting the release of calcium from non-mitochondrial agonist-releasable pools. In addition, when 1 μM thapsigargin were administrated to fura-2-loaded human lymphocytes, it generated a slow and sustained $[Ca^{2+}]_c$ increase, which reached a stable $[Ca^{2+}]_c$ plateau after 5 min of stimulation (Fig. 1c), again due to calcium release from intracellular stores. These increases induced by FMLP and thapsigargin were also observed in the presence of normal extracellular calcium (Fig. 1d–f), though the levels of calcium remained raised in comparison to those obtained in the absence of extracellular calcium. Furthermore, melatonin by itself had no effect on the calcium signal since when both neutrophils and lymphocytes were pretreated with 1 mM melatonin, indoleamine proved to be ineffective at modifying the thapsigargin- or FMLP-induced calcium signal (data not shown).

To further investigate the role of melatonin in mitochondrial apoptosis inhibition, we probed mPTP opening in human leukocytes loaded with calcein-AM in the presence of cobalt chloride to quench fluorescence from all cellular domains except from within mitochondria (Petronilli et al. 1999; Bejarano et al. 2009). Using this protocol, the addition of 10 nM FMLP to calcein-loaded neutrophils induced an abrupt decrease in mitochondrial calcein fluorescence (Fig. 2a). A similar result, although to a smaller extent, was obtained by incubating neutrophils with 1 μM thapsigargin (Fig. 2b). In addition, stimulation of calcein-loaded lymphocytes with 1 μM thapsigargin again provoked a marked decay in mitochondrial calcein

Table 1 Percentage cell viability in untreated cells assayed at different times (0–120 min) and in treated cells assessed in basal conditions (0 min) and after treatment with 1 μM thapsigargin (TG) or 10 nM FMLP for 60–120 min in the absence (−MEL) or presence (+MEL) of melatonin (1 mM for 60 min) in both human neutrophils and lymphocytes

	Neutrophils						Lymphocytes			
	Untreated		TG-treated		FMLP-treated		Untreated		TG-treated	
	−MEL	+MEL	−MEL	+MEL	−MEL	+MEL	−MEL	+MEL	−MEL	+MEL
0 min	96.3 ± 0.8	95.9 ± 0.9	95.8 ± 1.5	96.1 ± 0.7	96.2 ± 2.19	5.1 ± 1.3	94.6 ± 1.1	93.5 ± 0.7	93.8 ± 1.7	94.1 ± 0.7
10 min	95.4 ± 0.5	–	–	–	–	–	92.9 ± 0.6	–	–	–
30 min	95.6 ± 0.3	–	–	–	–	–	91.4 ± 0.5	–	–	–
60 min	92.3 ± 0.9	92.7 ± 0.3	81.8 ± 1.7*-***	90.5 ± 1.0*-**	86.4 ± 1.1*	93.0 ± 1.1**	88.4 ± 2.1*	91.3 ± 1.2*	78.4 ± 1.2*-***	86.2 ± 0.7*-**
120 min	87.5 ± 2.1*	89.6 ± 1.1*	71.8 ± 1.8*-***	86.5 ± 0.9*-**	76.6 ± 1.2*-***	89.4 ± 1.9*-**	85.6 ± 1.8*	87.9 ± 0.8*	70.7 ± 0.6*-***	89.7 ± 1.3*-**

Each value represents the mean ± SEM of five separate experiments

* $P < 0.01$ with respect to the values obtained in basal conditions (0 min). ** $P < 0.01$ with respect to the values obtained in the absence of melatonin (−MEL). *** $P < 0.01$ with respect to the values obtained in untreated cells

fluorescence (Fig. 2c). Although this protocol does not distinguish between calcein efflux and cobalt influx, it is consistent with induction of the permeability transition pore. Interestingly, when neutrophils and lymphocytes were preincubated with 1 mM melatonin for 60 min, the decay in fluorescence signal of mitochondrial calcein was significantly reduced from that recorded in cells treated with either FMLP (0.47 ± 0.04-fold increase in neutrophils; $P < 0.05$, Fig. 2a) or thapsigargin (0.26 ± 0.11- and 0.33 ± 0.08-fold increase in neutrophils and lymphocytes, respectively; $P < 0.05$, Fig. 2b, c), suggesting that melatonin inhibits mitochondrial apoptosis by blockade of mPTP opening. Similarly, the effects of FMLP and thapsigargin on mPTP opening were blocked by pretreatment of cells with the permeability transition pore inhibitor CsA (50 μM) for 30 min (Fig. 2).

This stimulatory effect of calcium signaling on mPTP opening was confirmed by determination of cytochrome *c* release from the mitochondria. We found that treatment of leukocytes with 1 μM thapsigargin for 120 min induced cytochrome *c* release, as revealed by the increase in levels of cytochrome *c* in the cytosolic fraction (2.17 ± 0.13- and 2.68 ± 0.31-fold increase in neutrophils and lymphocytes, respectively; $P < 0.05$, Fig. 3) and the decrease in the mitochondrial fraction (0.45 ± 0.09- and 0.47 ± 0.13-fold increase in neutrophils and lymphocytes, respectively; $P < 0.05$, Fig. 3). However, pretreatment of both neutrophils and lymphocytes for 60 min with 1 mM melatonin reversed the release of cytochrome *c* evoked by extensive depletion of the intracellular calcium pools using thapsigargin.

To examine the effect of melatonin on caspase-3 activation, 10 nM FMLP or 1 μM thapsigargin was again administered to human leukocytes for 1–120 min. Our results show that both FMLP (Fig. 4a) and thapsigargin (Fig. 4c) were able to increase caspase-3 activity in human neutrophils. The effect of stimulation with FMLP and thapsigargin on caspase-3 activity was time-dependent, reaching a maximal value after 120 min of stimulation (6.2 ± 0.6-fold increase and 7.9 ± 0.8-fold increase in FMLP- and thapsigargin-treated neutrophils, respectively; $P < 0.05$). Nevertheless, as shown in Fig. 4a, c, when neutrophils were preincubated with 1 mM melatonin for 60 min, FMLP- or thapsigargin-induced caspase-3 activity was significantly reduced at all times tested by us. Similar results were obtained when human lymphocytes were treated with thapsigargin (Fig. 4e). Extensive depletion of intracellular calcium stores, using 1 μM thapsigargin, induced a time-dependent caspase-3 activation similar to that observed in neutrophils, reaching an early activation within 5 min, with a 1.8 ± 0.3-fold increase, and then a late activation that was maintained for at least 120 min (4.5 ± 0.4-fold increase, $P < 0.05$, Fig. 4e). Once more,

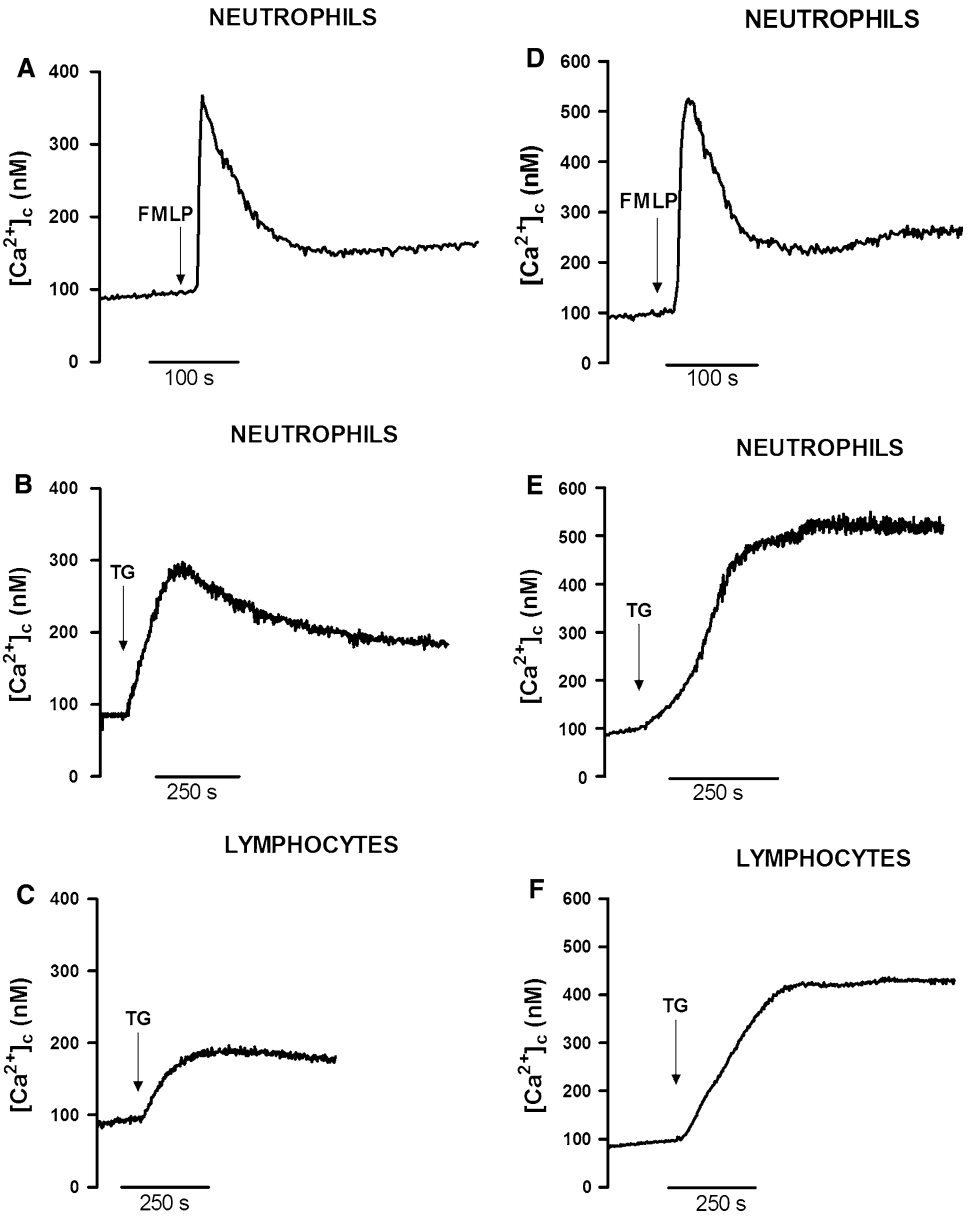


Fig. 1 Mobilization of calcium in human leukocytes. Fura-2-loaded human leukocytes were stimulated with 10 nM FMLP (**a**, **d**) or 1 μM thapsigargin (TG) (**b, c, e** and **f**), as indicated, in a calcium-free medium ($[Ca^{2+}]_o = 0 + 1$ mM EGTA was added) (**a–c**) or in the presence of normal extracellular calcium ($[Ca^{2+}]_o = 1.2$ mM) (**d–f**). Traces are representative of eight separate experiments

the presence of melatonin (1 mM for 60 min) was also able to reduce significantly the activation of caspase-3 evoked by administration of thapsigargin (Fig. 4e). It is worth noting that the spontaneous caspase-3 activation after 120 min showed no significant changes compared to caspase activity at the beginning of incubation (time = 0). Moreover, melatonin alone was unable to significantly modify spontaneous caspase-3 activation at either 60 or 120 min of incubation.

The results obtained by determination of caspase-3 activity were confirmed by Western blotting. Caspase activation was analyzed using a specific monoclonal anti-caspase-3 antibody, which detects the full-length inactive (procaspase) form (35 kDa) and the active large caspase-3 fragment (17 kDa) resulting from cleavage at Asp^{175} (Nicholson et al. 1995). Treatment of neutrophils with 10 nM FMLP (Fig. 4b) or 1 μM thapsigargin (Fig. 4d) for 120 min in medium containing 1.2 mM calcium resulted in significant activation of caspase-3 as revealed by the increase in the amount of the active form (2.0 ± 0.18- and 1.92 ± 0.05-fold increase in FMLP- and thapsigargin-treated neutrophils, respectively; $P < 0.05$) and the decrease in the inactive procaspase form. Similar results were obtained when lymphocytes were stimulated with 1 μM thapsigargin (Fig. 4f). However, pretreatment of leukocytes with 1 mM melatonin for 60 min significantly reduced FMLP- and thapsigargin-evoked activation of caspase-3, as can be estimated by the content of the active form of caspase-3 (Fig. 4b, d, f). Additionally, treatment with 1 mM melatonin alone did not affect the caspase-3 content.

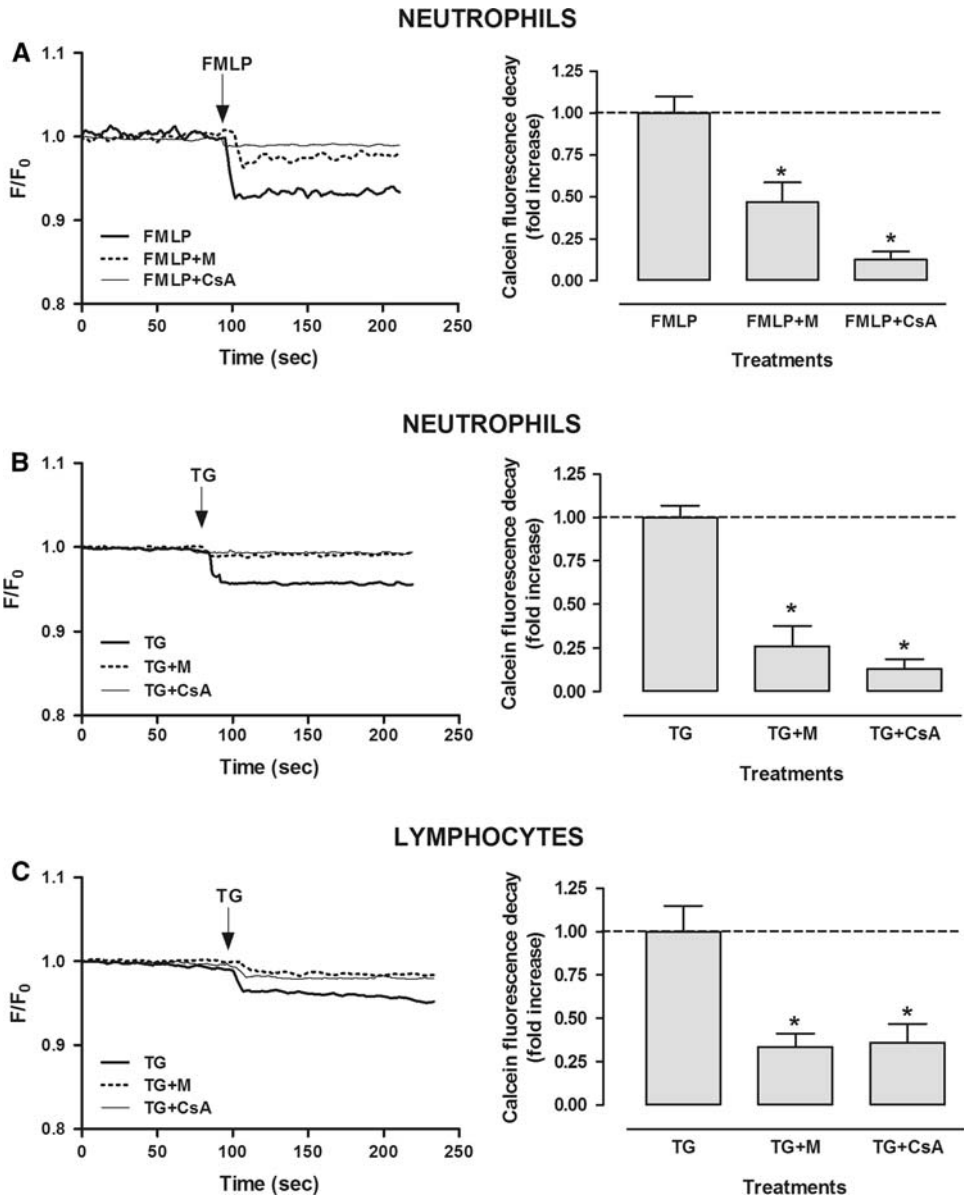


Fig. 2 Effect of melatonin on mPTP opening in human leukocytes. Calcein-loaded cells in the presence of 1 mM $CoCl_2$ were preincubated with 1 mM melatonin (M) for 60 min or 50 μM CsA for 30 min or the vehicle, then stimulated with 10 nM FMLP (**a**) or 1 μM thapsigargin (TG) (**b**, **c**). Changes in calcein fluorescence were determined as described under "Materials and Methods" and are expressed as fractional changes of emitted fluorescence (F/F_0). Traces are representative of five separate experiments. Histograms show the decay in mitochondrial calcein fluorescence and are expressed as fold increase. Data are presented as mean ± SEM of five independent experiments. *$P < 0.05$ compared to FMLP or TG alone

On the other hand, caspase-9 is an initiator caspase that is involved in the initial steps of mitochondrial apoptosis (Li et al. 1997). To investigate whether the activation of caspase-3 by FMLP or thapsigargin is a mitochondrial apoptosis, we checked caspase-9 activity in the presence of FMLP or thapsigargin. As shown in Fig. 5a, treatment of neutrophils with 10 nM FMLP induced a significant increase of caspase-9 activity.The effect of stimulation with FMLP on caspase-9 activity was again time-dependent, reaching a maximum after 120 min of stimulation with a 6.1 ± 0.6-fold increase ($P < 0.05$) (Fig. 5a). In addition, 1 μM thapsigargin was able to induce activation of caspase-9 in human neutrophils, reaching a maximum after 120 min of stimulation (5.2 ± 0.8-fold increase, $P < 0.05$, Fig. 5c). However, as shown in Fig. 5a, c, when neutrophils were preincubated with 1 mM melatonin for 60 min, FMLP- or thapsigargin-induced caspase-9 activity was significantly reduced at all times assayed. Similar results were obtained when human lymphocytes were stimulated with 1 μM thapsigargin in the absence or presence of melatonin (1 mM for 60 min) (Fig. 5e). The spontaneous caspase-9 activation after 120 min showed no significant changes compared to caspase activity at the beginning of incubation (time = 0). Again, melatonin alone proved to be ineffective at significantly modifying the spontaneous caspase-9 activation at either 60 or 120 min of incubation.

Similarly to caspase-3, caspase-9 activation was also analyzed by Western blotting using a monoclonal anti-caspase-9 antibody that recognizes the full-length inactive

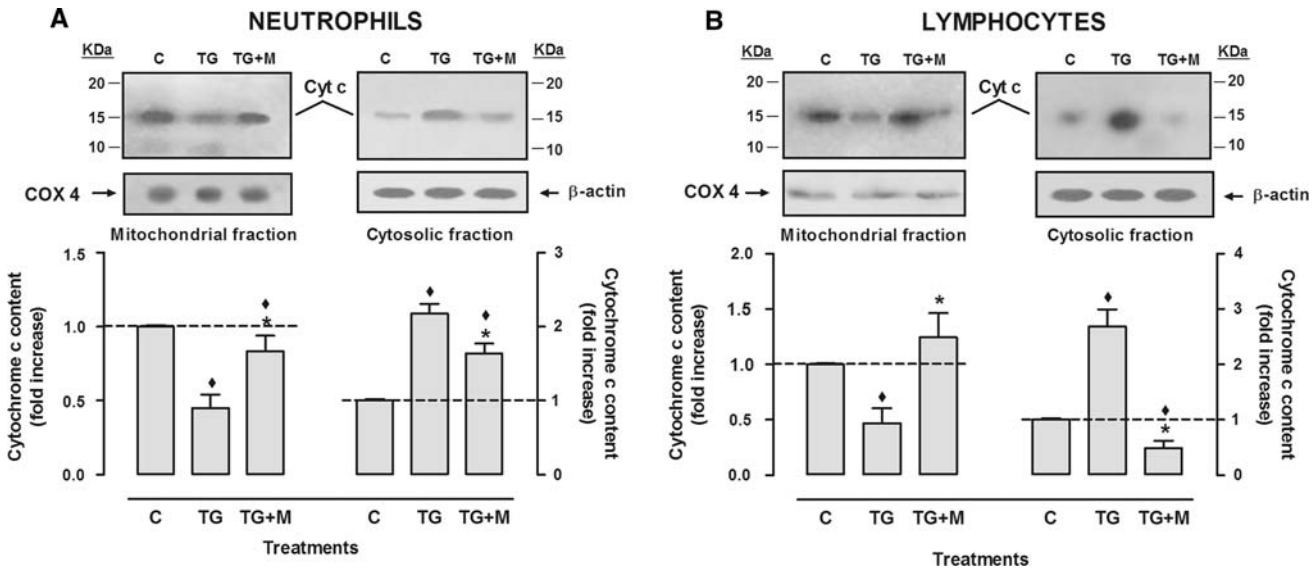
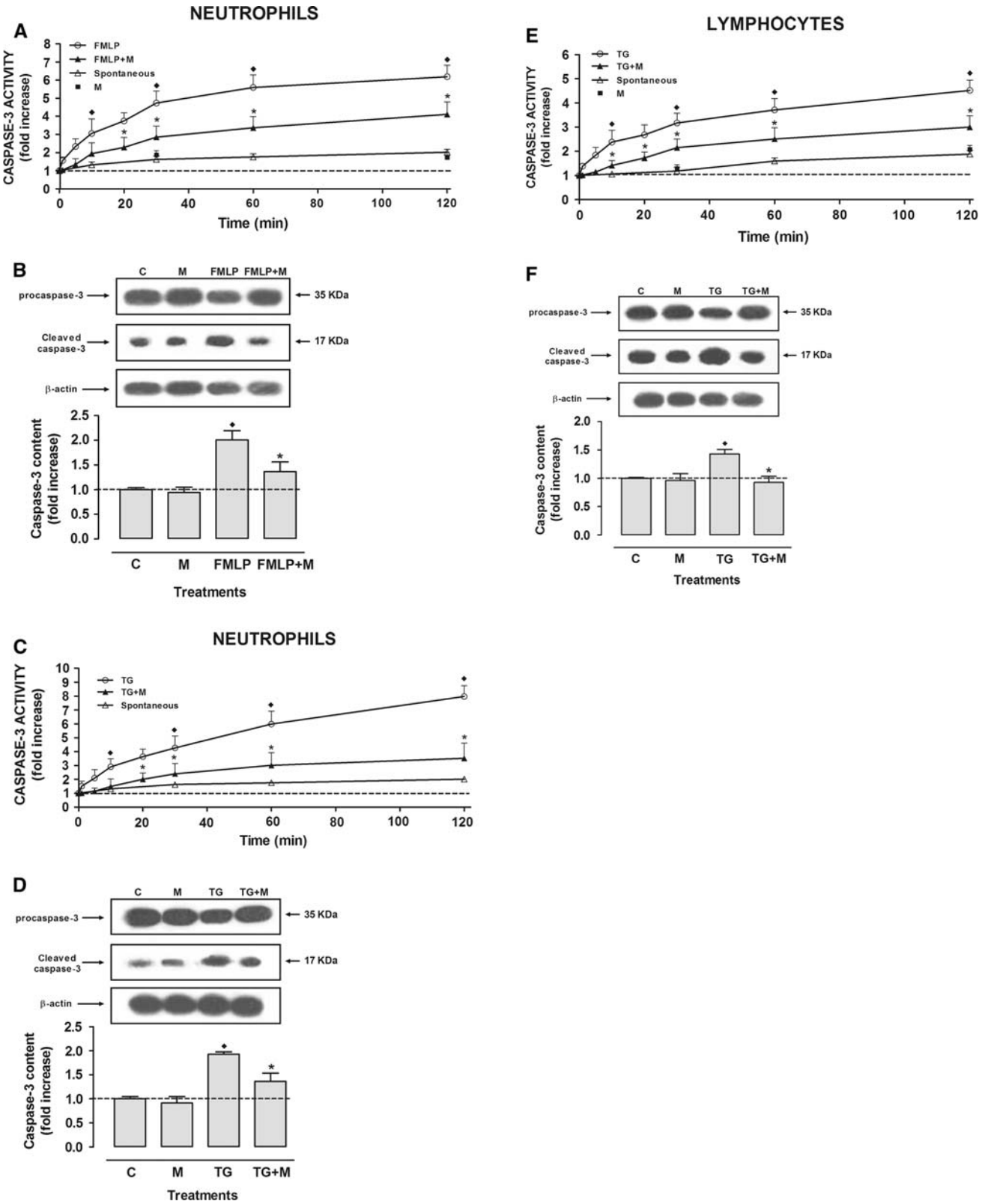


Fig. 3 Melatonin protects against thapsigargin-induced cytochrome *c* release. Human neutrophils (**a**) and lymphocytes (**b**) were preincubated with 1 mM melatonin (M) or vehicle for 60 min, then stimulated with 1 μM thapsigargin (TG) for 120 min. The cytochrome *c* content in the cytosolic and mitochondrial fractions was determined by Western blotting using anti-cytochrome *c* antibody as described in "Materials and Methods" and reprobed with both anti-Cox 4 and anti-*β*-actin antibody for checking the appropriate subcellular fractionation and protein loading control, respectively. The panels show a blot representative of five others. Histograms represent the change in cytochrome *c* content and are presented as fold increase over the pretreatment level (experimental/control). Data are presented as mean ± SEM of four independent experiments. ◆$P < 0.05$ compared to control (C) values, *$P < 0.05$ compared to TG alone

(procaspase) form (47 kDa) and the active large caspase-9 fragment (35 kDa) (Zou et al. 1999). Western blot analysis revealed that treatment of leukocytes with 10 nM FMLP (Fig. 5b) or 1 μM thapsigargin (Fig. 5d, f) significantly increased the amount of the active form of caspase-9 again (1.46 ± 0.13- and 1.35 ± 0.11-fold increase in FMLP- and thapsigargin-treated neutrophils, respectively, and 1.73 ± 0.21-fold increase in thapsigargin-treated lymphocytes; $P < 0.05$), and pretreatment of leukocytes with 1 mM melatonin for 60 min was able to revert the stimulatory effect of FMLP and thapsigargin, as revealed by the decrease in the amount of the active form and the increase in the inactive procaspase form of caspase-9 (Fig. 5b, d, f). Taken together, these findings strongly suggest that depletion of intracellular calcium pools by both thapsigargin and the physiological agonist FMLP induces activation of caspase-3 and -9 in a time-dependent manner and that melatonin is able to reduce such caspase activation evoked by calcium signals.

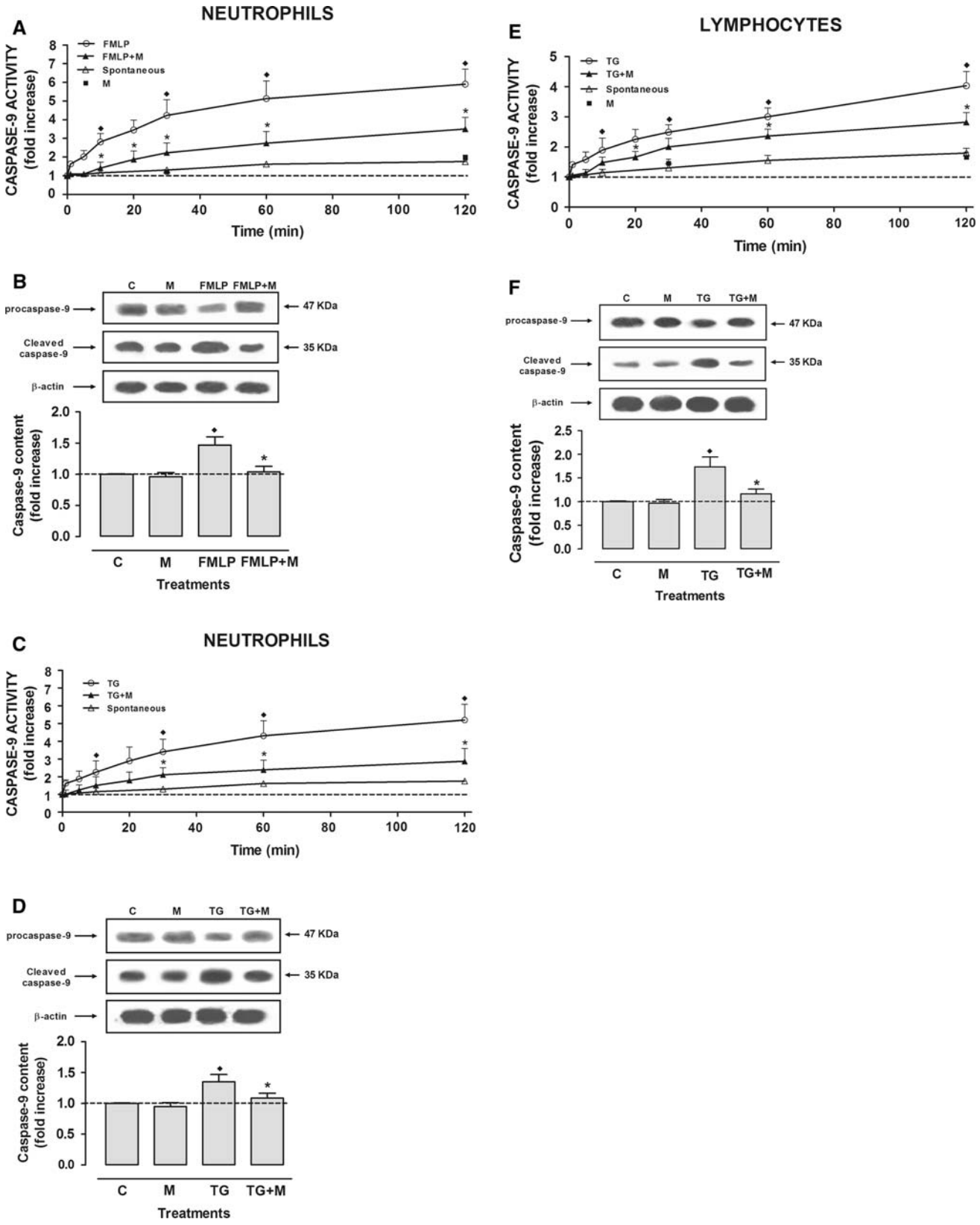
Finally, in order to identify possible intracellular apoptotic targets of calcium signaling during the promotion of apoptosis in human leukocytes, we investigated the activation of Bax after treatment with thapsigargin. Active Bax was detected by immunoprecipitation with the anti-Bax antibody (clone 6A7), which reacts only with Bax in its conformationally active state, followed by Western blotting with the same antibody as previously described (Phillips et al. 2007). Thapsigargin (1 μM for 120 min) caused a significant activation of Bax both in neutrophils (1.28 ± 0.1-fold increase, $P < 0.05$, Fig. 6a) and in lymphocytes (1.23 ± 0.09-fold increase, $P < 0.05$, Fig. 6b).In addition, the effect of thapsigargin on Bax activation was significantly attenuated when the leukocytes were preincubated with 1 mM melatonin for 60 min (Fig. 6). Melatonin alone was unable to modify the Bax activation in neutrophils or in lymphocytes.

Fig. 4 Effect of melatonin on caspase-3 activation in human leukocytes. Cells were preincubated with 1 mM melatonin (M) or vehicle for 60 min, then stimulated with 10 nM FMLP (**a**) or 1 μM thapsigargin (**c**, **e**) for various periods of time to check caspase-3 activity. For comparison, the effect of treatment with 1 mM melatonin (■) for 60 and 120 min is shown in **b** and **e**. Caspase-3 activity was estimated as described in "Materials and Methods." Values are presented as means ± SEM of six separate experiments and expressed as fold increase over the pretreatment level. To determine the caspase-3 content, cells were preincubated as described above, then stimulated with 10 nM FMLP (**b**) or 1 μM thapsigargin (**d**, **f**) for 120 min. Samples were lysed, subjected to gradient Tris–glycine isolation and subsequent Western blotting with a specific anti-caspase 3 (8G10) antibody and reprobed with anti-*β*-actin antibody for protein loading control. Histograms represent the quantification of the 17-kDa fragment of cleaved caspase-3 expressed as fold increase over the pretreatment level (experimental/control). Results are presented as mean ± SEM of four to six independent experiments. ◆$P < 0.05$ compared to control (C) values, *$P < 0.05$ compared to FMLP or thapsigargin alone



Discussion

Cytosolic calcium has been presented as a key regulator of cell survival, but this ion may also induce apoptosis in response to a number of pathological conditions (Hajnoczky et al. 2003; Orrenius et al. 2003). In addition, the mitochondria act as calcium buffers by sequestering excess calcium from the cytosol. Calcium overloading in



mitochondria may induce an apoptotic program by stimulating the release of apoptosis-promoting factors like cytochrome *c* and by generating reactive oxygen species due to respiratory chain damage (Brookes et al. 2004; Hajnoczky et al. 2006). Furthermore, mitochondria have been found to play an important role in calcium signaling

◀**Fig. 5** Effect of melatonin on caspase-9 activation in human leukocytes. Cells were preincubated with 1 mM melatonin (M) or vehicle for 60 min, then stimulated with 10 nM FMLP (**a**) or 1 μM thapsigargin (**c**, **e**) for various periods of time to check caspase-9 activity. For comparison, the effect of treatment with 1 mM melatonin (■) for 60 and 120 min is shown in **b** and **e**. Caspase-9 activity was estimated as described in "Materials and Methods." Values are presented as means ± SEM of six separate experiments and expressed as fold increase over the pretreatment level. To determine the caspase-9 content, cells were preincubated as described above, then stimulated with 10 nM FMLP (**b**) or 1 μM thapsigargin (**d**, **f**) for 120 min. Samples were lysed, subjected to gradient Tris–glycine isolation and subsequent Western blotting with a specific anti-caspase 9 (C9) antibody and reprobed with anti-β-actin antibody for protein loading control. Histograms represent the quantification of the 35-kDa fragment of cleaved caspase-9 expressed as fold increase over the pretreatment level (experimental/control). Results are presented as mean ± SEM of four to six independent experiments. $^{\blacklozenge}P < 0.05$ compared to control (C) values, $^{*}P < 0.05$ compared to FMLP or thapsigargin alone

(Hajnoczky et al. 2003). Here, we report the effect of the calcium-mobilizing agonist FMLP on caspase activation in human leukocytes, which evoked a time-dependent increase in caspase-3 (a key downstream effector of apoptosis) and initiator caspase-9 activities detected by two different means: Western blotting, which upon cell stimulation with FMLP shows an increase in the active form of both caspases, and a fluorimetric assay for caspase activity, which confirmed the results observed by Western blotting.

To further investigate the role of calcium on caspase activation, we used the specific inhibitor of calcium reuptake thapsigargin, which fully depletes the intracellular calcium stores after a few minutes. Incubation of human leukocytes with thapsigargin also induced time-dependent caspase activation. Similarly, we have previously shown that depletion of non-mitochondrial calcium stores in human platelets and spermatozoa is accompanied by caspase activation (Rosado et al. 2006; Bejarano et al. 2008). The caspase activation caused by depletion of intracellular calcium pools is probably due to induction of the mPTP. Evidence for the permeability transition pore opening was obtained with the fluorescence probe calcein (Petronilli et al. 1999). Depletion of intracellular calcium stores by FMLP or thapsigargin caused loss of calcein fluorescence, which indicated that a large enough pore had been induced to allow either calcein efflux or cobalt influx. These results are consistent with activation of the proapoptotic protein Bax and subsequently an increase in the amount of the cytochrome *c* released from the mitochondria, which was previously obtained in pancreatic acinar cells (Gerasimenko et al. 2002). In this regard, activation and mitochondrial translocation of Bax induced by thrombin, a calcium-mobilizing agonist, have been previously reported in human platelets (López et al. 2008). Likewise, postischemic dopamine treatment of contractile dysfunction

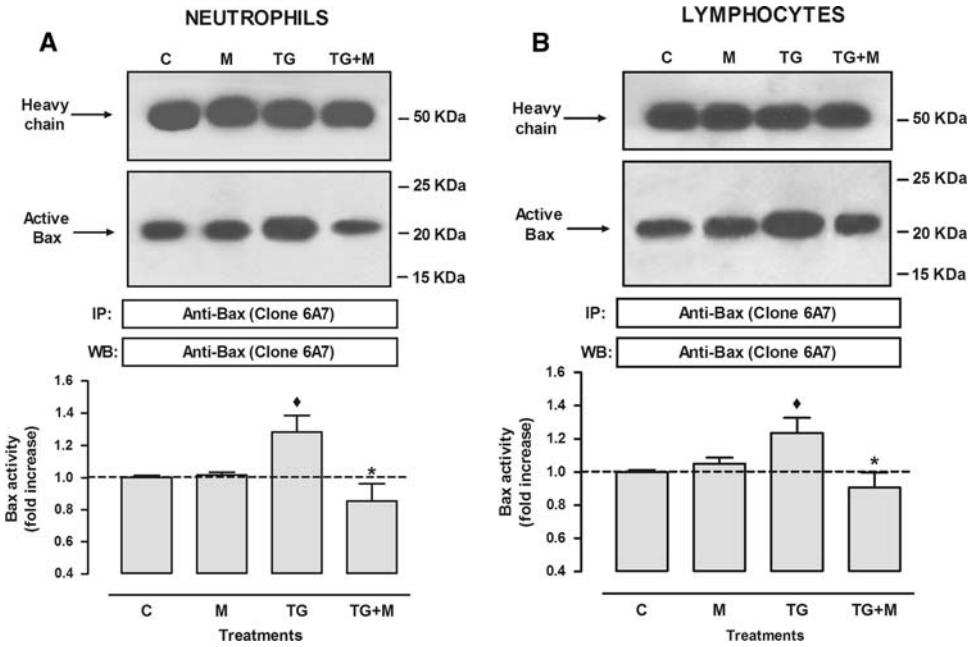


Fig. 6 Melatonin reduces Bax activation induced by thapsigargin. Human neutrophils (**a**) and lymphocytes (**b**) were preincubated with 1 mM melatonin (M) or vehicle for 60 min. Subsequently, cells were stimulated with 1 μM thapsigargin (TG) for 120 min and then lysed. Samples were immunoprecipitated with anti-Bax antibody (clone 6A7). Immunoprecipitates were analyzed by Western blotting using anti-Bax antibody as described in "Materials and Methods." Histograms show Bax activation expressed as fold increase over the pretreatment level (experimental/control). Data are mean ± SEM of four independent experiments. $^{\blacklozenge}P < 0.05$ compared to control (C) values, $^{*}P < 0.05$ compared to TG alone

rapidly induced calcium-dependent proapoptotic signal cascades, including an increased cellular content of proapoptotic Bax (Stamm et al. 2002). In fact, it is well established that treatment with agents that initiate endoplasmic reticulum stress, such as tunicamicyn or brefeldin A, can induce conformational changes and oligomerization of Bax on the endoplasmic reticulum as well as on mitochondria (Zong et al. 2003).

Melatonin is an indoleamine that is involved in many important physiological functions, and its role has been extensively examined both in vivo and in vitro. A role for melatonin in terms of immune function has been known for years (Rodríguez et al. 1999, 2005; Barriga et al. 2005); however, in recent years, interest in melatonin has markedly increased because of its influence on the process of apoptosis. The exact mechanism by which melatonin influences apoptosis has not been clarified as melatonin has been reported to have both pro- and antiapoptotic actions (for review, see Sainz et al. 2003). While several mechanisms have been proposed to explain the antiapoptotic actions of melatonin in immune cells, none has been definitively proven (Sainz et al. 2003). Here, we demonstrate for the first time that melatonin is able to reduce caspase-3 and -9 activities induced by calcium signal in human leukocytes, in both neutrophils and lymphocytes. In addition, our results demonstrate that melatonin induces reductions in the active forms of both caspases induced by intracellular calcium store depletion. This inhibitory effect of melatonin on caspase activation might not be a consequence of the opposing action of melatonin in the calcium pathway since melatonin proved to be ineffective at modifying FMLP- or thapsigargin-evoked calcium signal. However, we provide evidence supporting that the inhibitory effect of melatonin is probably due to blockade of mPTP opening since the loss of mitochondrial calcein fluorescence induced by depletion of intracellular calcium pools was reduced by preincubation with melatonin, in a similar manner to the permeability transition pore inhibitor CsA. To confirm this hypothesis, we tested the effect of melatonin on cytochrome *c* release after leukocyte stimulation with thapsigargin. Our results indicate that melatonin effectively reduced mitochondrial cytochrome *c* release evoked by thapsigargin. Our results are in agreement with previous reports which, using isolated heart mitochondria, demonstrated that the induction of mPTP opening and the release of cytochrome *c* after mitochondrial calcium overload were completely prevented by melatonin (Petrosillo et al. 2009b). Similar findings were obtained in an in vivo study where age-related mitochondrial dysfunction was prevented by melatonin (Petrosillo et al. 2008). These effects of melatonin seem to be due to its ability to inhibit cardiolipin peroxidation (Luchetti et al. 2007; Petrosillo et al. 2009a). Additionally, our results indicate that melatonin reduced activation of the proapoptotic protein Bax induced by depletion of intracellular calcium pools in both human neutrophils and lymphocytes. In this regard, Radogna and coworkers (2008) recently demonstrated that melatonin reduces apoptosis in U937 human tumor monocytes by impairing Bax activation due to melatonin-promoted Bcl-2 relocalization. Taken together, our findings indicate that the protective ability of melatonin against mitochondrial apoptosis is likely produced through the inhibition of both mPTP opening and Bax activation. Nonetheless, we cannot exclude the possibility that the inhibitory effects of melatonin on mitochondrial apoptosis are related to its well-known antioxidant capacity and free radical scavenger actions (Tan et al. 1993).

The melatonin-induced antiapoptotic effects presented here are in agreement with other studies showing that melatonin inhibits apoptosis in ischemic kidney (Kunduzova et al. 2003), amyloid β-peptide injury in hippocampal neurons (Shen et al. 2002), oxygen/glucose deprivation-induced apoptosis in cultures of embryonic mouse striatal neurons (Andrabi et al. 2004) and nitric oxide-induced cell death in PGT-β immortalized pineal cells (Yoo et al. 2002). It is interesting to note that melatonin is not protective in all models of apoptotic cell death (Harms et al. 2000), which may find its explanation in the fact that all the investigated noxious stimuli do not trigger the mPTP-mediated apoptotic pathways. In fact, in an early report, it was shown that melatonin promotes apoptosis assessed by DNA strand breaks in neutrophils from acute pancreatitis patients (Chen et al. 2005). In addition, this is, to our knowledge, the first demonstration of a protective role of melatonin on calcium signaling-induced apoptosis in primary human leukocytes, instead of cell lines or cells from other species, avoiding the common problem of translation into humans.

In the present investigation, we have shown that depletion of intracellular calcium stores stimulates caspase-3 and -9 in a time-dependent manner in human leukocytes. Calcium signal-induced caspase activation is associated with induction of mPTP opening, which is accompanied by an increase in the amount of mitochondrial cytochrome *c* released and activation of the proapoptotic protein Bax. We propose that melatonin is able to reduce this caspase-3 and -9 activation, which is mainly due to modulation of mPTP opening and Bax activation. This underlines the potential general interest of the role of melatonin as a controller of the life/death of immune cells within organisms.

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