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Melatonina y apoptosis en leucocitos humanos

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CERTIFICAN:

Que la presente Tesis Doctoral, presentada por D. Javier Espino Palma, con el título: "Melatonina y apoptosis en leucocitos humanos", ha sido realizado bajo nuestra dirección, en el Departamento de Fisiología de la Universidad de Extremadura, y entendiendo que reúne todos los requisitos establecidos, autorizamos su presentación para ser juzgada por el tribunal correspondiente.

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Espino J, Bejarano I, Paredes SD, Barriga C, Reiter RJ, Pariente JA, Rodríguez AB. Melatonin is able to delay endoplasmic reticulum stress-induced apoptosis in leukocytes from elderly humans. *Age (Dordr.)* 2011, 33: 497-507.

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Espino J, Bejarano I, Barriga C, Pariente JA, Rodríguez AB. Melatonin blocks intracellular calcium overload- and ROS-dependent caspase activation in human leucocytes. *Cell Membranes Free Radical Research* 2010, 2 (1): 37-38.

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4. Ponencias y comunicaciones orales:

Espino J, Bejarano I, Pariente JA. Calcium signalling and apoptosis: Role of melatonin. 4th International Congress on Cell Membranes and Oxidative Stress. Isparta (Turquía), 26-29 Junio (2012). Conference No. 8

Espino J, Rodríguez AB, Pariente JA. The inhibition of TNF-alpha-induced leucocyte apoptosis by melatonin involves membrane MT1/2 receptors. Joint FEPS and Spanish Physiological Society Scientific Congress 2012. Santiago de Compostela, 8-11 Septiembre (2012). Oral Presentation No. 113

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Espino J, Bejarano I, Barriga C, Pariente JA, Rodríguez AB. Melatonin blocks intracellular calcium overload- and ROS-dependent caspase activation in human leucocytes. 3rd International Congress on Cell Membranes and Oxidative Stress. Isparta (Turquía), 22-27 Junio (2010). Abstract No. 29

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Espino J, Rodriguez AB, Barriga C, Pariente JA. Melatonin and apoptosis induced by calcium signaling in human leukocytes. 1st International Meeting on Ion Channel Signalling Mechanisms: from basic science to clinical application. Marrakech (Marruecos), 30 Octubre - 4 Noviembre (2011). Abstract No. 137.

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6. Premios:

Second poster prize in 3rd International Congress on Cell Membranes and Oxidative Stress: Focus on Calcium Signaling and TRP channels (22-27 de Junio de 2010).

AA-NAT: Serotonina *N*-acetil-transferasa

ADNmt: ADN mitocondrial

AFMK: N^1-acetil-N^2-formil-5-metoxi-quinurenamina

AIF: Factor inductor de apoptosis

AMK: N^1-acetil-5-metoxi-quinurenamina

AMPc: Adenosín monofosfato cíclico

ANT: Transportador de nucleótidos de adenina

BH: Dominio de homología a Bcl-2

BIR: Baculoviral IAP Repeat

CARD: Dominio de reclutamiento de capasas

cFLIP: Proteína inhibitoria FLICE celular

CHX: Cicloheximida

cIAP: Proteína inhibidora celular de la apoptosis

COX-2: Ciclooxigenasa-2

CRD: Dominio rico en cisteínas

DD: Dominio de muerte

DED: Dominio efector de muerte

DISC: Complejo de señalización inductor de muerte

DTT: Ditiotreitol

EGF: Factor de crecimiento epidérmico

ERK: Quinasas reguladas por señales extracelulares

ERO: Especies reactivas de oxígeno

$FLIP_L$: FLIP largo

$FLIP_S$: FLIP corto

fMLP: *N*-formil- metionil-leucil-fenilalanina

GABA: Ácido γ-aminobutírico

GMPc: Guanosín monofosfato cíclico

GSH: Glutatión reducido

GSK-3: Glucógeno sintasa quinasa-3

HClO: Ácido hipocloroso

HIOMT: Hidroxi-indol-O-metiltransferasa

IAP: Proteína inhibidora de apoptosis

IP_3R: Receptor de inositol trifosfato

JNK: Quinasas c-Jun *N*-terminal

LDL: Lipoproteínas de baja densidad

MAPK: Proteína quinasa activada por mitógenos

MME: Membrana mitocondrial externa

MMI: Membrana mitocondrial interna

MCU: Uniporter de calcio mitocondrial

NAIP: Proteína inhibidora neuronal de la apoptosis

NF-κB: Factor nuclear-kappaB

NK: Natural Killer

NMDA: *N*-metil-D-aspartato

$^{\bullet}NO$: Óxido nítrico

nNOS: Óxido nítrico sintasa neuronal

NQO2: Quinona oxidorreductasa 2

$^1O_2$: Singlete de oxígeno

$O_2^{\bullet -}$: Anión superóxido

$^{\bullet}OH$: Anión hidroxilo

$ONOO^-$: Anión peroxinitrito

OPG: Osteoprotegerina

PARP: Poli ADP ribosa polimerasa

PI3K: Fosfoinositol 3-quinasa

PIDD: P53-Induced protein with a Death Domain

PKB: Proteína quinasa B

PLC: Fosfolipasa C

PPT: Poro de permeabilidad transitoria

PTEN: Homólogo de fosfatasa y tensina

RE: Retículo endoplasmático

ROR/RZR: Retinoid Orphan Receptors/ Retinoid Z Receptors

TGFβ-1: Factor de crecimiento transformante beta-1

THD: Dominios de homología a TNF

TM: Dominio transmembrana

TNF: Factor de necrosis tumoral

TPH: Triptófano-5-hidroxilasa

TRAIL: TNF-Related Apoptosis Inducing Ligand

UTP: Uracilo trifosfato

VDAC: Canal de aniones dependiente de voltaje

XIAP: X-linked Inhibitor of Apoptosis Protein

$[Ca^{2+}]_c$: Concentración de calcio citosólico

Índice

1. Introducción

1.1. Apoptosis o muerte celular programada

El término apoptosis, procedente del griego antiguo "apóptōsis", significa "algo que se cae o se desprende", como "los pétalos de las flores" o "las hojas de los árboles". Dicho término fue sugerido por J. Cormack, profesor de griego de la Universidad de Aberdeen, aunque fueron J.F.R. Kerr, A.H. Wyllie y A.R. Currie quienes, en 1972, propusieron su introducción en el lenguaje biológico para designar el mecanismo de la muerte celular programada (Kerr y cols., 1972).

La apoptosis se define como un proceso fisiológico de muerte celular iniciado por estímulos del desarrollo o ambientales que activan un programa genético que controla, diseña e inicia una cascada de eventos que dirige la destrucción ordenada y silenciosa de la célula, por lo que también se denomina muerte celular programada. Este fenómeno es un proceso activo, con requerimientos de energía en forma de ATP, no se propaga indiscriminadamente a las células vecinas y, por tanto, no induce una respuesta inflamatoria en los tejidos subyacentes, permitiendo así la eliminación de la célula apoptótica de un determinado tejido sin modificar la arquitectura o fisiología del mismo (Liu y cols., 1996; Strasser y cols., 2000).

La apoptosis es un mecanismo fisiológico primordial para la homeostasis tisular, el desarrollo de órganos y la eliminación de células defectivas o potencialmente peligrosas en organismos superiores. Además, este proceso de autodestrucción o "suicidio celular" tiene un papel crucial en el desarrollo y función del sistema inmune, así como en la protección antitumoral (Wyllie, 1997). Puesto que el proceso apoptótico depende de la integración de múltiples señales extracelulares e intracelulares, de la amplificación de dichas señales por parte de segundos mensajeros celulares y, en última instancia, de la activación de proteasas efectoras que llevan a término la muerte celular, cualquier defecto en el control de la apoptosis puede contribuir a una amplia variedad de enfermedades (Thompson, 1995). Así, una alteración del equilibrio homeostático puede conducir a situaciones patológicas como el cáncer cuando la proliferación se encuentra aumentada, mientras que puede causar enfermedades neurodegenerativas y autoinmunes cuando la tasa de muerte celular está incrementada (Hetts, 1998).

La apoptosis se caracteriza por una seria de cambios bioquímicos y morfológicos (Wyllie y cols., 1980) como son la retracción de pseudópodos o la reducción del volumen celular y nuclear (picnosis) debido a la condensación del citoplasma, por un lado, y a la compactación de la cromatina y fragmentación nuclear (cariorrexis), por otro lado. Esto último provoca la aparición de densos agregados que se deslocalizan para situarse junto a la membrana nuclear y que, posteriormente, serán degradados en fragmentos oligonucleosomales de 180 pares de bases o múltiplos de éstos por la acción de endonucleasas. Paralelamente, tiene lugar la dilatación del retículo endoplasmático (RE) dando lugar a la formación de vesículas, aunque sin pérdida de su integridad, que confieren a la célula el fenotipo característico en forma de "burbujas" (zeiosis). Por último, la célula se fragmenta en los denominados cuerpos apoptóticos que son fagocitados por macrófagos y otras células circundantes, evitando así la liberación del material intracelular y su exposición al sistema inmunitario, lo cual conllevaría al desarrollo de una respuesta inflamatoria (Figura I). Las células fagocíticas pueden reconocer a las células apoptóticas ya que éstas exponen en su superficie marcadores como la fosfatidilserina, fosfolípido situado en la cara interna de la membrana plasmática que se transloca a la cara externa en la fase temprana de la apoptosis (Fadok y cols., 1992). Otros marcadores reconocidos por las células fagocíticas son motivos oxidados tipo LDL (lipoproteínas de baja densidad), moléculas expresadas *de novo* como la trombospondina-1, o cambios en la composición de azúcares y en la carga eléctrica de la superficie celular.

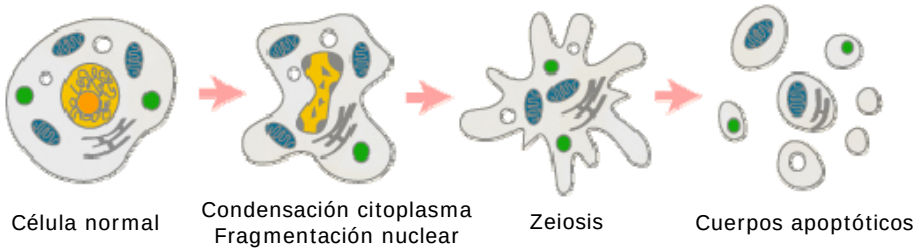

Figura I. Representación esquemática del proceso de muerte por apoptosis: condensación de los distintos orgánulos y fragmentación nuclear, formación de burbujas o "blebs" característicos de la apoptosis, fragmentación celular y formación de cuerpos apoptóticos.

De entre los mencionados eventos apoptóticos, la fragmentación del ADN y la externalización de la fosfatidilserina son dos características muy relevantes desde el punto de vista diagnóstico, ya que permiten detectar la activación de un programa

apoptótico mediante una amplia variedad de técnicas moleculares. En este sentido, quizás el cambio más característico de la apoptosis es la condensación de la cromatina nuclear, que comienza en la periferia nuclear. Seguidamente, se desintegra la envuelta nuclear, la laminina sufre desintegración proteolítica y, por último, se produce la fragmentación nuclear (Douglas y cols., 1995; Li y cols., 1995). El ADN en la cromatina condensada presenta hipercromasia y se marca intensamente con sondas fluorescentes de ADN. Además, los productos de la degradación del ADN debido a la acción de endonucleasas son fragmentos nucleosomales u oligonucleosomales que generan el característico patrón en escalera en electroforesis de geles de agarosa (Arends y cols., 1990; Compton, 1993). Por otro lado, la exposición de la fosfatidilserina en la cara extracelular de la membrana plasmática es también un indicador importante para la detección de la apoptosis (Balasubramanian y Schroit, 2003). De hecho, la fosfatidilserina expuesta en la superficie celular funciona como una señal específica de reconocimiento, conocida como señal "*eat-me*", que permite que los macrófagos identifiquen y, posteriormente, degraden la célula apoptótica (Ravichandran, 2010). La pérdida de la asimetría de la membrana plasmática se detecta comúnmente mediante sondas fluorescentes unidas a proteínas que interaccionan específicamente con la fosfatidilserina, como es el caso de la anexina V (Boersma y cols., 2005), aunque en los últimos años se están desarrollando nuevas metodologías basadas en el uso de sondas ratiométricas (Demchenko, 2012).

1.1.1. Maquinaria implicada en la muerte celular: Caspasas

Las caspasas (acrónimo del inglés *Cysteine ASPartyl-specific proteASES*) constituyen una familia de enzimas muy conservadas en la evolución y son los componentes centrales de la maquinaria de inducción de apoptosis. Se trata de cisteín-proteasas, ya que presentan un residuo de cisteína en su centro catalítico, que median la ruptura de otras proteínas y se caracterizan por cortar sus sustratos al nivel de un residuo de aspártico, de donde deriva su nombre.

Las caspasas están constituidas por un prodominio N-terminal de longitud variable, seguido de una subunidad mayor de unos 20 kDa y una subunidad menor de

unos 10 kDa en el extremo C-terminal (Figura II). El residuo de cisteína del centro activo forma parte de una secuencia pentapeptídica conservada, QACXG, y se encuentra en la subunidad mayor. Por su parte, los residuos que constituyen el sitio de unión para el sustrato se localizan tanto en la subunidad mayor como en la menor, si bien el residuo dominante para la especificidad de sustrato se halla en la subunidad menor (Wolf y Green, 1999).

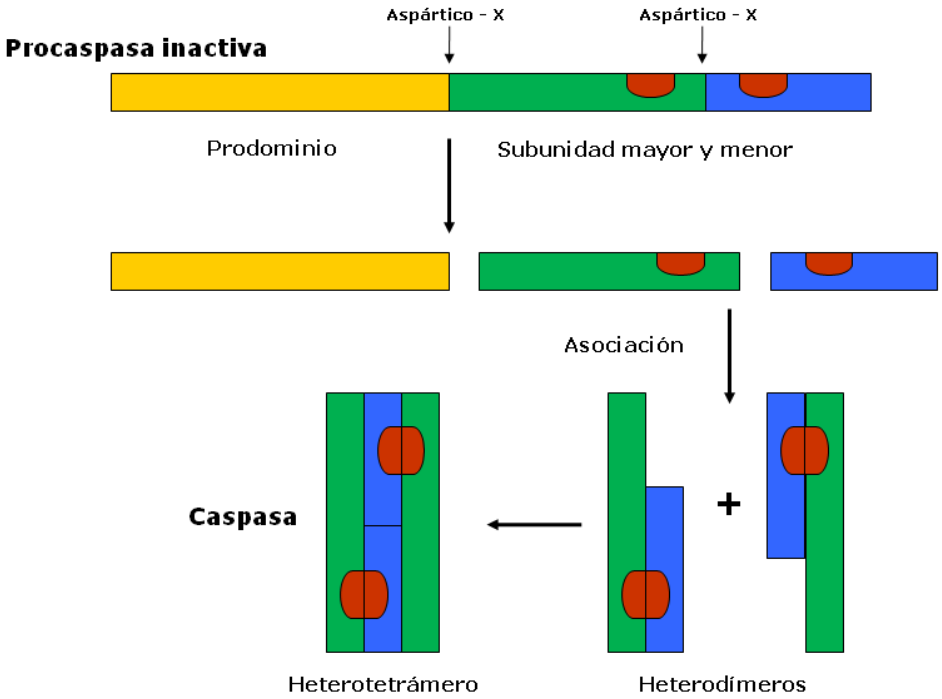

Figura II. Activación de las caspasas. La procaspasa contiene tres dominios: un prodominio, una subunidad mayor y otra menor. La procaspasa es inactiva y una vez que sufre dos roturas proteolíticas se separan las tres subunidades. La subunidad mayor y la menor forman un heterodímero por unión de sus centros activos. La unión de dos heterodímeros para formar un heterotetrámero activo tiene lugar entre las dos subunidades menores adyacentes rodeadas por las subunidades mayores.

La activación de las caspasas requiere de la escisión de las tres subunidades que las conforman, es decir, el prodominio, la subunidad mayor, y la subunidad menor. Debido a este procesamiento, se forma un tetrámero integrado por dos subunidades mayores y dos subunidades menores que presentará dos centros catalíticos (Wolf y Green, 1999). Esta proteólisis tiene lugar en dos fases: en la primera, la subunidad mayor, junto con el prodominio, se separa de la subunidad menor; en la segunda, el prodominio se escinde de la subunidad mayor (Figura II). La presencia de residuos de aspártico en los sitios de separación de las distintas subunidades se relaciona con la

capacidad de las caspasas de auto-activarse o ser activadas por otras caspasas dentro de la cascada apoptótica.

Las caspasas reconocen en sus sustratos una secuencia diana de, al menos, cuatro aminoácidos, P4-P3-P2-P1, y cortan después del aminoácido carboxi-terminal, P1, que es el residuo de aspártico. El residuo P3 suele ser una glutamina, mientras que los residuos P2 y P4 son variables. Por tanto, la secuencia de especificidad de corte de una caspasa será X-Glu-X-Asp. Los aminoácidos de la enzima que se unen al sustrato y, por tanto, reconocen la secuencia P4-P3-P2-P1, se denominan S4-S3-S2-S1. Los residuos S3 y S1 están muy conservados en las diferentes caspasas. Sin embargo, S2 y S4 varían significativamente de unas caspasas a otras y originan las distintas especificidades por el sustrato en función de los residuos situados en las posiciones P2 y P4.

En mamíferos, la familia de las caspasas está compuesta por catorce proteínas diferentes, aunque en humanos tan sólo se sabe que se expresen once, de la caspasa-1 a la -10 y la caspasa-14, que sólo se expresa en queratinocitos (Pistritto y cols., 2002). Las caspasas se dividen en tres grupos diferenciados en base a su función: caspasas iniciadoras, caspasas efectoras o ejecutoras, y caspasas implicadas en inflamación (Degterev y cols., 2003).

El grupo de las **caspasas iniciadoras** está formado por la caspasa-2, -8, -9 y -10, y son las primeras en activarse tras un estímulo apoptótico. En realidad, este estímulo provoca el ensamblaje de lo que se denominan complejos de activación, que están integrados por las formas inactivas de las propias caspasas y por proteínas adaptadoras, y constituyen una plataforma para la activación de las citadas caspasas iniciadoras. Las proteínas adaptadoras se unen de manera específica a dominios DED (acrónimo del inglés *Death Effector Domain*) o CARD (*CAspase Recruitment Domain*), los cuáles se sitúan en los prodominios de las caspasas. Existen varios complejos de activación para las caspasas iniciadoras (Figura III). Así, el complejo de señales inductoras de muerte o DISC (*Death-Inducing Signalling Complex*) recluta y activa a la caspasa-8 y -10 mediante los dominios DED (Juo y cols., 1998). Por su parte, el apoptosoma permite el reclutamiento y activación de la caspasa-9 a través de su dominio CARD (Liu y cols.,

1999). La caspasa-2 también tiene dominios CARD, pero su complejo de activación es conocido como PIDDosoma, ya que está constituido por PIDD (*P53-Induced protein with a Death Domain*) y la proteína adaptadora RAIDD, que también contiene un dominio de muerte (Bao y Shy, 2007).

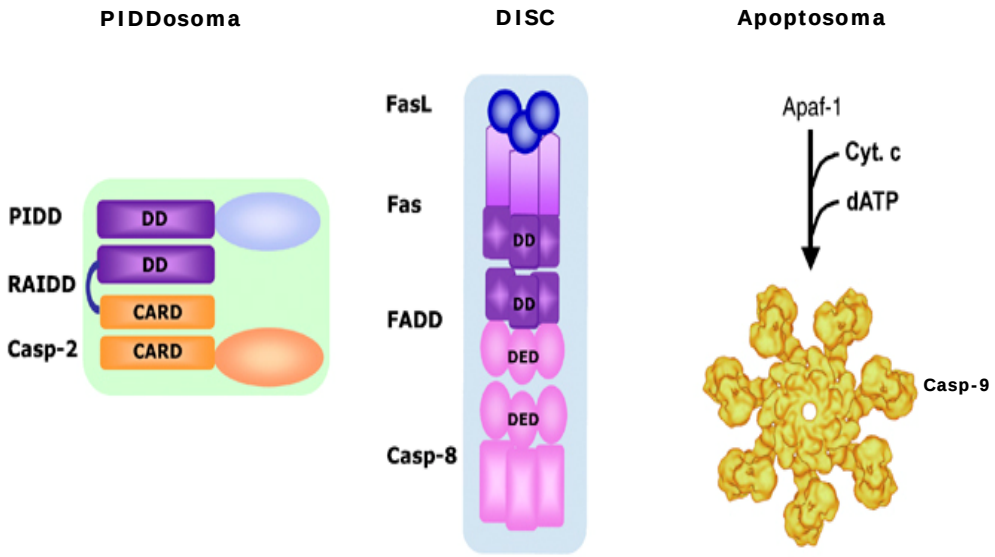

Figura III. Representación esquemática de los tres complejos de reclutamiento y activación de las caspasas iniciadoras (modificado de Bao y Shy, 2007).

El reclutamiento de las caspasas iniciadoras por proteínas adaptadoras, así como la formación de los complejos de activación, determinaron la aparición de la teoría de activación de dichas caspasas por un mecanismo de autocatálisis por proximidad (Salvesen, 1999). Estudios más recientes han demostrado, sin embargo, que la activación de caspasas iniciadoras puede ocurrir en ausencia de cortes proteolíticos, simplemente debido al cambio conformacional que tiene lugar tras la homodimerización de las formas proenzimáticas monoméricas en los complejos de activación, especialmente en el caso de la caspasa-9 (Boatright y cols., 2003).

El conjunto de **caspasas efectoras** está integrado por la caspasa-3, -6 y -7, que carecen de dominios DED y CARD y son activadas directamente por las caspasas iniciadoras. Las caspasas efectoras son las encargadas de degradar los distintos sustratos celulares que provocan los cambios morfológicos, estructurales y bioquímicos característicos de la muerte por apoptosis. Aparte de su implicación en la apoptosis, también se ha descrito una activación temprana de caspasas, especialmente de la

caspasa-3, independiente de apoptosis que es probablemente requerida para la función celular, incluyendo la agregación plaquetaria o la secreción pancreática (Rosado y cols., 2006).

La caspasa-1, -4 y -5 constituyen una clase particular dentro de esta familia de proteasas, ya que se relacionan con el control de ciertas **respuestas inflamatorias**, especialmente de la maduración de citoquinas. También pueden jugar un papel en la muerte por apoptosis en determinadas situaciones patológicas, como ocurre durante la isquemia, actuando probablemente tanto de caspasas iniciadoras como de caspasas efectoras. El complejo de activación de estas caspasas se conoce como inflamasoma.

1.1.2. Vías de inducción de la apoptosis

Existen dos tipos generales de vías de señalización que conducen a la activación de la muerte celular programada: **1)** la ruta de los receptores de muerte o extrínseca, que implica la activación de determinados miembros de la superfamilia de receptores del factor de necrosis tumoral (TNF), y **2)** la ruta mitocondrial o intrínseca, inducida por distintas formas de estrés celular, como puede ser el causado por la privación de factores de crecimiento en el medio, pérdida de adhesión al sustrato, daño al ADN, estrés en el RE, activación de oncogenes, infección viral, radiaciones ionizantes, radiaciones ultravioletas, etc. Ambas vías producen la activación de las caspasas iniciadoras y convergen en la activación de las caspasas ejecutoras, responsables últimas de las características morfológicas de la apoptosis.

1.1.2.1. Vía extrínseca o de los receptores de muerte

Una célula normal o patológica puede entrar en apoptosis en respuesta al estímulo desencadenado por la unión de los denominados ligandos de muerte a sus receptores específicos. Los ligandos de muerte son proteínas transmembrana tipo II pertenecientes a la superfamilia del TNF, incluyendo FasL/CD95L, TRAIL (acrónimo del inglés *TNF-Related Apoptosis Inducing Ligand*) o el propio TNF. Estos ligandos presentan dominios de homología a TNF (THD, *TNF Homology Domain*), a través de los cuales se asocian a sus receptores (Bodmer y cols. 2002). Dichos ligandos son

expresados por distintas células del sistema inmunológico tales como linfocitos T, células NK (*Natural Killer*), monocitos y células dendríticas, permitiendo la eliminación de células que han cumplido su función, células infectadas, células autorreactivas y/o células metaplásicas (Singh y cols., 1999; Perlman y cols., 2001; Yang y cols., 2001; Zhang y cols., 2004).

Los receptores de muerte a los que se unen estos ligandos son proteínas transmembrana tipo I de la superfamilia de receptores del TNF y reciben el nombre de receptores de muerte. Se caracterizan por presentar en su región intracelular los denominados dominios de muerte (DD, *Death Domain*), a través de los cuales se transmite el estímulo apoptótico. Existen, además, receptores anti-apoptóticos o "señuelo" para dichos ligandos, denominados así porque no transmiten la señal apoptótica (Gruss y Dower, 1995). Ambos tipos de receptores presentan en su región extracelular unos dominios ricos en cisteína (CRD, *Cysteine-Rich Domain*) que se asocian con las regiones THD de los ligandos de muerte por medio de puentes disulfuro, formando de este modo multímeros funcionales. La unión del ligando a su correspondiente receptor de muerte va a tener como consecuencia la trimerización del receptor y el reclutamiento de proteínas citoplasmáticas adaptadoras con dominios DD homólogos a los del receptor, entre las que destaca la proteína FADD. Estas proteínas, a su vez, reclutan a las caspasas iniciadoras (caspasa-8 y/o -10) a través de los dominios DED, conformando así el complejo DISC donde se activarán dichas caspasas (Kischkel y cols., 2000).

En función de las señales implicadas en la inducción de apoptosis tras la activación de los receptores de muerte, las células pueden clasificarse en tipo I, si el proceso apoptótico es independiente de la vía mitocondrial, o tipo II, si se requiere la participación de dicho orgánulo (Scaffidi y cols., 1998). En las células de tipo I, las caspasas iniciadoras activadas a nivel del complejo DISC, a su vez, activan de forma directa a las caspasas efectoras que, en última instancia, provocan la muerte celular por apoptosis. Por el contrario, en las células de tipo II, las caspasas iniciadoras provocan la activación de las señales mitocondriales características de la ruta intrínseca (ver apartado 1.1.2.2.) que, posteriormente, activan a las caspasas efectoras.

El TNF tiene un papel fundamental en inflamación e inmunidad. Se han descrito dos receptores para TNF: TNFR-1 y TNFR-2. Sólo TNFR-1 se considera un receptor de muerte, ya que TNFR-2 carece del dominio de muerte intracelular necesario para transmitir la señal apoptótica. A pesar de ello, TNFR-1 puede disparar la inducción de apoptosis o bien activar rutas de supervivencia celular, dependiendo del complejo de señalización que se active. Asimismo, la interacción de FasL/CD95L con su receptor de muerte Fas/CD95 da lugar, generalmente, a la inducción de apoptosis, aunque se han descrito algunas situaciones en las que Fas no tiene una acción citotóxica sino que induce proliferacíon, migración y producción de citoquinas (Aggarwal, 2003; Micheau y Tschopp, 2003).

Por otro lado, el ligando de muerte TRAIL puede unirse específicamente a cuatro receptores transmembrana (TRAIL-R1 a -R4) y, con menor afinidad, a un receptor soluble denominado osteoprotegerina (OPG), aunque se desconoce la relevancia de esta unión en condiciones fisiológicas (Almasan y Ashkenazi, 2003). Los receptores de membrana de TRAIL se clasifican en pro- y anti-apoptóticos, en función de su capacidad para inducir la activación de la cascada apoptótica. Los receptores TRAIL-R1/DR4 y TRAIL-R2/DR5 son receptores de muerte pro-apoptóticos, pues presentan en su región intracelular los dominios DD necesarios para transmitir la señal de apoptosis tras la unión del ligando (Pan y cols., 1997; Cha y cols., 2000). Los receptores TRAIL-R3/DcR1 y TRAIL-R4/DcR2 son receptores "señuelo" debido a su incapacidad para inducir apoptosis tras la unión del ligando. TRAIL-R3 carece de dominios transmembrana e intracitoplasmático, anclándose a la membrana a través de una cola de glicofosfatidil-inositol (Degli-Esposti y cols., 1997). Por el contrario, TRAIL-R4 presenta regiones citoplasmática y transmembrana, pero posee un dominio DD truncado que lo incapacita a la hora de ensamblar la maquinaria necesaria para la activación del proceso apoptótico, aunque le permite activar rutas de supervivencia celular tras la unión de TRAIL. Esto último también se ha descrito tras la unión de TRAIL a sus receptores pro-apoptóticos (Chaudhary y cols., 1997).

1.1.2.2. Vía intrínseca o mitocondrial

Esta ruta de inducción de apoptosis es conocida también como ruta mitocondrial ya que la mitocondria es el principal orgánulo implicado en este tipo de muerte celular. La ruta intrínseca es activada por diferentes estímulos, incluyendo daño al ADN, radiación ultravioleta y drogas quimioterápicas. Las vías de señalización activadas por estos agentes estresantes provocan la permeabilización de la membrana mitocondrial externa (MME) y, en consecuencia, la liberación de proteínas solubles desde el espacio intermembrana al citoplasma. Estos factores pro-apoptóticos producen la activación de caspasas, aunque algunos de ellos también pueden conducir a una muerte celular independiente de caspasas (Rosado y cols., 2006).

Se han propuesto dos mecanismos para explicar la permeabilización de la MME, pudiendo funcionar uno u otro según el contexto, el estímulo o el tipo celular:

- El primero de ellos, en el que participa la membrana mitocondrial interna (MMI), implica la formación de un poro que recibe el nombre de poro de permeabilidad transitoria (PPT). Este poro permite el paso de agua y moléculas de bajo peso molecular provocando la pérdida del equilibrio iónico, así como el incremento en el volumen de la matriz mitocondrial por exceso de hidratación, desencadenando de este modo la ruptura de la MME. El PPT está formado, entre otros componentes, por el transportador de nucleótidos de adenina (ANT) a nivel de la MMI y el canal de aniones dependiente de voltaje (VDAC) de la MME. En condiciones fisiológicas, el canal VDAC es necesario para el mantenimiento de la función energética mitocondrial ya que es la proteína más abundante de la MME y, junto con el transportador ANT, es el encargado del transporte de ATP y ADP dentro y fuera de la mitocondria (Mattson y Kroemer, 2003).

- En el segundo caso, la permeabilidad de la MME se atribuye a la formación de canales proteicos constituidos por la oligomerización de miembros pro-apoptóticos de la familia Bcl-2 que actúan directamente sobre la MME, o bien debido a la asociación de las citadas proteínas pro-apoptóticas con distintos componentes lipídicos de la MME que provocan la aparición de grandes poros (Green y Kroemer, 2004).

Existe un tercer modelo que surge de la asociación de los anteriores. Según dicho modelo, los miembros pro-apoptóticos de la familia Bcl-2 interaccionarían con las proteínas que constituyen el PPT. De este modo, VDAC permitiría la salida de factores mitocondriales a través de la MME y el transportador ANT conformaría canales iónicos en la MMI (Shimizu y cols., 1999).

En cualquier caso, una vez permeabilizada la MME, se producen una serie de eventos, todos ellos implicados en la muerte celular:

1) Liberación de proteínas del espacio intermembrana de la mitocondria. Estas proteínas pueden activar caspasas, nucleasas o pueden neutralizar la acción de ciertos inhibidores citosólicos del proceso apoptótico. Algunas de estas proteínas son:

a) Citocromo c: Una vez que éste es liberado al citoplasma ante un estímulo apoptótico, se une a la proteína pro-apoptótica Apaf-1, en presencia de ATP. Apaf-1 presenta tres dominios funcionales. Una secuencia CARD en el extremo amino que tiene un alto grado de homología con la secuencia CARD de la caspasa-9, un dominio de unión de ATP en la zona central y, finalmente, en el extremo carboxilo presenta el dominio WD-40, rico en dobletes de residuos triptófano y ácido aspártico que se repiten 40 veces. Este último está considerado como un dominio de unión proteína-proteína a través de cual el citocromo *c* interacciona con Apaf-1 (Shi, 2002). La unión del citocromo *c* y la molécula de ATP facilita un cambio conformacional en la estructura de Apaf-1 que permite la oligomerización de éste con otras moléculas de Apaf-1 para formar un heptámero (ver Figura III). De este modo, se constituye el mencionado apoptosoma, que recluta a la procaspasa-9 a través de interacciones entre los dominios CARD presentes tanto en Apaf-1 como en la procaspasa-9 (Fan y cols., 2005). La activación de la procaspasa-9 dentro del apoptosoma se produce por una proteólisis autocatalítica.

b) AIF (acrónimo del inglés *Apoptosis-Inducing Factor*): Es una flavoproteína que posee una señal de localización mitocondrial y otra nuclear. Normalmente se encuentra confinada en el espacio intermembrana de la mitocondria pero, una vez que es liberada al citoplasma en respuesta a

estímulos apoptóticos, se transloca al núcleo produciendo fragmentación del ADN y condensación periférica de la cromatina. AIF también provoca otros cambios celulares característicos de la apoptosis, como la disipación del potencial de membrana mitocondrial o la exposición de la fosfatidilserina en la superficie celular. Cabe destacar que ninguno de estos procesos, resultado de la activación de AIF, son dependientes de caspasas. Por tanto, esta proteína actúa de manera independiente del apoptosoma (Joza y cols., 2001).

c) Smac/DIABLO: Esta proteína se une e inactiva a una serie de proteínas inhibidoras de caspasas que pertenecen a la familia de los IAPs (proteínas inhibidoras de apoptosis) (Du y cols., 2000). Además, se ha demostrado que la proteína Smac/DIABLO tiene una capacidad pro-apoptótica independiente de su acción inhibidora sobre los IAPs (Fulda y cols., 2002).

d) Omi/HtrA2: Es una serín-proteasa que interacciona con las proteínas IAPs citosólicas de manera similar a Smac/DIABLO. Por tanto, puede promover la activación indirecta de caspasas, como la caspasa-3 o -9. Sin embargo, Omi/HtrA2 también posee capacidad de inducir apoptosis de un modo independiente de la activación de caspasas o de su interacción con proteínas IAPs, gracias a su dominio catalítico serín-proteasa (Suzuki y cols., 2001).

e) Endonucleasa G: Esta proteína, una vez liberada al citoplasma, se transloca al núcleo donde está involucrada en la fragmentación del ADN. Dicha fragmentación es independiente de la activación de caspasas. Asimismo, es probable que la endonucleasa G necesite de otras nucleasas o cofactores para poder realizar su función (Van Loo y cols., 2001).

2) Alteración de la cadena de transporte de electrones, de la fosforilación oxidativa y de la producción de ATP. El citocromo *c* transporta electrones del complejo III al complejo IV de la cadena de transporte electrónico, lo cual genera un potencial eléctrico a través de la MMI ($\Delta\psi m$). Este potencial es utilizado por el complejo V para generar ATP a partir de ADP y fosfato libre procedente de la respiración aeróbica, y también se utiliza para importar proteínas así como para la biogénesis mitocondrial (Waterhouse y cols., 2001). La liberación del citocromo *c* desde

el espacio intermembrana causa una pérdida transitoria de este potencial, aunque este hecho por sí solo es insuficiente para impedir completamente la fosforilación oxidativa y la producción de ATP. Sin embargo, cuando las caspasas se activan debido a la permeabilización de la MME, éstas cortan la subunidad p75 del complejo I de la cadena de transporte electrónico (Ricci y cols., 2004). Este hecho provoca una pérdida persistente del potencial de membrana y una alteración en la producción de ATP, principal forma de energía de la célula y esencial para el mantenimiento de la viabilidad, por lo que se ve afectada la supervivencia celular.

3) Alteración del potencial reducción-oxidación (redox) de la célula. La mitocondria es la principal fuente de producción de especies reactivas de oxígeno (ERO) dentro de la célula. Como consecuencia de las alteraciones mitocondriales mencionadas, se genera un aumento en la producción de ERO que a su vez provoca modificaciones oxidativas en diferentes macromoléculas, incluyendo lípidos, proteínas y ácidos nucléicos, que alteran su estructura y función. Este tipo de reacciones inducen la formación de agregados proteicos que facilitan la formación del PPT provocando, al mismo tiempo, la pérdida del $\Delta\psi m$ y la liberación de proteínas pro-apoptóticas, como el citocromo *c*, y la consiguiente apoptosis o muerte celular programada (Brookes y cols., 2004; Uguz y cols., 2009; Bejarano y cols., 2011a). El citocromo *c* está cargado positivamente y se encuentra unido a la cardiolipina, cargada negativamente, en la cara exterior de la MMI. La salida de citocromo *c* requiere la peroxidación de la cardiolipina, un proceso mediado por las ERO, lo cual provoca un cambio en sus propiedades físicas que posibilita la ruptura de la unión entre el citocromo *c* y la cardiolipina (Ott y cols., 2002).

1.1.2.3. Proteínas de la familia Bcl-2

Como ya se ha mencionado anteriormente, miembros de la familia Bcl-2 regulan la permeabilización de la MME. Dentro de esta familia existen miembros anti-apoptóticos, los cuales preservan la integridad de la MME e impiden que se dispare el proceso de muerte celular por apoptosis, y miembros pro-apoptóticos, encargados de promover la permeabilización de la MME y todos los eventos mitocondriales que conducen a la muerte de la célula.

Los miembros de la familia Bcl-2 pueden clasificarse en tres grupos en función de su actividad pro- o anti-apoptótica, así como del número de dominios de homología a Bcl-2 que posean, denominados dominios BH (Figura IV).

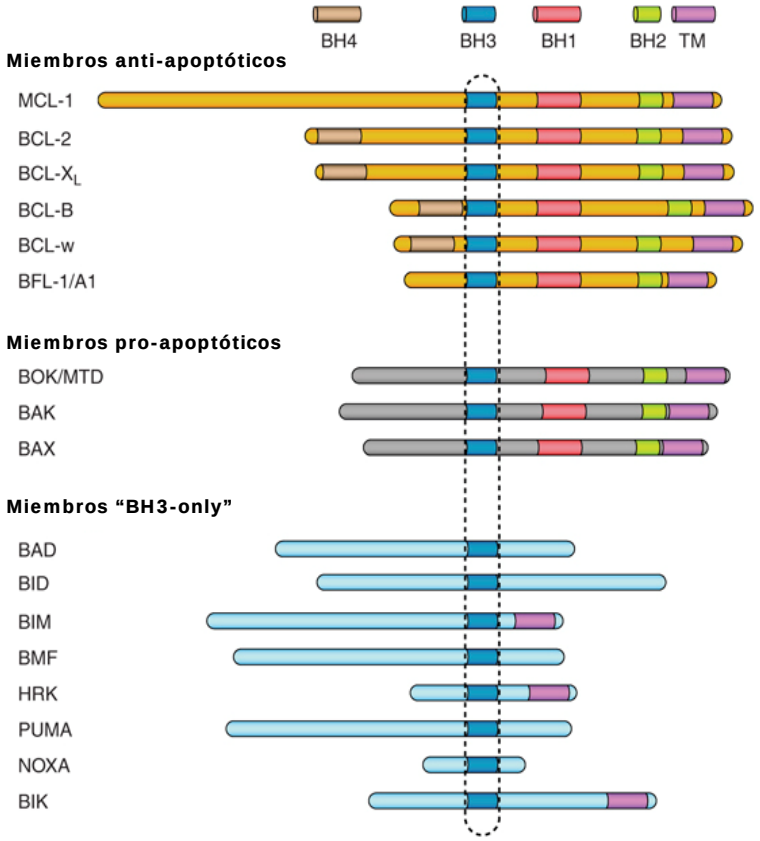

Figura IV. Clasificación de la familia de proteínas Bcl-2 basada en la organización de los dominios y en su función. Están resaltados los distintos dominios de homología a Bcl-2 (BH) así como el dominio transmembrana (TM) (modificado de Danial, 2009).

Miembros anti-apoptóticos: Las proteínas de esta clase presentan, por lo general, cuatro dominios de homología: BH1, BH2, BH3 y BH4. Dentro de este grupo se encuentran las proteínas Bcl-2, Bcl-x_L, Bcl-w, Boo/Diva, y sus homólogos más divergentes Mcl-1 y Bfl-1/A1, que carecen de dominio BH4 (Figura IV) (Sattler y cols., 1997). Los cuatro dominios BH intervienen en las interacciones con otras proteínas de la familia y con moléculas que están localizadas en la cara citoplasmática de las membranas intracelulares, como por ejemplo la MME, la membrana del RE y la envuelta nuclear. Los dominios BH1, BH2 y BH3 de las proteínas anti-apoptóticas son los responsables de formar una región hidrofóbica de interacción con los dominios BH3 de las proteínas pro-apoptóticas, provocando su inhibición (Willis y Adams, 2005). La

importancia de esta familia en la regulación de la muerte celular se hizo evidente a partir de estudios que mostraron que la sobreexpresión de Bcl-2 contribuía al desarrollo de determinados tipos de linfomas. Por este motivo, Bcl-2 da nombre a esta familia de proteínas (Leibowitz y Yu, 2010).

Miembros pro-apoptóticos: Son proteínas multiméricas y se caracterizan por presentar dominios BH1, BH2, y BH3. Dentro de esta clase se encuadran las proteínas Bax, Bak y Bok (Figura IV). Estas proteínas juegan un papel importante en la apoptosis ya que modifican el $\Delta\psi m$ y la permeabilidad de la MME promoviendo, de este modo, la liberación de moléculas pro-apoptóticas tales como citocromo *c* y Smac/DIABLO (Letai y cols., 2002; López y cols., 2008). Las proteínas pro-apoptóticas multiméricas se expresan de forma constitutiva y sólo inducen la permeabilidad de la MME tras un estímulo apoptótico, lo que sugiere que permanecen inactivas hasta que la célula recibe un estímulo apoptótico (Wolter y cols., 1997). Estas proteínas también presentan un dominio transmembrana (TM) en su extremo carboxilo que les permite anclarse a las membranas celulares. Dicho dominio TM se encuentra plegado hacia el bolsillo hidrofóbico, constituido por los dominios BH1, BH2 y BH3, evitando así su inserción en las membranas. La retracción de este dominio TM tras un estímulo apoptótico resulta en la translocación e inserción de estas proteínas en la membrana mitocondrial (Nechushtan y cols., 2001; Borner, 2003). Una vez adheridos en la membrana mitocondrial, Bak y Bax oligomerizan y alteran la permeabilidad de la MME formando canales (Zamzami y Kroemer, 2001).

Miembros pro-apoptóticos "BH3-only": Los miembros de esta clase comparten sólo, entre ellos y con el resto de miembros de la familia, el dominio BH3, de ahí su denominación. Dentro de este grupo se encuentran las proteínas Bad, Bim, Bid, Bmf, Bik, Noxa, Puma, Blk, Hrk, Nip3 y BNip3 (Figura IV). Estas proteínas pro-apoptóticas juegan un papel importante en la activación de Bax y Bak, aunque el mecanismo de activación no está completamente claro. La mayoría de miembros "*BH3-only*" lo que hacen es "desreprimir" o permitir la actividad intrínseca de Bax y Bak interaccionando e inhibiendo a los miembros anti-apoptóticos de la familia. Así, la proteína Bad, que se encuentra secuestrada en el citoplasma por proteínas 14-3-3, es activada por un estímulo apoptótico y se transloca a la MME donde se une a los miembros anti-apoptóticos de la

familia inhibiendo su función (Zha y cols., 1997). Del mismo modo, Bmf permanece secuestrada en el citoplasma hasta que un estímulo apoptótico permite su liberación y unión a las proteínas anti-apoptóticas (Puthalakath y cols., 2001). Sin embargo, algunas proteínas "*BH3-only*" pueden unirse y activar Bax y Bak directamente. Este es el caso de Bid y Bim que pueden interaccionar a través del dominio BH3 con Bax y Bak, provocando su dimerización y activación (Luo y cols., 1998; Puthalakath y Strasser, 2002), aunque también pueden activarlas indirectamente al unirse a las proteínas Bcl-2 o Bcl-x_L, inhibiendo la función anti-apoptótica de estas últimas (Puthalakath y cols., 1999).

1.1.3. Regulación de la apoptosis

La apoptosis es un proceso que se encuentra altamente regulado, ya que sólo puede activarse en situaciones y momentos determinados. Por ello, existen numerosas proteínas que, ya sea inhibiendo directamente la señalización de apoptosis o bien activando señales y rutas de supervivencia, evitan la posible entrada de la célula en apoptosis de forma accidental. Entre los mecanismos reguladores de la apoptosis podemos señalar el balance entre los niveles de receptores pro- y anti-apoptóticos o de proteínas pro- y anti-apoptóticas de la familia Bcl-2. Existen otros mecanismos de control que favorecen o impiden la señal de apoptosis, entre los que podemos destacar los que a continuación se describen.

1.1.3.1. Inhibidores de caspasas: IAPs y c-FLIP

IAPs: Esta familia de proteínas se identificó en baculovirus, observándose su capacidad para impedir la entrada de la célula infectada en apoptosis mediante inactivación de las caspasas. Se han descrito hasta ocho proteínas IAPs en mamíferos, entre las que se encuentran XIAP (acrónimo del inglés *X-linked Inhibitor of Apoptosis Protein*), cIAP-1 y cIAP-2 (*cellular Inhibitor of Apoptosis Protein*), ILP2 (*IAP-Like Protein-2*), ML-IAP (asociado a melanoma), NAIP (*Neuronal Apoptosis Inhibitory Protein*), survivina y Bruce (Wright y cols., 2005).

Estas proteínas inhibidoras de la apoptosis, con capacidad de unión e inactivación de caspasas tanto iniciadoras como efectoras, llevan a cabo su acción de inhibición a través de los denominados dominios BIR (*Baculoviral IAP Repeat*). Estos dominios consisten en aproximadamente 80 aminoácidos plegados alrededor de un átomo de zinc. Muchas de estas proteínas contienen también un dominio contiguo denominado RING encargado de la destrucción de la caspasa, ya que actúa como una ligasa de ubiquitina que promueve la degradación por el proteosoma del propio IAP asociado a la caspasa. Junto al dominio RING, tanto en cIAP-1 como en cIAP-2 se localiza un dominio CARD que sugiere que estas IAPs podrían regular directa o indirectamente el procesamiento de las caspasas a través de interacciones con dicho dominio (Yang y Li, 2000).

c-FLIP (*Flice Inhibitory Protein*): Esta proteína se identificó originalmente como producto de un gen viral, mediante el cual el virus trataba de mantener con vida a la célula infectada para lograr con éxito su etapa de replicación, evitando la apoptosis mediada por receptores de muerte (Thome y cols., 1997). Se trata de una proteína que inhibe la activación de la caspasa-8 y -10. Se ha visto que se expresa constitutivamente en diferentes tipos de células normales como cardiomiocitos, células endoteliales, queratinocitos, células dendríticas, células madre hematopoyéticas $CD34^+$, y espermatocitos. Además, se ha encontrado una elevada expresión de c-FLIP en células tumorales que suelen ser resistentes a la apoptosis inducida por receptores de muerte (Hu y cols., 1997). En humanos, encontramos dos isoformas de la proteína obtenidas por procesamiento alternativo del ARNm y denominadas por su longitud FLIP largo ($FLIP_L$), de 55 kDa, y FLIP corto ($FLIP_S$), de 26 kDa (Hu y cols., 1997). Ambas isoformas se caracterizan por presentar en su estructura dos dominios DED, análogos a los encontrados en los prodominios de la caspasa-8 y -10. Estos dominios son necesarios para cumplir su función inhibidora de apoptosis puesto que, a través de ellos, pueden asociarse por interacciones homotípicas con la proteína adaptadora FADD y ser reclutados en el complejo DISC en lugar de dichas caspasas iniciadoras. Si bien ésta constituye la estructura básica de la isoforma $FLIP_S$, en el caso de $FLIP_L$ encontramos también un dominio pseudocaspasa inactivo que va a ser degradado a nivel del complejo DISC como si de la auténtica caspasa-8/-10 se tratase. Como consecuencia, se

genera un fragmento de 43 kDa que queda anclado al complejo DISC impidiendo el reclutamiento de la auténtica caspasa (Krueger y cols., 2001).

1.1.3.2. Ruta de las MAPKs

Los diferentes miembros de la familia de proteínas quinasas activadas por mitógenos (MAPKs) participan en múltiples vías de señalización conservadas a lo largo de la evolución, regulando importantes procesos biológicos relacionados con la supervivencia celular y la inducción de la apoptosis. La cascada de señalización de las MAPKs puede ser activada por una gran variedad de estímulos tanto extracelulares como intracelulares, los cuales incluyen factores de crecimiento, citoquinas, hormonas, estrés oxidativo y estrés del RE, entre otros. Tras su activación, las MAPKs actúan sobre una serie de moléculas efectoras, como son otras quinasas, fosfolipasas, proteínas del citoesqueleto, y factores de transcripción, para finalmente regular distintas funciones celulares, como por ejemplo el control de la hematopoyesis (Platanias, 2003).

Los tres grupos principales de MAPKs son:

- Familia ERK (*Extracellular signal-Regulated Kinases*). Dentro de este grupo se distinguen dos miembros: ERK1 y ERK2. Estas proteínas son activadas en respuesta a ciertas citoquinas y factores de crecimiento, y producen mayoritariamente señales mitogénicas y anti-apoptóticas, por lo que se asocian con supervivencia celular. Bajo ciertas circunstancias también pueden promover la diferenciación celular (Arthur y Ley, 2013).

- Familia p38. Está compuesta por cuatro isoformas que son p38α, p38β, p38γ y p38δ. Esta familia es activada principalmente por señales de estrés, pero también en respuesta a la interacción de diferentes receptores de citoquinas con sus correspondientes ligandos. Estas quinasas son necesarias para la regulación de la apoptosis, bloqueo del ciclo celular, inducción de la diferenciación celular, así como producción de citoquinas e inflamación. La activación de esta ruta puede inducir, bajo ciertas condiciones, señales anti-apoptóticas, dependiendo del tejido y la isoforma que se active (Arthur y Ley, 2013).

- Familia JNK (*c-Jun N-terminal Kinases*). Esta familia está constituida por tres miembros: JNK1, JNK2 y JNK3. Estas proteínas son también activadas en respuesta a señales de estrés y factores de crecimiento, y están relacionadas con la inducción de apoptosis, producción de citoquinas y progresión del ciclo celular. La principal diana celular de estas proteínas es c-Jun que, junto a c-Fos, conforma el factor de transcripción AP1. De este modo, la mayoría de los efectos de JNK derivan de la expresión de los genes regulados por AP1 (Arthur y Ley, 2013).

Para que se produzca la activación de las MAPKs, éstas deben ser fosforiladas en residuos de treonina y tirosina que están situados en motivos específicos (ThrXaaTyr) para cada grupo de quinasas. Esta fosforilación está mediada por unas quinasas denominadas MAPKKs y la activación de éstas, a su vez, es llevada a cabo por otras quinasas llamadas MAPKKKs, las cuales fosforilan a las MAPKKs en residuos específicos de serina. La activación de las diferentes MAPKKKs depende de las diferentes señales recibidas (Figura V).

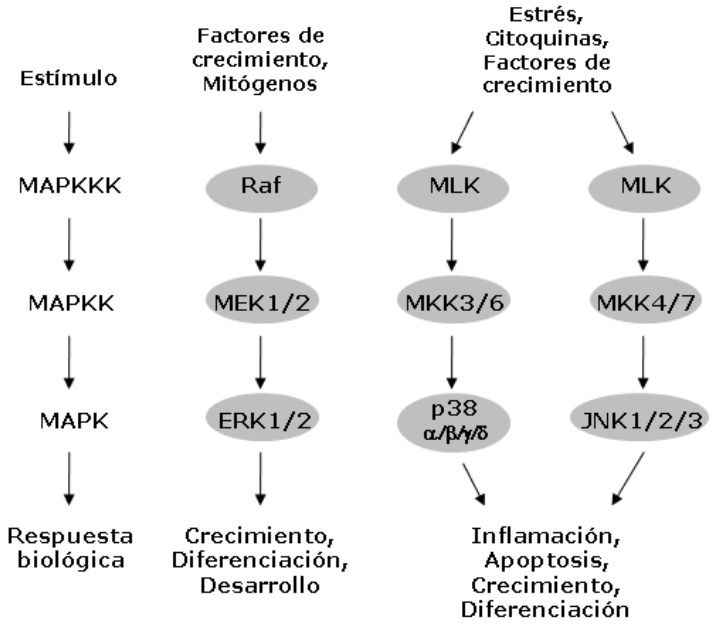

Figura V. Representación esquemática de las distintas rutas de las MAPKs.

Estudios realizados sobre las rutas de señalización de las MAPKs han demostrado que estas proteínas tienen un papel muy importante en la regulación de la apoptosis y el crecimiento de células tumorales hematopoyéticas, por lo cual, los

investigadores han tratado de caracterizar el mecanismo preciso implicado en estos fenómenos. La ruta Raf/MEK/ERK está implicada en la generación de respuestas mitogénicas encargadas de regular procesos tan dispares como la hematopoyesis fisiológica o el desarrollo de diversos tumores hematológicos (Chung y Kondo, 2011). Además, las proteínas ERK1/2 regulan la actividad y los niveles de proteínas de la familia Bcl-2 tales como la proteína pro-apoptótica Bim y la proteína anti-apoptótica Mcl-1. En concreto, ERK media la fosforilación del factor de transcripción FoxO3A, que regula la expresión de Bim, para que sea degradado por el proteosoma (Shankar y cols., 2008). En cuanto a Mcl-1, ERK fosforila a esta proteína estabilizándola y promoviendo la supervivencia de la célula tumoral (Domina y cols., 2004). Por todo ello, la ruta de señalización ERK es considerada como una prometedora diana terapéutica para el desarrollo de drogas antitumorales (Park y cols., 2010).

Las rutas de JNK y p38 parecen mediar señales responsables de la sensibilidad o resistencia a ciertos agentes farmacológicos que se utilizan en terapia antitumoral. Existen reguladores comunes de ambas rutas, de modo que diversos estímulos pueden activarlas simultáneamente. Por otro lado, los efectos de ambas vías de señalización pueden ser similares pero también antagónicos, según el contexto, existiendo una interacción entre ellas. Así, se ha demostrado que p38 puede regular negativamente la actividad JNK por diferentes mecanismos. En general, los efectos producidos por las rutas de JNK y p38 dependen del tipo celular y de la isoforma implicada en el proceso. De hecho, además de promover la apoptosis y actuar como supresores de tumores, también pueden activar fenómenos anti-apoptóticos, proliferativos, y producir señales de supervivencia bajo ciertas condiciones (Wagner y Nebreda, 2009).

Todos estos conocimientos han dado lugar al desarrollo de inhibidores específicos de las diferentes MAPKs, algunos de los cuales están siendo evaluados actualmente en la práctica clínica. Dado que la actividad ERK1/2 se encuentra aumentada en ciertos tumores, estas proteínas se han convertido en diana de fármacos inhibidores que presentan escasa o nula toxicidad en células no transformadas, como los compuestos PD-184352 o PD-98059. También se han desarrollado inhibidores específicos de JNK y de p38, algunos de los cuales han sido probados en ensayos clínicos para el tratamiento de determinados tipos de tumores (Sebolt-Leopold, 2000).

1.1.3.3. Ruta de PI3K

La ruta de supervivencia iniciada por la enzima fosfoinositol 3-quinasa (PI3K) se inicia por la unión de factores de crecimiento, como el factor de crecimiento epidérmico (EGF), a sus receptores específicos, o bien directamente a través del proto-oncogen Ras (Rodriguez-Viciana y cols., 1996). Esta ruta determina la actividad de la quinasa Akt, también conocida como proteína quinasa B (PKB), la cual se encuentra regulada en condiciones no patológicas por una tirosina fosfatasa que actúa como supresora de tumores y es conocida como PTEN (acrónimo del inglés *Phosphatase and TENsin homolog*) (Li y cols., 1997). La expresión de Akt se encuentra incrementada en un elevado número de tumores, siendo capaz de bloquear señales intracelulares apoptóticas mediante fosforilación de distintos sustratos:

- Bad, que pierde así su capacidad de unión e inhibición de Bcl-x_L (Matheny y Adamo, 2009).
- Caspasa-9, inhibiendo su activación (Cardone y cols., 1998).
- Mdm2, una ligasa de ubiquitina que se une a la proteína p53 y, por tanto, induce la degradación proteasomal de dicha proteína supresora de tumores (Mayo y Donner, 2002).
- IKK, potenciando la ruta de supervivencia mediada por el factor nuclear-kappa B (NF-κB) (Mitsiades y cols., 2004)

El hecho de que PI3K participe en un gran número de procesos celulares que afectan a la proliferación, supervivencia, e incluso a la resistencia a quimioterapia y radioterapia, ha fomentado la búsqueda de inhibidores específicos de esta ruta como potenciales agentes antitumorales. De hecho, LY-294002, un bloqueante específico de PI3K, se ha usado en combinación con otros compuestos citotóxicos en ensayos *in vivo* e *in vitro*, y se ha descrito su efecto como agente sensibilizador a la apoptosis mediada por diversos estímulos, como el ligando TRAIL (Alladina y cols., 2005).

1.1.4. Implicaciones clínicas de la apoptosis

1.1.4.1. Apoptosis y cáncer

Más del 50% de las neoplasias presentan defectos en la maquinaria apoptótica. Entre los defectos mejor caracterizados se encuentran el aumento en la expresión de algunas proteínas anti-apoptóticas de la familia Bcl-2 y mutaciones en el gen supresor de tumores *TP53*, que codifica para la proteína pro-apoptótica p53 (Vaux y cols., 1988; Strasser y cols., 1990; Vazquez y cols., 2008). Este gen, llamado el "guardián del genoma", inicia el proceso apoptótico en respuesta a daños en el ADN inducidos por radiación, agentes químicos, estrés oxidativo y otros agentes, mediante la activación transcripcional de una gran variedad de proteínas pro-apoptóticas tales como Puma, Noxa y Bax. Los defectos en el gen *TP53* heredados por la siguiente generación, como en el caso del síndrome de Li-Fraumeni, resultan en la aparición de numerosos neoplasmas, incluyendo gliomas y sarcomas (Vazquez y cols., 2008).

La mayoría de agentes quimioterapéuticos induce la apoptosis de las células tumorales (Uguz y cols., 2012). Así, el fármaco imanitib, un inhibidor de la tirosina quinasa, se usa en el tratamiento de la leucemia mieloide crónica ya que provoca la muerte de las células tumorales incrementando los niveles de las proteínas pro-apoptóticas Bim y Bad (Kuroda y cols., 2006). Por otro lado, el inhibidor selectivo de las proteínas Bcl-2 y Bcl-$_{XL}$, ABT-737, se ha mostrado altamente eficaz en el tratamiento de ciertos tumores tanto por sí sólo como en combinación con otros fármacos antineoplásicos (Lessene y cols., 2008; Cragg y cols., 2009). Asimismo, el fármaco ABT-263, un análogo del ABT-737, se está usando en ensayos clínicos para el tratamiento de algunos tipos de leucemia y como terapia coadyuvante en tumores sólidos (Cragg y cols., 2009). Otros agentes quimioterapéuticos pro-apoptóticos que se emplean en ensayos clínicos están dirigidos contra las proteínas survivina y XIAP, inhibidores endógenos de la apoptosis (Tolcher y cols., 2008).

1.1.4.2. Apoptosis y sistema inmune

Las alteraciones en el proceso de apoptosis pueden ocasionar una mayor susceptibilidad a enfermedades autoinmunes (Oliveira y Gupta, 2008). De hecho, tanto una tasa de apoptosis aumentada como una tasa de eliminación de cuerpos apoptóticos

disminuida pueden desembocar en procesos inflamatorios y deficiencia en linfocitos T reguladores, también denominados linfocitos T supresores, puesto que es una subpoblación especializada de linfocitos T que actúa inhibiendo la activación del sistema inmunitario, manteniendo así la homeostasis de este sistema y favoreciendo la tolerancia hacia autoantígenos. Por el contrario, una tasa de apoptosis disminuida puede ocasionar una eliminación de células autorreactivas defectiva, lo cual puede ocasionar enfermedades autoinmunes órgano-específicas o sistémicas como es el caso del síndrome linfoproliferativo autoinmune, una enfermedad congénita y hereditaria que se desarrolla en pacientes que muestran defectos en el ligando o en el receptor de muerte Fas (Oliveira y Gupta, 2008). Además, actualmente se sabe que, durante su desarrollo en el timo, aquellos linfocitos T que expresan receptores de reconocimiento de autoantígenos son eliminados mediante un proceso apoptótico que parece ser dependiente de la proteína pro-apoptótica Bim, ya que los linfocitos T carentes de dicha proteína son insensibles a la apoptosis inducida por la estimulación de los receptores de reconocimiento de autoantígenos (Bouillet y cols., 2002). Del mismo modo, la eliminación de los linfocitos T y B activados por antígeno durante el cese de la respuesta inflamatoria está mediada tanto por la proteína pro-apoptótica Bim como por el receptor de muerte Fas (Hughes y cols., 2008). Por último, en el caso de la diabetes de tipo I, se ha planteado que la pérdida de células β pancreáticas podría estar mediada por el receptor de muerte Fas, pues recientemente se ha comprobado que los linfocitos T que expresan el ligando Fas interaccionan con el receptor Fas presente en la membrana plasmática de las células β pancreáticas induciendo su muerte por apoptosis (Foulis, 2008).

1.1.4.3. Apoptosis y enfermedades neurológicas

Cada vez es más evidente que la apoptosis neuronal juega un papel fundamental en las alteraciones neurológicas neonatales (Ferriero, 2004), ya que las neuronas en desarrollo son particularmente vulnerables a la apoptosis en respuesta a estímulos nocivos, como la hipoxia, durante el período de sinaptogénesis (Barinaga, 2000). El síndrome alcohólico fetal, un trastorno permanente provocado por la exposición del embrión y del feto al alcohol ingerido por la madre durante el periodo de gestación, es debido a la neurodegeneración apoptótica que resulta tanto de la acción inhibitoria del

etanol sobre el receptor *N*-metil-D-aspartato (NMDA), un receptor de glutamato, como de la acción estimuladora del etanol sobre los receptores del ácido γ-aminobutírico (GABA) (Ikonomidou y cols., 2000). Análogamente, los anestésicos generales también modulan tanto los receptores NMDA como los receptores GABA y, lo que es más importante, recientes estudios en modelos animales han demostrado que los anestésicos generales inducen una extensa apoptosis neuronal en neonatos y, consecuentemente, pueden causar defectos cognitivos a largo plazo (Loepke y cols., 2009).

1.1.4.4. Apoptosis y hepatitis

Los hepatocitos son especialmente propensos a la apoptosis en respuesta a distintos tipos de estímulos, incluyendo la infección viral (Lemasters, 2005). Así, un ensayo clínico de un potente inhibidor de caspasas (IDN-6556) en pacientes con hepatitis C crónica mostró que dicho fármaco reduce significativamente los niveles séricos de las transaminasas alanina aminotransferasa y aspartato aminotransferasa, indicando una disminución concomitante en el daño celular hepático dado que dichas transaminasas son liberadas al torrente sanguíneo como consecuencia de un aumento de la permeabilidad celular debido al daño hepático (Pockros y cols., 2007).

1.1.4.5. Apoptosis y patologías cardiovasculares

El daño isquémico es debido a una obstrucción del riego sanguíneo al músculo cardíaco que causa un déficit en el suministro de oxígeno y de sustratos, lo cual provoca la muerte masiva de las células que constituyen la zona afectada del tejido cardíaco. Mayoritariamente, las células mueren por necrosis, aunque a menudo se observan células apoptóticas en las zonas hipóxicas después del daño isquémico. En este sentido, la pérdida celular se puede prevenir bloqueando la apoptosis, por ejemplo, mediante el uso de inhibidores de caspasas. Además, la ciclosporina inhibe la apoptosis bloqueando la apertura del PPT mitocondrial y, de manera interesante, es capaz de disminuir el área infartada en pacientes con infarto de miocardio agudo. De hecho, un estudio piloto llevado a cabo en 58 pacientes con infarto de miocardio agudo demostró que la administración de un bolo intravenoso de ciclosporina justo antes de la intervención percutánea coronaria fue capaz de reducir significativamente el área de tejido infartado (Piot y cols., 2008).

1.1.4.6. Apoptosis y sepsis

Se conoce como sepsis al síndrome de respuesta inflamatoria sistémica provocado por una infección, generalmente, grave. La sepsis es probablemente el contexto clínico más relevante en el que está implicada la apoptosis. De hecho, en pacientes con sepsis se produce una apoptosis masiva de células inmunes efectoras y células epiteliales gastrointestinales (Hotchkiss y cols., 1999; Hotchkiss y cols., 2000; Hotchkiss y cols., 2001). La amplia pérdida de células inmunes efectoras durante la sepsis inhibe la habilidad del sistema inmune de erradicar la infección primaria y, como consecuencia, los pacientes son más susceptibles frente a infecciones nosocomiales. En este contexto, numerosos estudios en animales han destacado la importancia de la apoptosis en el agravamiento de la sepsis, ya que la supresión de la apoptosis inducida por sepsis mejora la tasa de supervivencia (Hotchkiss y Nicholson, 2006; Ayala y cols., 2008).

1.2. Papel del calcio y las especies reactivas de oxígeno en la apoptosis

La fosforilación oxidativa mitocondrial es la principal ruta de síntesis de ATP en células eucariotas. Durante este proceso, los electrones liberados por los sustratos reductores son transportados a través de una serie de complejos que sufren reacciones de oxido-reducción hasta transferir los electrones al aceptor final, el oxígeno molecular (O_2). Esta transferencia de electrones está acoplada a la generación de un gradiente electroquímico de H^+ a lo largo de la MMI, el cual se emplea para la síntesis de ATP por el complejo V de la cadena transportadora de electrones, la ATP sintasa.

El proceso de reducción del O_2 ($O_2 \rightarrow O_2^{\bullet -} \rightarrow H_2O_2 \rightarrow {}^{\bullet}OH \rightarrow H_2O$), desde un punto de vista químico, procede mediante la generación de varias ERO, las cuales pueden dañar componentes celulares como proteínas, lípidos, y ADN. Por el contrario, se ha demostrado que las ERO mitocondriales desempeñan también un papel crucial en la homeostasis celular (Fleury y cols., 2002; Ueda y cols., 2002). Para lograr un equilibrio óptimo en el metabolismo aeróbico, la fosforilación oxidativa mitocondrial permite la reducción de O_2 a H_2O al tiempo que aumenta al máximo la síntesis de ATP y mantiene la producción de ERO sólo a los niveles requeridos para la señalización celular (Inoue y cols., 1999; Brookes y Darley-Usmar, 2002).

Además de la síntesis de ATP, las mitocondrias son los orgánulos donde tienen lugar otras importantes reacciones metabólicas, incluyendo la síntesis de porfirinas y hormonas esteroideas, el ciclo de la urea, el metabolismo de los lípidos, y la interconversión de aminoácidos (Darley-Usmar y cols., 1987). Las mitocondrias también juegan un papel central en la regulación de los niveles de insulina (Maechler, 2002) y en la homeostasis del calcio celular (Gunter y cols., 2004), el cual a su vez afecta a otra gran cantidad de vías de señalización celular.

A pesar de que las funciones de la mitocondria parecían estar completamente definidas, diferentes observaciones llevadas a cabo en la última década han impulsado un renovado interés por la investigación mitocondrial: 1) las ERO mitocondriales no son sólo productos de desecho de la respiración celular, sino también importantes mensajeros para la señalización celular (Pariente y cols., 2001; Brookes y cols., 2002; Redondo y cols., 2004; Rosado y cols., 2004); 2) la liberación de factores

mitocondriales, como el citocromo *c*, es un paso clave en la muerte celular programada (Liu y cols., 1996); 3) el óxido nítrico ($NO^{\bullet}$) es un potente regulador de la función mitocondrial (Brookes y Darley-Usmar, 2002; Brookes y cols., 2002); 4) las mitocondrias son estructuras dinámicas sujetas a eventos de fusión, fisión y movimientos intracelulares en una escala temporal de nanosegundos (Karbowski y Youle, 2003); y 5) las mitocondrias organizan activamente los perfiles espacio-temporales de calcio intracelular (Camello-Almaraz y cols., 2002; González y cols., 2003; Gunter y cols., 2004). En conjunto, todas estas observaciones sugieren que las mitocondrias ejercen un papel regulador prominente en la función celular tanto en condiciones normales como patológicas.

1.2.1. El calcio mitocondrial regula la supervivencia celular

El calcio es un importante segundo mensajero que participa en numerosas funciones celulares. Se ha sugerido que las alteraciones en la homeostasis del calcio intracelular constituyen una señal de muerte celular (Cerella y cols., 2010). En este sentido, la captación de calcio por las mitocondrias afecta a las diferentes vías de muerte celular. De hecho, la sobrecarga de calcio mitocondrial se asocia desde hace tiempo con la necrosis que ocurre en respuesta a la isquemia/reperfusión del tejido cardíaco, así como con la excitotoxicidad neuronal (Orrenius y cols., 2003). Durante la isquemia/reperfusión, la sobrecarga mitocondrial de calcio, junto con la acumulación de ERO, favorece la apertura prolongada del PPT. Este hecho causa de manera súbita el colapso del potencial de membrana y el hinchamiento o "*swelling*" de la mitocondria, con la consecuente liberación de nucleótidos de piridina y citocromo *c*. La crisis bioenergética y el agotamiento de las reservas de ATP resultantes desembocan en la muerte celular por necrosis de los cardiomiocitos (O'Rourke, 2000; Di Lisa y Bernardi, 2009).

En el caso de la excitotoxicidad neuronal, la activación de los receptores de NMDA por parte del glutamato genera un primer incremento en la concentración de calcio citosólico ($[Ca^{2+}]_c$), debido a la entrada de calcio en el citoplasma a través de dichos receptores (Nicholls, 2009; Pivovarova y Andrews, 2010). Asimismo, la

despolarización induce la apertura de los canales de calcio activados por voltaje, al tiempo que inhibe la actividad del intercambiador Na^+-Ca^{2+} de la membrana plasmática. Durante este primer aumento en la $[Ca^{2+}]_c$, las mitocondrias acumulan y retienen calcio para tamponar la carga citosólica. Sin embargo, este primer aumento de la $[Ca^{2+}]_c$ dependiente de glutamato promueve una extensa acumulación de calcio varias horas después del estímulo tóxico. Se ha demostrado que la necrosis se inicia por esta entrada retardada de calcio, la cual es independiente de la liberación de calcio desde la mitocondria pero está relacionada con un descenso en la actividad de los mecanismos de aclaramiento de calcio citosólico. Tras el inicio del proceso necrótico, las mitocondrias se sobrecargan de calcio, se produce el colapso del gradiente electroquímico de H^+ y, finalmente, se induce la muerte celular por necrosis (Bano y cols., 2005).

La fase de iniciación de la vía intrínseca de la apoptosis requiere la liberación de los componentes del apoptosoma, tales como el citocromo *c*, desde la mitocondria al citoplasma. En este proceso parece estar implicada la fragmentación mitocondrial debida a la apertura del PPT (Rasola y Bernardi, 2011). A pesar de carecer de una explicación acerca del mecanismo subyacente, numerosos estudios revelan que el principal desencadenante de la apertura del PPT es el calcio, el cual actúa en conjunto con una amplia gama de señales apoptóticas en la célula. Así, estímulos sub-apoptóticos como el estrés oxidativo o la producción de C2-ceramida, un análogo permeable de la ceramida, actúan de manera sinérgica con el calcio citosólico liberado en respuesta a un estímulo fisiológico, provocando de este modo la apertura del PPT y la muerte por apoptosis de la célula en última instancia (Szalai y cols., 1999; Jacobson y Duchen, 2002; Davidson y cols., 2012).

A pesar de que el mecanismo involucrado en la liberación de las proteínas pro-apoptóticas desde la mitocondria aún se desconoce, se ha demostrado que la apertura del PPT causa una reorganización de las crestas mitocondriales que permite la liberación del citocromo *c* (Scorrano y cols., 2002). Asimismo, se ha observado también que los incrementos en la $[Ca^{2+}]_c$ inducen modificaciones morfológicas de tipo pro-apoptótico en las mitocondrias. De hecho, la translocación de la proteína de fisión mitocondrial-1 debida a la activación de la calcineurina, una proteína dependiente de calcio, causa la fragmentación mitocondrial (Cribbs y Strack, 2007; Cereghetti y cols., 2008) y la

consiguiente liberación de citocromo *c* (Frank y cols., 2001; Martinou y Youle, 2006). Diversos estudios llevados a cabo por nuestro grupo de investigación resaltan el papel del calcio en el proceso apoptótico. Así, por ejemplo, la exposición prolongada de espermatozoides humanos a progesterona, un agonista fisiológico, conduce a la muerte celular por apoptosis mediante un proceso dependiente de la señal de calcio citosólico y de la recaptación de calcio por parte de las mitocondrias (Bejarano y cols., 2008). Del mismo modo, en la línea celular de leucemia mieloide aguda HL-60, el uracilo trifosfato (UTP) actúa como agonista capaz de movilizar calcio desde los depósitos intracelulares e induce despolarización de la membrana mitocondrial y la consecuente activación de caspasas. La atenuación de esta respuesta tras el tratamiento con BAPTA, un quelante de calcio intracelular, o rojo de rutenio, un inhibidor del uniporter de calcio mitocondrial (MCU), sugieren que dicho proceso apoptótico se inicia debido a un aumento en la concentración de calcio en la mitocondria (González y cols., 2010a). Además, se ha descrito que los quelantes de calcio reticular inducen estrés del RE y, consecuentemente, apoptosis en plaquetas humanas, un proceso dependiente de la activación de caspasas (-8, -9 y -3) así como de la externalización de fosfatidilserina (López y cols., 2009).

El papel de las señales de calcio en la apoptosis fueron ratificadas tras probarse que las proteínas anti-apoptóticas, como Bcl-2, eran capaces de reducir los niveles de calcio en el RE, y disminuir por tanto las señales citosólicas y mitocondriales de calcio en respuesta a estímulos extracelulares, debido a un aumento en la fuga de calcio del RE (Pinton y cols., 2000; Palmer y cols., 2004). Por el contrario, las proteínas pro-apoptóticas, como por ejemplo Bax y Bak, ejercen el efecto opuesto (Scorrano y cols., 2003). De manera interesante, en células T que carecen de dichas proteínas (Bax y Bak), la liberación de calcio desde el RE esta disminuída, y este fenómeno se correlaciona con una menor tasa de proliferación celular (Jones y cols., 2007). Por otro lado, la proteína anti-apoptótica Bcl-x_L interacciona directamente y sensibiliza al receptor de inositol trifosfato (IP_3R), provocando de este modo un vaciado parcial del RE que previene la captación de calcio por la mitocondria tras un estímulo, y manteniendo además la función bioenergética mediante la estimulación tónica de las deshidrogenasas de la matriz mitocondrial (White y cols., 2005). En algunos tipos

celulares o bajo ciertas condiciones experimentales, no se detecto disminución alguna en los niveles de calcio en el RE en presencia de Bcl-2 (Chen y cols., 2004; Hanson y cols., 2008), suscitando la posibilidad de que el control de la fuga de calcio del RE mediado por Bcl-2 podría depender del subtipo de IP_3R o de modificaciones post-traduccionales de los distintos IP_3R (Bassik y cols., 2004; Oakes y cols., 2005). Además, en algunos de estos estudios se mostró una reducción en la cinética de liberación de calcio desde el RE, lo cual sugiere una disminución en la captación de calcio por parte de las mitocondrias (Chen y cols., 2004; Rong y cols., 2008; 2009). En conjunto, estos resultados destacan un papel dual del calcio mitocondrial en la provisión energética de la célula y la inducción de la muerte celular.

En general, se considera que la sobrecarga de calcio mitocondrial tiene un papel fundamental en el proceso apoptótico, permitiendo que diferentes estímulos tóxicos produzcan la liberación de los cofactores de las caspasas desde el interior de la organela, lo cual resulta en la muerte celular programada (Rizzuto y cols., 2012). Además, parece ser que la alteración de esta respuesta celular, por ejemplo, mediante proteínas tumorales o virales, desempeña un papel importante en la patogénesis de diversas enfermedades (Pinton y cols., 2001; Chami y cols., 2003; Scorrano y cols., 2003; Campanella y cols., 2004). Al mismo tiempo, la prolongada apertura del PPT conlleva el colapso total del potencial de membrana mitocondrial y la liberación de calcio desde la organela, lo cual resulta en la pérdida total de la función mitocondrial y la muerte celular por necrosis. En consecuencia, la supresión genética del regulador del PPT, la ciclofilina D, reduce la necrosis inducida por sobrecargas de calcio y daño oxidativo (Baines y cols., 2005).

1.2.2. ERO mitocondriales en la regulación de la apoptosis

En condiciones fisiológicas, existe un equilibrio entre la generación de ERO y su neutralización mediante el sistema antioxidante de la célula. Sin embargo, cuando ese equilibrio se rompe y se produce un nivel elevado de pro-oxidantes en detrimento de los antioxidantes, se origina una situación de estrés oxidativo (Farrugia y Balzan, 2012). El estrés oxidativo, por lo general, supone una situación que causa un daño severo en los

principales componentes celulares y que, en última instancia, compromete la viabilidad celular (Sies, 1991; Halliwell y Cross, 1994).

Numerosas investigaciones han probado que la apoptosis va acompañada de un estallido en la producción de ERO (Castilho y cols., 1995; Green y Reed, 1998; Kowaltowski y cols., 1998; Grijalba y cols., 1999). Además, estudios llevados a cabo en mitocondrias aisladas han mostrado una compleja interrelación entre la apertura del PPT y la generación de ERO en respuesta a calcio (Castilho y cols., 1995; Green y Reed, 1998; Kowaltowski y cols., 1998). Sin embargo, todavía no se ha determinado si la producción de ERO es una simple consecuencia de la apertura del PPT y la liberación de citocromo *c* o si, por el contrario, es parte integral de la maquinaria de señalización implicada en la apertura del PPT. Aunque existen bastantes evidencias de que la adición exógena de ERO puede desencadenar la apertura del PPT (Packer y Murphy, 1994; Castilho y cols., 1995; Brookes y Darley-Usmar, 2004), no está totalmente claro si las ERO generadas endógenamente pueden desempeñar esa función. En este sentido, se ha demostrado que los antioxidantes o los agentes reductores, como el ditiotreitol (DTT), son capaces de inhibir la apertura del PPT mediada por calcio, lo cual apoya la hipótesis acerca del papel de las ERO endógenas en la regulación del PPT (Crompton, 1999). Asimismo, se ha descrito que la apertura del PPT es sensible a la concentración de O_2 (el sustrato para la generación de anión superóxido ($O_2^{\bullet -}$)) y es un proceso inhibido por acción de la catalasa, lo cual sugiere una dependencia de la generación de H_2O_2 en dicho mecanismo (Castilho y cols., 1995). Estudios llevados a cabo por nuestro grupo de investigación también ponen de manifiesto la estrecha relación entre las ERO y la apoptosis. Así, se ha observado en plaquetas humanas que el tratamiento prolongado con el agonista fisiológico trombina genera un estallido de ERO asociado a la despolarización de la membrana mitocondrial que, en última instancia, induce apoptosis. Además, todos estos eventos fueron revertidos en presencia de catalasa (López y cols., 2007). Por otro lado, en células de pancreatoma de rata AR42J, se mostró que el tratamiento con H_2O_2 exógena induce un aumento en la $[Ca^{2+}]_c$, despolarización mitocondrial, liberación de citocromo *c*, y activación de caspasa-3 a través de un mecanismo que requiere la recaptación de calcio (Morgado y cols., 2008). En espermatozoides humanos, la exposición a H_2O_2 exógena produce un aumento en la

actividad enzimática de la caspasa-3 y -9, así como la posterior externalización de fosfatidilserina, en un proceso dependiente de calcio (Bejarano y cols., 2008). Además, el H_2O_2 exógena afecta de un modo similar a la línea celular de leucemia humana HL-60, puesto que induce la despolarización de la membrana mitocondrial y la posterior activación de la caspasa-3 y -9 mediante un proceso que implica la participación del calcio (González y cols., 2010b). De manera similar, el tratamiento con TNF provoca la activación de caspasas y la consecuente muerte celular en distintas líneas de leucemia mieloide humana (HL-60 y K562), en un proceso dependiente de la señalización de calcio y la generación de ERO intracelulares (González-Flores y cols., 2014).

Teóricamente, la liberación de citocromo *c* a través del PPT provocaría un aumento en la producción de ERO debido a la inhibición del complejo III de la cadena de transporte de electrones, aunque los datos experimentales en este sentido son limitados. No obstante, parece ser que la respiración mitocondrial y la síntesis de ATP se mantienen durante la apoptosis (Waterhouse y cols., 2001), presumiblemente para permitir que se lleve a cabo el programa apoptótico, el cual requiere ATP (Nicotera y cols., 1998). Es importante resaltar que el citocromo *c* no es la única molécula liberada desde las mitocondrias tras la apertura del PPT. De hecho, unas cien proteínas son liberadas desde el espacio intermembrana (Patterson y cols., 2000), así como glutatión reducido (GSH) y otros soluto presentes en la matriz mitocondrial. Cualquiera de estos componentes, especialmente el GSH, podría ser responsable del aumento en la generación de ERO durante la apoptosis. Otra idea propuesta en este contexto es aquella que sugiere que el citocromo *c* actúa como un antioxidante, puesto que esta molécula neutraliza de manera efectiva el $O_2^{\bullet -}$, y es fácilmente difusible y reciclado en la célula (Pereverzev y cols., 2003). Este hecho suscita la posibilidad de que la liberación del citocromo *c* durante el desacoplamiento mitocondrial mediado por calcio o ERO podría haber evolucionado como una estrategia de defensa para secuestrar las ERO extramitocondriales antes de que éstas inflijan daños a las organelas.

Las mitocondrias constituyen la principal fuente de ERO dentro de la célula, pero a menudo se convierten en la diana de acción de las ERO con consecuencias fatales, como el daño oxidativo al ADN mitocondrial (ADNmt) (Orrenius y cols., 2007; Circu y cols., 2009; Rachek y cols., 2009). En este sentido, se ha postulado

recientemente que los elevados niveles de anión hidroxilo ($^{\bullet}OH$) y $O_2^{\bullet -}$ asociados al daño en el ADNmt desempeñan un papel crucial en la apoptosis (Ricci y cols., 2008), aunque el mecanismo por el cual el daño en el ADNmt media la señalización apoptótica no se conoce por completo (Circu y Aw, 2010). El ADNmt es especialmente susceptible al ataque de ERO debido a su proximidad a la cadena de transporte de electrones y a la ausencia de histonas protectoras. El daño oxidativo en el ADNmt es la principal fuente de inestabilidad genómica de la mitocondria y conlleva defectos en la respiración mitocondrial y uno de los factores más importantes en el envejecimiento (Mammucari y Rizzuto, 2010).

Por último, la oxidación e inactivación de proteínas hierro azufre (Fe-S), como las aconitasas, y la consecuente liberación de hierro y H_2O_2 es otro importante mecanismo de toxicidad inducida por $O_2^{\bullet -}$ en las mitocondrias (Orrenius y cols., 2007), ya que el H_2O_2 reacciona directamente con los lípidos de la membrana mitocondrial modificando sus propiedades estructurales y funcionales (Khawaja y cols., 2008). Además, el hierro y el H_2O_2 liberados son los ingredientes de las reacciones de Fenton y Haber-Weiss que dan lugar al radical $^{\bullet}OH$, el cual provoca modificaciones oxidativas en ADN, proteínas y lípidos mitocondriales, y amplifica así el daño iniciado por $O_2^{\bullet -}$ (Orrenius y cols., 2007).

1.3. El indol melatonina

En 1960, Lerner y colaboradores aislaron la que se considera la sustancia secretora más importante de la glándula pineal: la melatonina. Se denominó así debido a su acción blanqueadora cuando se aplicaba en algunas especies nocturnas de vertebrados inferiores. En mamíferos, no se han observado cambios diarios en el color, aunque la pineal y la melatonina se han relacionado con los cambios estacionales en la pigmentación cutánea.

La melatonina es un indol de bajo peso molecular presente de manera muy ubicua en todos los organismos vivos. A pesar de tener una estructura simple, es una molécula pleiotrópica con importantes funciones biológicas desde bacterias hasta mamíferos (Hardeland y cols., 2011). A este respecto, la melatonina ha demostrado ser un potente antioxidante y secuestrador de radicales libres (Bonnefont-Rousselot y Collin, 2010). Además, este indol está implicado en el control de los ritmos biológicos (Arendt y Skene, 2005), en la regulación de la reproducción (Reiter y cols., 2009a), y en la inmunomodulación (Guerrero y Reiter, 2002).

1.3.1. Estructura y síntesis

La melatonina es una molécula lipofílica principalmente sintetizada y secretada por la glándula pineal, un órgano de secreción interna que modula la producción y liberación rítmica de muchos compuestos bioactivos en función de la luz ambiental, como es el caso del indol melatonina (Reiter y cols., 2010). Este indol pineal se sintetiza siguiendo un patrón circadiano con niveles circulantes más elevados durante la fase oscura del fotoperiodo y mínimos durante la fase luminosa, de tal forma que mantiene informado al individuo de las variaciones luz/oscuridad (Vandewalle y cols., 2009).

La biosíntesis de melatonina (Figura VI) comienza con la captación de su precursor, el aminoácido esencial *L*-triptófano, que es tomado de la sangre por los pinealocitos, a través de un mecanismo de transporte activo que está bajo control adrenérgico (Romero y Axelrod, 1974). A continuación, el triptófano se transforma en 5-hidroxitriptófano mediante una hidroxilación llevada a cabo por la enzima triptófano-

5-hidroxilasa (TPH). Posteriormente, el 5-hidroxitriptófano es descarboxilado por la enzima 5-hidroxitriptófano descarboxilasa para producir serotonina. Los niveles de serotonina en la glándula pineal, que son de manera general mucho mayores que en el resto del cerebro, son especialmente elevados durante el día, y caen marcadamente durante la noche como consecuencia de su conversión a melatonina. Esta conversión nocturna implica un proceso enzimático de dos pasos. Inicialmente, la enzima serotonina *N*-acetiltransferasa (AA-NAT), que muestra un incremento de actividad de 30 a 70 veces mayor durante la noche, transforma la serotonina en *N*-acetilserotonina, aumentando ésta su concentración a valores entre 10 y 30 veces mayores de los que existen durante el día. Después, la enzima hidroxi-indol-O-metiltransferasa (HIOMT), que también incrementa su actividad durante la noche, metila la *N*-acetilserotonina para producir *N*-acetil-5-metoxitriptamina, más comúnmente conocida como melatonina.

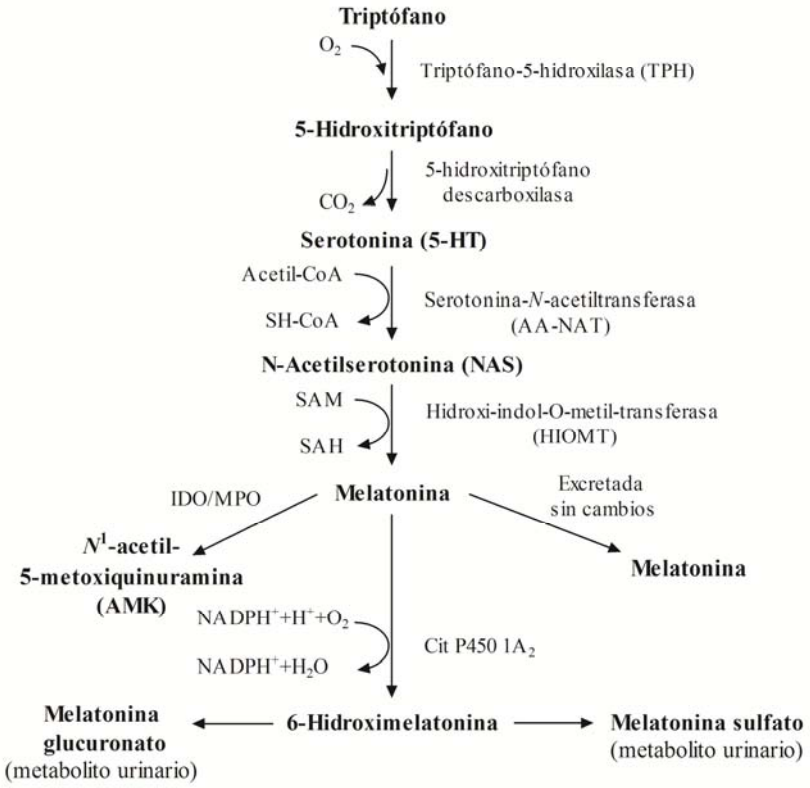

Figura VI. Ruta biosintética de la melatonina a partir del aminoácido triptófano. Acetil-CoA: acetil coenzima A; SH-CoA: coenzima A; SAM: S-adenosil metionina; SAH: S-adenosil homocisteína; IDO: indolamina 2,3-dioxigenasa; MPO: mieloperoxidasa.

La melatonina no se almacena en el lugar de síntesis, sino que se secreta directamente tanto al líquido cefalorraquídeo como al sistema cardiovascular (Reiter y cols., 2010). Además del ritmo diario secretor de esta hormona, aparecen cambios estacionales y circa-anuales que la implican como un factor central en la regulación de los procesos rítmicos (Reiter y col., 2010). Así, si se invierten las condiciones de luz ambiental se invierten de modo paralelo las actividades enzimáticas y la biosíntesis pineal de la indolamina. Debido a su alto grado de solubilidad en lípidos, una gran cantidad de melatonina se libera a la circulación a medida que se sintetiza, probablemente a través de un mecanismo de difusión pasiva, aunque existen pruebas de la existencia de secreción pulsátil en alguna especie.

La síntesis de este indol no es exclusiva de la glándula pineal, sino que también existe producción de melatonina en la retina y el hipocampo (Cogburn y cols., 1987; Grace y cols., 1991), el cuerpo ciliado del iris (Aimoto y cols., 1985), la glándula lacrimal (Mhatre y cols., 1988), la glándula de Harder (Vakkuri y cols., 1985), el tejido ovárico (Itoh y cols., 1999), las plaquetas (Launay y cols., 1982), las células mononucleares periféricas (Finocchiaro y cols., 1995), las células de la médula ósea (Conti y cols., 2000), el aparato digestivo (Tan y cols., 1999), el sistema inmune (Barriga-Ibars y cols., 2005), y el sistema portal hepático (Huether y cols., 1992). Sin embargo, a diferencia de la melatonina sintetizada en la glándula pineal, la melatonina producida por estos tejidos no pasa a la sangre en cantidades significativas sino que funciona localmente como una señal autocrina o paracrina (Pandi-Perumal y cols., 2006), siendo los niveles tisulares de melatonina cientos de órdenes de magnitud mayores que los sanguíneos (Reiter y Tan, 2003). Además, la melatonina también ha sido identificada en el reino vegetal, pues son muchas las plantas comestibles (Manchester y cols., 2000), frutas (González-Gómez y cols., 2009) y plantas medicinales (Reiter y Tan, 2002) en las que se han encontrado cantidades detectables de melatonina.

La vida media de la melatonina circulante varía entre 20-40 minutos (Boutin y cols., 2005), lo que indica que es una hormona de acción rápida. Transcurrido este tiempo, la mayor parte de la melatonina circulante es inactivada mediante su conversión hepática en 6-hidroximelatonina y es excretada en la orina en forma de sulfatos (75%) o

glucurónidos (5%) (Alonso, 1999). En la glándula pineal y cerebro también es posible su transformación en quinureninas, en presencia de la indolamina 2,3-dioxigenasa (Hirata y cols., 1974), o su transformación de forma no enzimática mediante la reacción con $^{\bullet}OH$, $O_2^{\bullet -}$, o H_2O_2 (Tan y cols., 1998a; Tan y cols., 2000). Entre las quinureninas destacan la N^1-acetil-N^2-formil-5-metoxi-quinurenamina (AFMK) y la N^1-acetil-5-metoxi-quinurenamina (AMK). Estas quinureninas, al igual que la melatonina, presentan importantes efectos como secuestradoras de radicales libres (Reiter y cols., 2008).

1.3.2. Mecanismos de acción

Una de las funciones mejor conocidas de la melatonina es quizás su habilidad de modular los ritmos circadianos y las respuestas estacionales. Sin embargo, numerosas investigaciones han destacado su papel en la regulación de otras muchas funciones fisiológicas y bioquímicas (Pandi-Perumal y cols., 2013). Algunas de estas acciones parecen depender de la potente capacidad antioxidante y secuestradora de radicales libres que posee la melatonina. No obstante, los mecanismos de acción de la melatonina en mamíferos también pueden ser mediados por la interacción de ésta con proteínas intracelulares, como la calmodulina, o por su unión a receptores específicos (Ekmekcioglu, 2006). Por este motivo, tanto los agonistas como los antagonistas de los receptores de melatonina son compuestos muy útiles para definir los múltiples mecanismos mediante los cuales la melatonina regula los procesos fisiológicos y patológicos. Desde 1994, cuando Reppert y colaboradores identificaron por primera vez la expresión de un receptor de alta afinidad para la melatonina en melanocitos de *Xenopus laevis* (Ebisawa y cols., 1994), se han descrito, clonado y estudiado en profundidad distintos receptores de melatonina tanto de membrana como citosólicos y nucleares.

Los receptores MT1 y MT2, anteriormente designados como Mel1a y Mel1b, son dos clases de receptores de membrana plasmática para la melatonina identificados en diversos tejidos humanos (sistema nervioso central, tracto gastrointestinal, intestino, piel, glándula adrenal, gónadas, riñón, corazón, vasos sanguíneos, tejido adiposo,

neutrófilos, linfocitos y tejidos linfoides) (Dubocovich y Markowska, 2005). Además, su expresión se ha demostrado en distintas líneas tumorales de diferente origen (Chan y cols., 2002; Imbesi y cols., 2008; Ramracheya y cols., 2008). Los receptores MT1 y MT2 pertenecen a una superfamilia de receptores acoplados a proteínas G constituidos por siete dominios transmembrana, que presentan un alto grado de homología en su secuencia de aminoácidos, y que difieren principalmente en su distribución y localización. La activación de estos receptores promueve la disociación de las proteínas G en los dímeros Gα y Gβγ, e inhibe la enzima adenilato ciclasa, reduciendo de este modo los niveles de adenosín monofosfato cíclico (AMPc) y activando la fosfolipasa C (PLC) (Dubocovich y Markowska, 2005). Asimismo, la activación del receptor MT1 incrementa la fosforilación de MEK1/2 y ERK1/2 (Pala y cols., 2013), potencia la producción de ATP y prostaglandina F2α (Barrett y cols., 2003), y regula las respuestas funcionales de la melatonina modulando los niveles intracelulares de calcio (Vanecek, 1998). Por su parte, el receptor MT2 se asocia con la activación de las mismas vías de señalización, aunque parece estar mucho más relacionado con la inhibición del nucleótido guanosín monofosfato cíclico (GMPc) que con la inhibición de AMPc (Dubocovich y Markowska, 2005).

Aparte de los citados receptores de membrana, también se ha identificado un receptor putativo, denominado MT3, en órganos de hámster. Dicho receptor presenta menor afinidad por la melatonina (rango nanomolar) y una rápida cinética de asociación/disociación (Nosjean y cols., 2000). Además, esta proteína presenta un 95% de homología con la quinona oxidorreductasa 2 (NQO2) humana, una enzima involucrada en la detoxificación de radicales libres, toxinas y metales pesados. No obstante, el papel fisiológico de este receptor MT3 no se conoce aún por completo (Volkova y cols., 2012).

Debido a su habilidad de cruzar las membranas lipídicas, la melatonina es capaz de alcanzar directamente el núcleo, mediando sus acciones a través de un grupo de receptores nucleares hormonales, llamado ROR/RZR (acrónimo del inglés *Retinoid Orphan Receptors/Retinoid Z Receptors*) (Becker-Andre y cols., 1994). La estructura de esos receptores consiste de un dominio N-terminal, una secuencia de unión a ADN, y una región de unión al ligando en el dominio C-terminal (Becker-Andre y cols., 1994).

Los diversos receptores ROR/RZR se distribuyen de un modo diferente dependiendo del subtipo al que pertenecen. Así, el receptor RZRβ se encuentra principalmente en los tejidos neurales, mientras que el receptor RZRα se distribuye más ampliamente y podemos encontrarlo en tejido adiposo, piel, testículos, cartílago, e hígado (Kobayashi y cols., 2003). Aunque la aparente interacción directa de la melatonina con estos receptores nucleares es un tema controvertido, el descubrimiento de una vía de señalización nuclear para la melatonina ha contribuido enormemente a la comprensión de los efectos tan variopintos y profundos que ostenta este indol. Por tanto, la activación de estas vías nucleares puede inducir la unión de la melatonina a regiones específicas de ciertos genes promoviendo de este modo la transcripción de genes diana involucrados en supervivencia, proliferación y diferenciación celular (Sánchez-Barceló y cols., 2003). Además, algunos estudios establecen una correlación entre los receptores nucleares para la melatonina y las funciones de la melatonina en el sistema inmune (Steinhilber y cols., 1995). De hecho, a través de su interacción con el receptor RORα, la melatonina reprime la expresión de la enzima 5-lipooxigenasa en linfocitos B, una enzima clave en las reacciones alérgicas e inflamatorias (Steinhilber y cols., 1995).

Por otro lado, los mecanismos de acción de la melatonina pueden ser mediados por la interacción de ésta con proteínas intracelulares, como por ejemplo la calmodulina (Benítez-King y cols., 1993). Ésta es una interacción de baja afinidad que permite la regulación de la actividad de algunas proteínas quinasas. Mediante este mecanismo, la melatonina puede inhibir la actividad de la calmodulina quinasa II (Benítez-King y cols., 1996) y de la óxido nítrico sintasa neuronal (nNOS) (Pozo y cols., 1997), así como participar en la regulación del citoesqueleto (Benítez-King y Antón-Tay, 1993), aunque en esta última función también pueden están involucrados los receptores de membrana (Benítez-King y cols., 2009). Por último, se ha descrito que la calreticulina, una proteína residente del RE que une calcio, parece constituir un sitio de unión de alta afinidad para la melatonina que puede estar involucrado en algunas funciones de la indolamina, incluyendo la regulación genómica (Macías y cols., 2003).

Adicionalmente, la melatonina posee acciones que no dependen de la interacción con sus receptores debido a su capacidad de secuestrar radicales libres y moléculas reactivas relacionadas. El mecanismo por el cual la melatonina detoxifica los radicales

libres es la donación de electrones (Tan y cols., 2002). Las acciones relacionadas con su capacidad antioxidante únicamente requiere la proximidad de la melatonina al radical libre en el lugar y el momento en que éste es formado. Moléculas como el ADN nuclear, las proteínas y los lípidos son protegidas del estrés oxidativo gracias a la ubicua distribución de la melatonina. En concreto, se ha demostrado que la melatonina es capaz de neutralizar $^{\bullet}OH$, anión peroxinitrito ($ONOO^{-}$), $NO^{\bullet}$, singlete de oxígeno ($^{1}O_2$), $O_2^{\bullet -}$, H_2O_2, y ácido hipocloroso (HClO). Asimismo, ciertos metabolitos formados tras la interacción de la melatonina con los radicales libres, tales como AFMK y AMK, conservan una alta capacidad antioxidante y secuestradora de radicales libres (Galano y cols., 2013), algo que se conoce como cascada antioxidante de la melatonina (Tan y cols., 2002). Además de estas acciones, la melatonina incrementa la eficiencia del transporte electrónico entre los complejos de la cadena respiratoria mitocondrial, uno de los principales focos de generación de ERO dentro de la célula, evitando así la formación de ERO a este nivel (Hardeland, 2005). Adicionalmente, la melatonina actúa indirectamente como antioxidante ya que estimula la actividad de una gran cantidad de enzimas antioxidantes, como la catalasa o la superóxido dismutasa, que convierten los radicales libres en moléculas inocuas (Rodríguez y cols., 2004). Por último, la melatonina estimula la síntesis de glutatión, un importante antioxidante intracelular (Urata y cols., 1999), y presenta también efectos sinérgicos potenciando la capacidad de antioxidantes clásicos, como las vitaminas C y E (Tan y cols., 2013).

1.3.3. Melatonina y apoptosis

En 1994, Maestroni y colaboradores describieron por vez primera el papel que ejerce la melatonina como regulador del proceso de apoptosis. Desde entonces, la investigación en torno a este campo ha crecido enormemente y se ha centrado principalmente en tres categorías: el papel de la melatonina en la inhibición de la apoptosis de células inmunes; el papel de la melatonina en la prevención de la muerte celular neuronal; y, finalmente, el papel de la melatonina en la inducción de apoptosis en células tumorales. Sin embargo, los mecanismos por los que la melatonina regula el programa apoptótico y si su papel en la apoptosis depende de la línea celular o del

estado oxidativo de la célula son dos cuestiones importantes que aún permanecen sin resolver.

1.3.3.1. Efectos anti-apoptóticos de la melatonina

La apoptosis es un mecanismo fisiológico primordial para la homeostasis tisular, de manera que una desregulación del proceso apoptótico puede conducir a desórdenes hiperproliferativos (por una disminución en la tasa de apoptosis), como por ejemplo el cáncer, o hipoproliferativos (por un aumento en la tasa de apoptosis), como son las enfermedades neurodegenerativas y autoinmunes (Thompson, 1995). Además, el proceso apoptótico desempeña un papel crucial en las respuestas inmune e inflamatoria, ya que regula la tasa de maduración de las células B y T, y prolonga la viabilidad de aquellas células inmunes presentes en los focos de inflamación (Wesche-Soldato y cols., 2007; Bolitho y cols., 2007). En este sentido, investigaciones relativamente recientes han demostrado que los leucocitos poseen toda la maquinaria enzimática necesaria para sintetizar melatonina a partir de triptófano, así como los receptores de membrana para la indolamina, lo cual pone de manifiesto la implicación de la melatonina en las respuestas inmune e inflamatoria (Carrillo-Vico y cols., 2006). Y lo que es más importante, varios estudios han destacado el control que ejerce la melatonina sobre la apoptosis de las células inmunes, lo cual se considera una de las funciones no neurológicas más sorprendentes de esta indolamina (Miller y cols., 2006). Así, por ejemplo, los primeros estudios en esta área revelaron que la melatonina reduce la fragmentación de ADN inducida por glucocorticoides en timocitos en cultivo (Sainz y cols., 1995). El posible mecanismo para explicar el papel inhibitorio de la melatonina en la apoptosis inducida por glucocorticoides parece ser una reducción en los niveles de expresión del receptor de glucocorticoides, tal y como se demostró en timo y tiomocitos en cultivo (Sainz y cols., 1999). Posteriores estudios señalaron que la melatonina, además de inhibir la fragmentación de ADN, también impide la liberación del citocromo *c* desde la mitocondria al citoplasma en timocitos de ratón tratados con dexametasona. De hecho, la melatonina puede inhibir la vía mitocondrial de la apoptosis, presumiblemente a través de la regulación de los niveles de la proteína pro-apoptótica Bax (Hoijman y cols., 2004).

Aparte de estos estudios, diversos resultados han documentado la capacidad de la melatonina de modular la apoptosis inducida experimentalmente mediante una amplia gama de agentes farmacológicos. Así, la indolamina actúa como agente anti-apoptótico en células inmunes (Sainz y cols., 1995, 1999) y tejidos periféricos (Nava y cols., 2000; Meki y cols., 2001), y previene la muerte celular neuronal en distintos modelos de párkinson (Mayo y cols., 1998a; Chuang y Chen, 2004), alzhéimer (Pappolla y cols., 1997; Lahiri y cols., 2004) y daño cerebral por isquemia-reperfusión (Cheung, 2003; Pei y Cheung, 2003). Los mecanismos mediante los cuales la melatonina inhibe la apoptosis parecen estar relacionados con sus propiedades antioxidantes y como secuestrador de radicales libres. Por ejemplo, la melatonina atenúa el daño por isquemia/reperfusión disminuyendo el daño oxidativo mitocondrial mediante la activación de la vía de señalización JAK2/STAT3, la cual está implicada en la prevención del daño miocárdico por isquemia/reperfusión (Yang y cols., 2013). Adicionalmente, la melatonina ejerce acciones anti-apoptóticas en células epiteliales de la retina disminuyendo la producción de ERO a nivel de la mitocondria y evitando, en última instancia, daños en el ADN mitocondrial y la liberación del citocromo *c* al citoplasma (Fu y cols., 2012).

El reciente descubrimiento de que las mitocondrias constituyen una diana para la melatonina ha abierto nuevas perspectivas en relación a los mecanismos de acción de la indolamina (Acuña-Castroviejo y cols., 2002). De hecho, la melatonina regula directamente la homeostasis mitocondrial (Martín y cols., 2000; 2002a), lo cual podría explicar el efecto protector de esta indolamina en desórdenes como el párkinson, el alzhéimer, la epilepsia, la sepsis e, incluso, el envejecimiento; todos ellos debidos a una disfunción mitocondrial como causa primaria o secundaria (Acuña-Castroviejo y cols., 2001). En este sentido, se ha sugerido que los efectos anti-apoptóticos de la melatonina podrían explicarse por una interacción directa de la indolamina con el PPT de la mitocondria (Andrabi y cols., 2004). Además, estudios más minuciosos han puesto de manifiesto que los efectos inhibitorios de la melatonina sobre el PPT están probablemente relacionados con la habilidad de la indolamina de prevenir la peroxidación de la cardiolipina (Paradies y cols., 2010; Jou, 2011; Peng y cols., 2012), un lípido que se encuentra de forma exclusiva en la membrana mitocondrial interna y

cuya oxidación parece desencadenar la permeabilización de la MME, quizá, a través de su interacción con proteínas pro-apoptóticas de la familia Bcl-2 (Kagan y cols., 2004; Jiang y cols., 2008).

Por otro lado, diferentes estudios experimentales han revelado que la melatonina es capaz de promover rutas de supervivencia celular como otro mecanismo de modulación del proceso apoptótico. De este modo, se ha sugerido que la melatonina puede ejercer sus efectos citoprotectores a través de diferentes vías dependientes de las MAPKs. Por ejemplo, se ha observado que la activación de la ruta de supervivencia Raf/MEK/ERK es necesaria para que la melatonina contrarreste la apoptosis inducida por luz ultravioleta en células inmunes (Luchetti y cols., 2009; 2010) o por H_2O_2 exógena en espermatozoides humanos (Espino y cols., 2010a; 2011a). Igualmente, las propiedades neuroprotectoras de la melatonina en cultivos primarios de astrocitos son mediadas a través de la activación de la vía de señalización de PI3K/Akt (Kong y cols., 2008). Además, el papel neuroprotector de la indolamina también parece estar relacionado con la activación de la ruta de las sirtuinas, desacetilasas de histonas involucradas en vías de supervivencia y protección celular (Tajes y cols., 2009). Finalmente, se ha observado que la administración de melatonina reduce el daño inflamatorio en ratas con colitis inhibiendo la activación de NF-κB (Li y cols., 2005), así como en ratas con una pancreatitis aguda inducida por ceruleína (Carrasco y cols., 2013).

1.3.3.2. Efectos pro-apoptóticos de la melatonina

En células tumorales, la melatonina presenta dos efectos principales: inhibición de la proliferación celular y activación de la apoptosis. Aunque en determinados tipos de cáncer la melatonina puede producir ambos efectos, se asume de manera general que la melatonina, a bajas concentraciones (en el rango nanomolar y normalmente referidas como concentraciones "fisiológicas"), sólo podría ejercer una acción citostática; mientras que los efectos apoptóticos se observan a menudo a concentraciones más elevadas (Bizarri y cols., 2013; Rodríguez y cols., 2013).

Las bajas concentraciones de melatonina pueden inhibir de manera efectiva el crecimiento celular en un amplio rango de líneas celulares, aunque también se han

documentado algunas excepciones. Además, algunas líneas tumorales presentan baja o nula sensibilidad a concentraciones de melatonina en el rango nanomolar, mientras que la misma línea celular responde a mayores concentraciones de la indolamina reduciendo su tasa de proliferación. Así, tumores de próstata tanto sensibles (LNCaP) como insensibles (PC3) a andrógenos no responden a concentraciones de melatonina en el rango nanomolar, pero concentraciones milimolar inducen una significativa inhibición del crecimiento (Sainz y cols., 2005). Un patrón similar se he demostrado para la línea celular de glioma de rata C6 (Martín y cols., 2006), para las células de glioblastoma humano A172 (Sánchez-Sánchez y cols. 2011), así como para el cáncer de ovario de hámster chino (Sainz y cols., 2003a) o el cáncer de colon (Farriol y cols., 2000).

De manera general, el efecto citotóxico de la melatonina a concentraciones del rango milimolar se ha observado tan sólo en un conjunto restringido de líneas tumorales. Sin embargo, este comportamiento no puede ser tomado como una regla, dado que la apoptosis puede ser inducida a concentraciones de melatonina en el rango nanomolar en líneas tumorales que previamente habían sido consideradas como insensibles a las bajas concentraciones de melatonina. De hecho, se ha demostrado que la melatonina, a bajas concentraciones (10^{-9} M), puede reducir de manera efectiva la viabilidad de células de cáncer de mama (MCF-7), activando una respuesta apoptótica bifásica (Cucina y cols., 2009; Proietti y cols., 2011). Asimismo, Jung-Hynes y colaboradores descubrieron que la melatonina, a través de la inhibición de la sirtuina-1, causa una reducción sustancial de la viabilidad de células de cáncer de próstata, tanto *in vivo* como *in vitro*, incluso a bajas concentraciones de la indolamina (Jung-Hynes y cols., 2011). Estos resultados son de suma importancia, puesto que proporcionan una base convincente para explicar los efectos antineoplásicos ejercidos *in vivo* por la melatonina a concentraciones fisiológicas, tal y como se ha recogido mediante estudios clínicos (Vijayalaxmi y cols., 2002) y epidemiológicos (Davis y Mirick, 2006).

A pesar de que la inhibición del crecimiento de células tumorales por parte de la melatonina es un fenómeno bastante aceptado, los mecanismos que regulan la apoptosis dependiente de melatonina en el cáncer aún deben ser esclarecidos. No obstante, las evidencias acerca de las vías de muerte activadas por la melatonina están aumentando notablemente en la actualidad (Rodríguez y cols., 2013). En relación a los diferentes

cánceres hematológicos, la melatonina induce apoptosis en células de linfoma de Burkitt mediante un mecanismo que implica la activación de caspasa-3, liberación del citocromo *c*, y desregulación de la proteína anti-apoptótica Bcl-2 (Trubiani y cols., 2005). Un efecto similar se ha observado en células de leucemia mieloide aguda tratadas con melatonina. En este caso particular, la indolamina incrementa significativamente la apoptosis, lo cual está asociado con una elevada liberación de citocromo *c*, activación de caspasas (-3 y -9) y Bax, así como una reducida expresión de Bcl-2 (Rubio y cols., 2007; Bejarano y cols., 2009). Por otro lado, Casado-Zapico y colaboradores llevaron a cabo un estudio con una amplia gama de tumores hematológicos (linfoma, leucemia mieloide aguda y crónica, y leucemia linfoide aguda). En estas células, la melatonina causa un pronunciado aumento en la activación de la caspasa-8 y su sustrato molecular, la proteína pro-apoptótica Bid, que además está relacionado con un aumento paralelo en la expresión del receptor Fas y su ligando, FasL. Todo esto sugiere que la vía extrínseca se activa de manera preferente durante la muerte celular inducida por la melatonina, al menos en determinados cánceres hematológicos (Casado-Zapico y cols., 2011). No obstante, estudios más recientes han observado que la melatonina induce apoptosis en células leucémicas Molt-3 mediante la activación de la vía intrínseca, sin participación de la caspasa-2, la caspasa-8 o la proteína Bid (Perdomo y cols., 2013). Además, Sánchez-Hidalgo y colaboradores han referido que la melatonina provoca un bloqueo en la fase G1 del ciclo celular y una reducción simultánea en la proporción de células en las fases S y G2/M, así como un aumento sustancial en la tasa de apoptosis a través de la activación de la caspasa-3 y el consiguiente procesamiento de la poli ADP ribosa polimerasa (PARP) en una amplia gama de tumores hematológicos (Sánchez-Hidalgo y cols., 2012).

Por otro lado, los efectos antitumorales de la melatonina se han descrito en tumores mamarios positivos para el receptor de estrógenos. De este modo, se ha observado que la melatonina, a concentraciones del rango nanomolar, induce una respuesta apoptótica bifásica en células MCF-7 (Cucina y cols., 2009). La apoptosis temprana inducida por la indolamina es un proceso independiente de caspasas fundamentalmente activado por la proteína AIF. Por el contrario, la apoptosis tardía (inducida tras 96 horas de tratamiento con melatonina) es el resultado de una vía de

señalización más compleja, que implica una desregulación en el ratio Bcl-2/Bax así como la activación de caspasas iniciadoras (-9 y -7) y el consiguiente procesamiento de PARP. Además, parece ser que la proteína p53 se activa durante la apoptosis temprana inducida por la melatonina, mientras que la proteína p73, el homólogo de p53, está involucrado en la apoptosis tardía dependiente de caspasas. Todos estos resultados fueron confirmados posteriormente en un estudio que mostraba que la apoptosis tardía inducida por la metonina en células MCF-7 estaba además asociada a la activación de la vía del factor de crecimiento transformante beta-1 (TGFβ-1), una proteína implicada en el control de la diferenciación y la proliferación (Proietti y cols., 2011). Aparte de esto, investigaciones recientes indican que la indolamina también muestra propiedades pro-apoptóticas en líneas celulares de cáncer de mama insensibles a estrógenos (Wang y cols., 2012). De hecho, en células MDA-MB-231 (negativas para el receptor de estrógenos), las dosis farmacológicas de melatonina inhiben significativamente la expresión de la ciclooxigenasa-2 (COX-2) y la producción de prostaglandina E2, reducen drásticamente la actividad histona acetiltransferasa del cofactor p300 y la acetilación mediada por p300 del factor nuclear NF-κB y, por tanto, bloquean la unión y reclutamiento de NF-κB al promotor de COX-2. Asimismo, la melatonina inhibe marcadamente la fosforilación de las proteínas PI3K, Akt y la glucógeno sintasa quinasa-3 (GSK-3), inactivando de este modo la vía de señalización PI3K/Akt. En última instancia, la melatonina desencadena la activación de Apaf-1 y la consiguiente cascada de caspasas (Wang y cols., 2012).

En cuanto al cáncer de próstata, se ha demostrado que la indolamina activa el receptor MT1 para inhibir la proliferación de las células 22Rv1 (insensibles a andrógenos) mediante la sobreexpresión de la proteína p27Kip1, un inhibidor de ciclinas dependientes de quinasas implicado en la regulación del ciclo celular, a través de la activación simultánea de las vías de señalización de PKA y PKC (Tam y cols., 2007). La estimulación de la apoptosis por acción de la melatonina también ha sido descrita en células de cáncer de próstata sensibles a andrógenos (LNCaP), lo cual implica la activación de la caspasa-8 y la reducción en el ratio Bcl-2/Bax (Joo y Yoo, 2009; Kim y Yoo., 2010). En relación al cáncer de hígado, es muy probable que los efectos pro-apoptóticos de la melatonina estén mediados por diferentes vías de

señalización, incluyendo la regulación positiva del factor de transcripción FoxO3A que induce la activación de la proteína pro-apoptótica Bim (Carbajo-Pescador y cols., 2012), la inhibición de la expresión de COX-2, la reducción de los niveles de Bcl-2, o la elevación de los niveles del factor de transcripción pro-apoptótico CHOP (Zha y cols., 2012). Distintos estudios han evaluado el papel de la melatonina como inductor de apoptosis en cánceres de páncreas experimentales. Leja-Szpak y colaboradores (2010) encontraron que la indolamina, a concentraciones fisiológicas, presentaba acciones pro-apoptóticas en las células de cáncer de páncreas humano PANC-1 mediante la modulación del ratio Bcl-2/Bax y la activación de la caspasa-9. En contraposición, González y colaboradores (2011) describieron que la melatonina, a dosis en el rango milimolar, actuaba como inductor de apoptosis mediante una activación de la caspasa-3 dependiente de calcio en células de cáncer de páncreas de rata AR42J. Por último, en osteosarcoma, la melatonina incrementa la acetilación de la proteína p53 mediante la regulación negativa de la sirtuina-1. La acetilación de p53 evita su degradación proteasomal y activa la vía intrínseca de la apoptosis aumentando la expresión de Bax y citocromo *c*, y reduciendo los niveles de Bcl-2 (Cheng y cols., 2013).

Finalmente, puesto que la melatonina a altas concentraciones presenta propiedades antioxidantes, varios trabajos de investigación han intentado establecer una relación entre el efecto pro-apoptótico de la indolamina en células tumorales y el estado oxidativo de la célula. Frente a lo esperado, se observó un aumento temprano en el estrés oxidativo en aquellas células cancerosas donde la melatonina es capaz de inducir apoptosis, además del aumento tardío de oxidantes intracelulares asociado al proceso apoptótico en sí mismo. Así, Osseni y colaboradores determinaron que tan sólo 45 minutos después del tratamiento con melatonina (concentraciones superiores a 100 μM) se produjo un incremento en la producción de ERO y un descenso simultáneo en la viabilidad de células HepG2 (Osseni y cols., 2000). Por su parte, Buyukavci y colaboradores encontraron una correlación entre el incremento en la producción de ERO y la inducción de apoptosis por acción de la melatonina en varias líneas de cánceres hematológicos (Buyukavci y cols., 2006). También usando células leucémicas (HL-60 y K562), Bejarano y colaboradores observaron que el aumento en la generación de ERO dos horas después del tratamiento con melatonina (1 mM) se correspondía con el

descenso en la viabilidad celular y la activación de la caspasa-9 y la caspasa-3. Todos estos cambios fueron prevenidos por antioxidantes como el trolox, la catalasa, el glutatión o la N-acetilcisteína (Bejarano y cols., 2011b). Del mismo modo, Casado-Zapico y colaboradores mostraron un incremento en la producción de ERO previo a distintos eventos apoptóticos en diferentes líneas de cánceres hematológicos (HL-60, K562 y CA46) tratadas con melatonina (1 mM). En concreto, observaron un incremento en los niveles de distintos receptores de muerte y sus ligandos, así como activación de caspasas tanto de la vía extrínseca como intrínseca (Casado-Zapico y cols., 2011). Los mismos autores obtuvieron resultados similares en varias líneas celulares de sarcoma de Ewing, mostrando además que el uso de antioxidantes prevenía la producción de ERO y el descenso en la viabilidad celular (García-Santos y cols., 2012).

2. Justificación y Objetivos

La apoptosis es un modo de muerte celular activa y fisiológica, codificada genéticamente, y con potenciales implicaciones patológicas. Este modo de muerte celular constituye un mecanismo relevante en diversos procesos fisiológicos del sistema inmunitario como los implicados en el desarrollo de la tolerancia linfocitaria y en el control de la expansión clonal tras estimulación antigénica. Las alteraciones en la tasa de apoptosis de los leucocitos se han relacionado con el desarrollo de diferentes procesos patológicos, tanto de naturaleza inflamatoria como neoplásica (Oliveira y Gupta, 2008).

Tradicionalmente, se han descrito dos vías generales en el proceso apoptótico. Una es conocida como vía extrínseca y se activa por la unión de un ligando de muerte extracelular, como puede ser FasL, a su receptor de muerte en la superficie celular, como es el receptor Fas (Ashkenazi y Dixit, 1998). La segunda vía se conoce como vía intrínseca y está mediada por alteraciones mitocondriales. Así, en respuesta a estímulos apoptóticos, diferentes proteínas son liberadas desde el espacio intermembrana de la mitocondria al citoplasma (Green y Reed, 1998). Entre las proteínas mejor caracterizadas se encuentra el citocromo *c*, involucrado en la activación de la caspasa-9 (Li y cols., 1997), lo cual dispara una cascada de activación de caspasas que incluye la caspasa-3 que, a su vez, promueve la autodestrucción de la célula.

La hormona pineal melatonina es conocida por regular los ritmos circadianos y estacionales en mamíferos (Tan y cols., 1993), pero pruebas más recientes sugieren que está implicada en otros procesos importantes tales como la protección de leucocitos humanos y otros tipos celulares contra la apoptosis inducida por daño celular (Luchetti y cols., 2006; Radogna y cols., 2008). Algunos resultados indican que la denominada vía intrínseca podría representar la diana principal de la melatonina para modular el proceso apoptótico en células sanas (Radogna y cols., 2008; Espino y cols., 2010a), así como en líneas tumorales y modelos *in vivo* (Feng y Zhang, 2004; Acuña-Castroviejo y cols., 2007; Bejarano y cols., 2009; 2011b). De hecho, existen numerosas evidencias que establecen una relación entre la melatonina y la mitocondria, incluyendo las propiedades anti-apoptóticas de la indolamina a través de su interacción con el PPT (Andrabi y cols., 2004; Jou, 2011). No obstante, la melatonina también puede ejercer sus acciones anti-apoptóticas mediante la interacción con sus receptores específicos,

incluyendo los receptores de membrana MT1 y MT2 (Das y cols., 2010; Espino y cols., 2011a).

Por otra parte, el calcio es un regulador esencial para la supervivencia celular, puesto que la concentración de calcio citosólico libre es un factor regulador primordial para un gran número de procesos celulares como la contracción muscular, el metabolismo, la secreción, o incluso la diferenciación celular y la apoptosis. Sin embargo, la elevación sostenida de la concentración de calcio intracelular desempeña un papel en la muerte celular (Demaurex y Distelhorst, 2003). Los efectos pro-apoptóticos del calcio están mediados por un diverso rango de factores sensibles al calcio que están compartimentarizados en diferentes organelas intracelulares, incluyendo el RE y las mitocondrias (Hajnoczky y cols., 2003). La sobrecarga de calcio en la mitocondria puede inducir apoptosis, tanto estimulando la liberación de factores promotores de la apoptosis desde el espacio intermembrana de la mitocondria al citoplasma, como alterando la función mitocondrial (Wang, 2001).

Conforme a lo expuesto, el **Objetivo General** de la presente Tesis Doctoral ha sido estudiar los mecanismos implicados en las propiedades anti-apoptóticas de la melatonina en células inmunocompetentes no tumorales, como son los leucocitos humanos, valorando la participación de los receptores de membrana de la melatonina en dichos mecanismos, así como la acción directa de la indolamina a través de su capacidad antioxidante.

Este Objetivo General ha sido desglosado a su vez en los siguientes **Objetivos Específicos**:

1. Evaluar el papel de la melatonina en la apoptosis de leucocitos humanos inducida por sobrecargas de calcio intracelular con tapsigargina o *N*-formil-metionil-leucil-fenilalanina (fMLP), analizando la inducción de la apertura del PPT, la liberación del citocromo *c* desde la mitocondria, así como la activación de la caspasa-9, la caspasa-3 y la proteína pro-apoptótica Bax.

2. Determinar si las acciones protectoras de la melatonina en la apoptosis de leucocitos humanos inducida por aumentos sostenidos en la $[Ca^{2+}]_c$ dependen de la capacidad antioxidante de la indolamina.

3. Investigar la implicación de los receptores de membrana de la melatonina en el papel protector ejercido por la indolamina en la apoptosis de leucocitos humanos inducida por el ligando de muerte TNF-α.

4. Establecer los efectos de la melatonina sobre la apoptosis y el estrés oxidativo en leucocitos humanos procedentes de individuos mayores de 65 años.

2. Rationale and Objectives

Apoptosis is a form of active, genetically encoded and physiological cell death with potential pathological implications. This mode of cell death is an important mechanism involved in various physiological processes of the immune system such as development of lymphocyte tolerance and control of clonal expansion following antigen stimulation. Interestingly, the alterations in the rate of leukocyte apoptosis have been implicated in the development of several pathological processes, of both inflammatory and neoplastic nature (Oliveira and Gupta, 2008).

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 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.

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 healthy cells (Radogna *et al*., 2008; Espino *et al*., 2010a), as well as in tumor cell lines and *in vivo* models (Feng and Zhang, 2004; Acuña-Castroviejo *et al*., 2007; Bejarano *et al*., 2009; 2011b). In fact, increasing evidence for a melatonin–mitochondria relationship includes the anti-apoptotic properties of the indoleamine through its interaction with the mitochondrial permeability transition pore (Andrabi *et al*., 2004; Jou, 2011). Nevertheless, melatonin may also exert its anti-apoptotic actions by binding specific receptors/interactors, such as the plasma membrane receptors MT1/MT2 (Das *et al*., 2010; Espino *et al*., 2011a).

Moreover, calcium is a key regulator of cell survival since cytosolic free-calcium concentration 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 pro-apoptotic 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 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).

Based on the above, the **General Objective** of this PhD Thesis was to study the mechanisms involved in the anti-apoptotic properties of melatonin in immunocompetent non-tumor cells, evaluating the involvement of melatonin membrane receptors in these mechanisms and the direct action of the indoleamine through its antioxidant capacity.

This General Objective has been broken down in turn in the following **Specific Objectives**:

1. To evaluate the role of melatonin in human leukocyte apoptosis induced by thapsigargin or *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), analyzing the induction of the mitochondrial permeability transition pore, the release of mitochondrial cytochrome *c*, and the activation of caspase-9, caspase-3 and Bax.

2. To determine whether the protective actions of melatonin on human leukocyte apoptosis induced by sustained cytosolic calcium concentration increases depend on the antioxidant capacity of the indoleamine.

3. To investigate the involvement of melatonin membrane receptors on the protective role exerted by the indoleamine on human leukocyte apoptosis induced by the death ligand TNF-α.

4. To establish the effects of melatonin on apoptosis and oxidative stress in human leukocytes from aged individuals.

3. Materiales y Métodos y Resultados

PREÁMBULO

Los materiales y métodos empleados así como los resultados obtenidos durante el desarrollo experimental de esta Tesis Doctoral se exponen a continuación en el formato de las publicaciones a las que han dado lugar. Con esta forma de exponer los resultados se pretende aportar una mayor concisión y fidelidad al trabajo experimental desarrollado. Además de la discusión que contiene cada artículo, la cual favorece la interpretación de cada objetivo de investigación planteado, hemos incluido, tras el apartado de resultados, una discusión general de nuestra investigación con la finalidad de dar una visión más completa de nuestros resultados.

PREFACE

The materials and methods used and the results obtained during the experimental development of this PhD Thesis are presented below in the format of the publications in which they are collected. The aim of displaying the results in this way is to provide brevity and fidelity to the experimental work developed. Despite the fact that each article has its own discussion, we have decided to include, after the results section, a general discussion of our research in order to give a more comprehensive view of our results.

3.1. La melatonina reduce la apoptosis inducida por sobrecargas de calcio en leucocitos humanos: Evidencias para la implicación de la mitocondria y la activación de Bax.

3.1. Melatonin reduces apoptosis induced by calcium signaling in human leukocytes: Evidence for the involvement of mitochondria and Bax activation.

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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,

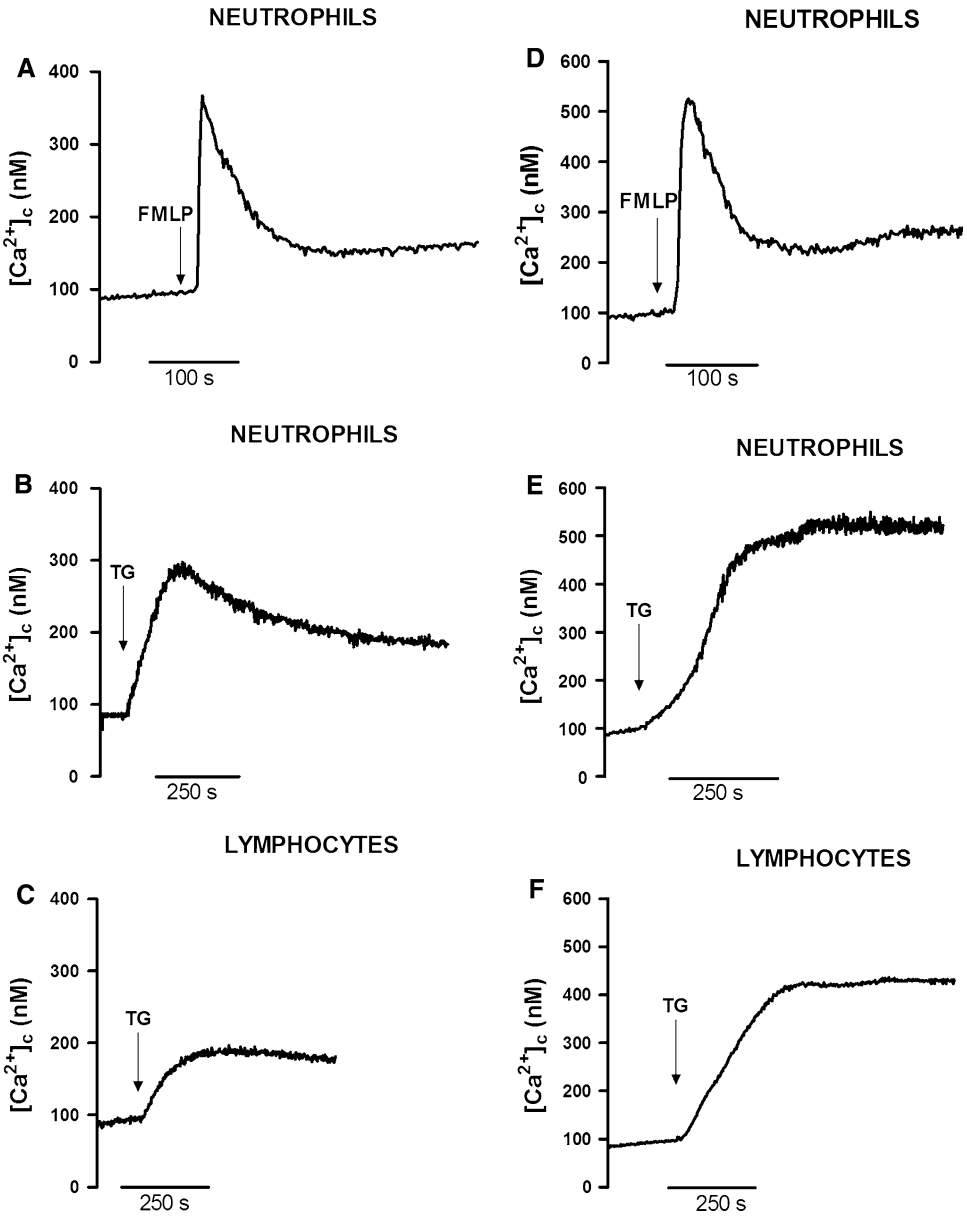


Fig. 1 Mobilization of calcium in human leukocytes. Fura-2-loaded human leukocytes were stimulated with 10 nM FMLP (**a**, **d**) or 1 μM thapsigargin (TG) (**b, c, e** and **f**), as indicated, in a calcium-free medium ($[Ca^{2+}]_o = 0 + 1$ mM EGTA was added) (**a–c**) or in the presence of normal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

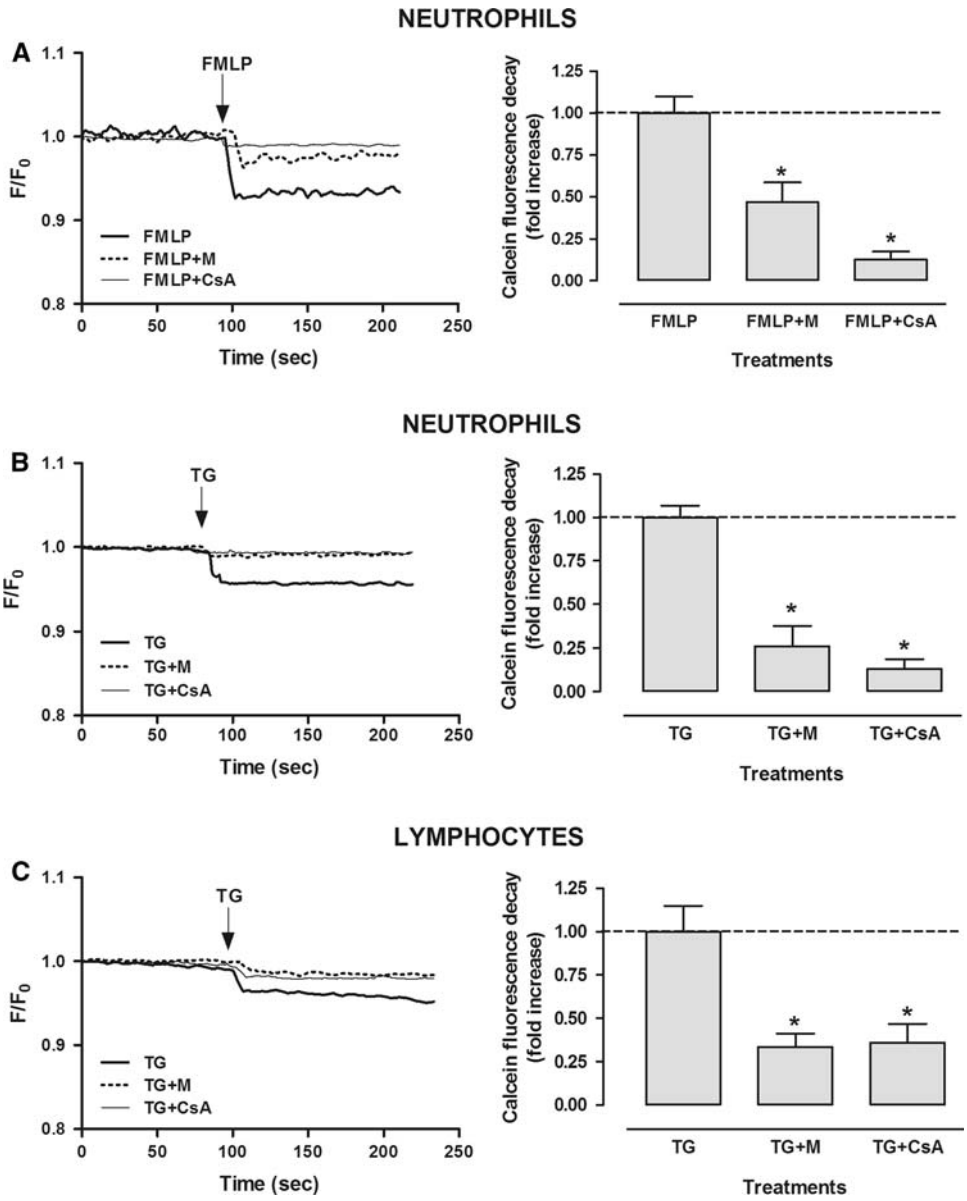


Fig. 2 Effect of melatonin on mPTP opening in human leukocytes. Calcein-loaded cells in the presence of 1 mM $CoCl_2$ were preincubated with 1 mM melatonin (M) for 60 min or 50 μM CsA for 30 min or the vehicle, then stimulated with 10 nM FMLP (**a**) or 1 μM thapsigargin (TG) (**b**, **c**). Changes 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

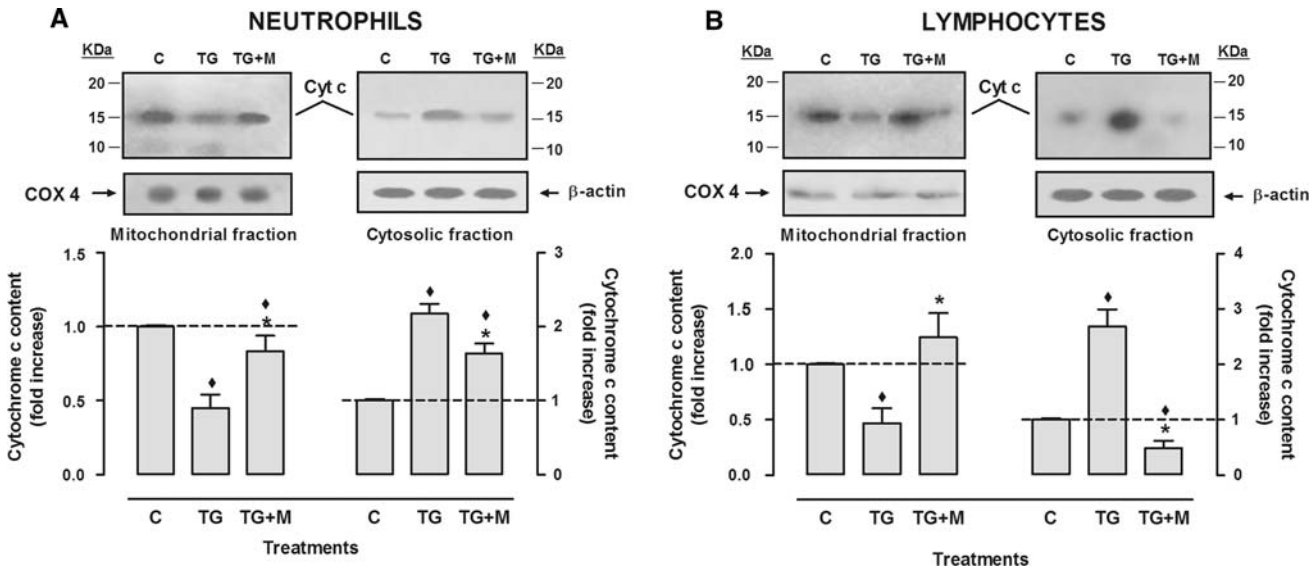
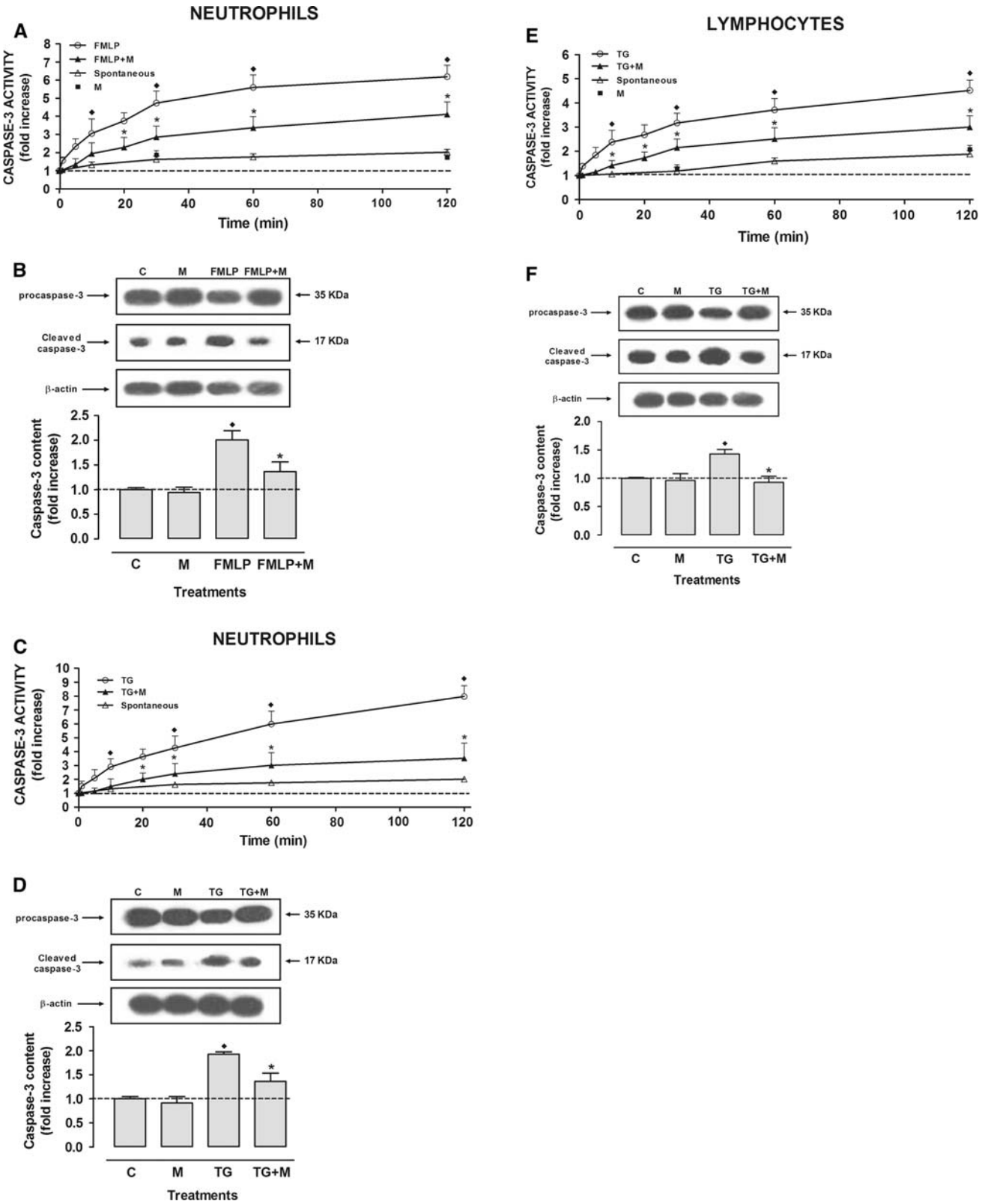


Fig. 3 Melatonin protects against thapsigargin-induced cytochrome *c* release. Human neutrophils (**a**) and lymphocytes (**b**) were preincubated with 1 mM melatonin (M) or vehicle for 60 min, then stimulated with 1 μM thapsigargin (TG) for 120 min. The cytochrome *c* content in the cytosolic and mitochondrial 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.

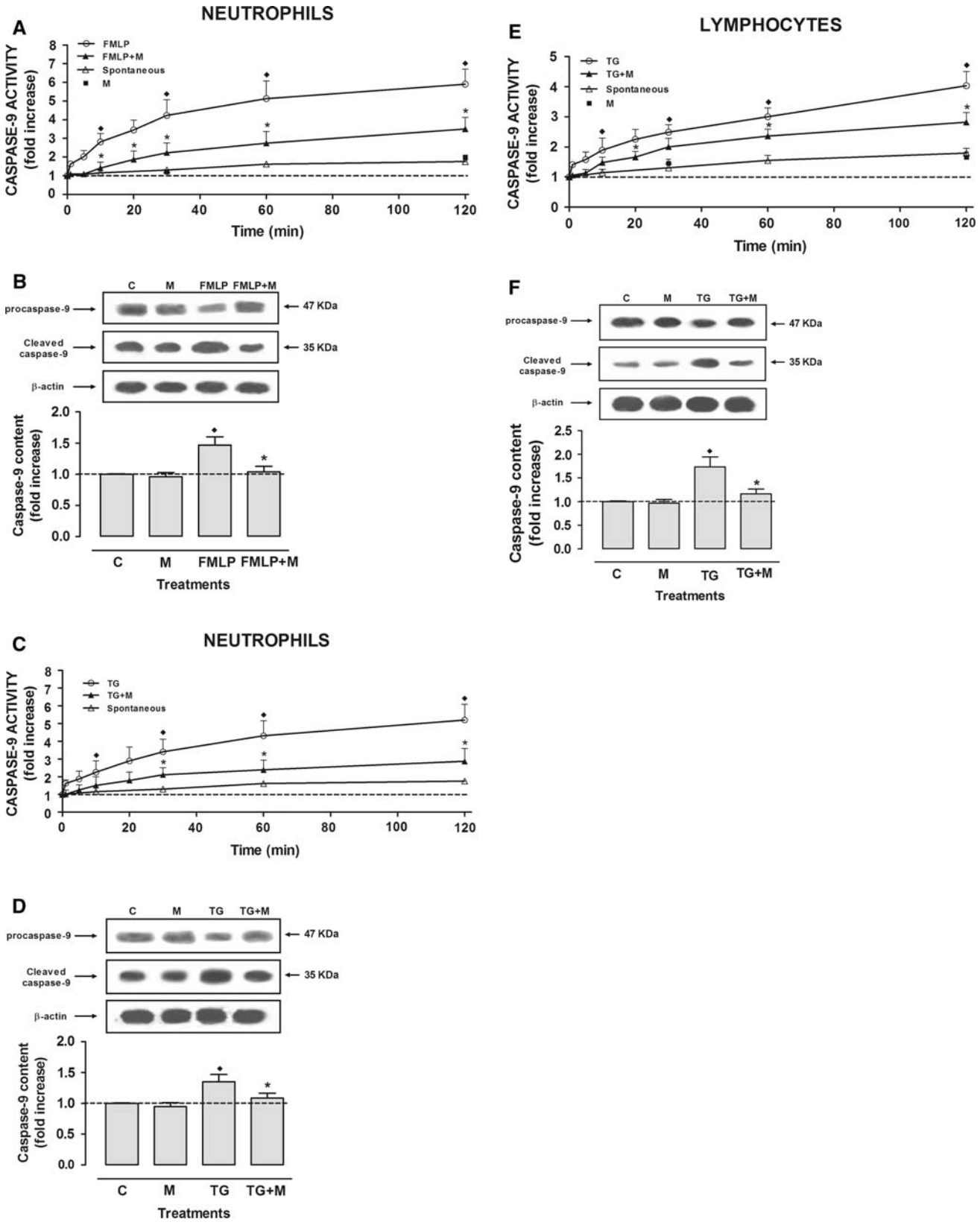
Finally, in order to identify possible intracellular apoptotic targets of calcium signaling during the promotion of apoptosis in human leukocytes, we investigated the activation of Bax after treatment with thapsigargin. Active Bax was detected by immunoprecipitation with the anti-Bax antibody (clone 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

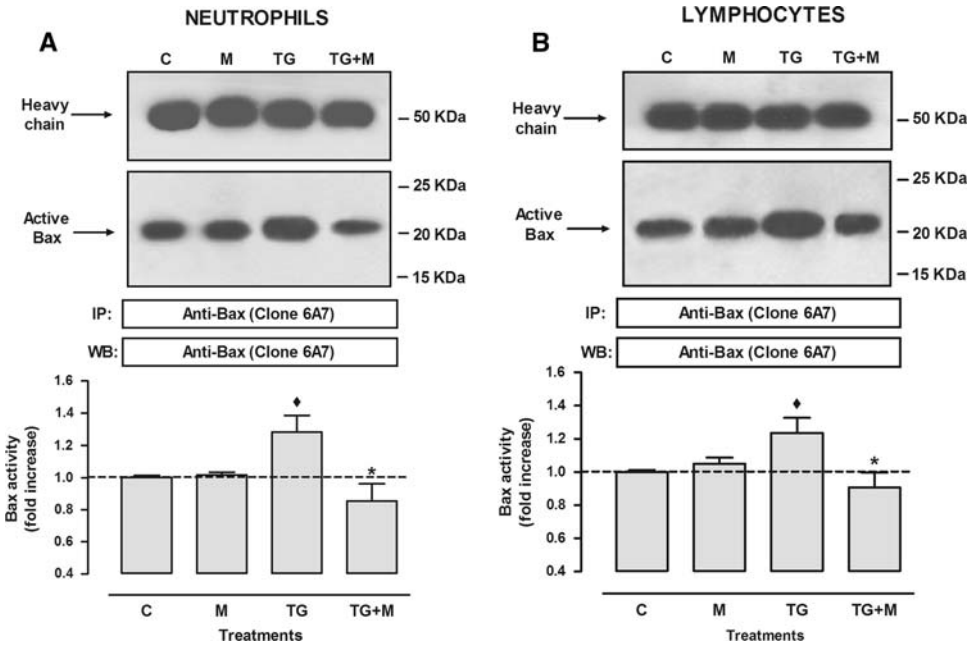


Fig. 6 Melatonin reduces Bax activation induced by thapsigargin. Human neutrophils (**a**) and lymphocytes (**b**) were preincubated with 1 mM melatonin (M) or vehicle for 60 min. Subsequently, cells were stimulated with 1 μM thapsigargin (TG) for 120 min and then lysed. Samples were immunoprecipitated 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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3.2. Efecto protector de la melatonina frente a la apoptosis de leucocitos humanos inducida por sobrecargas de calcio intracelular: relación con sus acciones antioxidantes.

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

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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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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

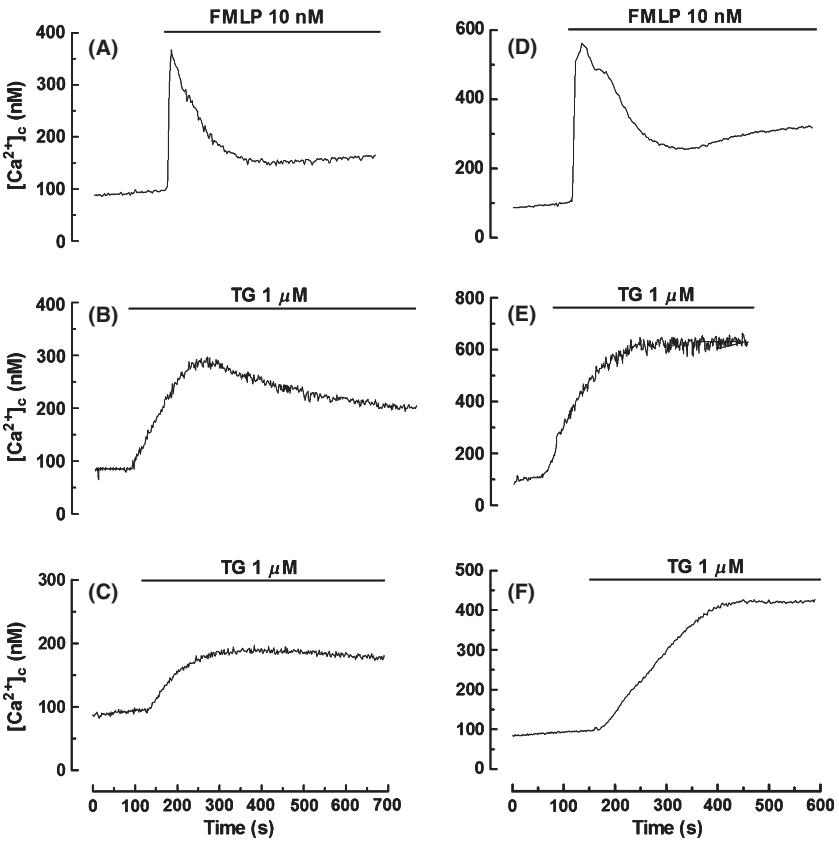

Fig. 1. Calcium mobilization in human leukocytes. Fura-2-loaded human leukocytes were stimulated with 10 nM *N*-formyl-methionyl-leucyl-phenylalanine (panels A and D) or 1 μM thapsigargin (panels B, C, E and F), as indicated, in a calcium-free medium ($[Ca^{2+}]_o$ = 0 mM + 1 mM EGTA was added; panels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

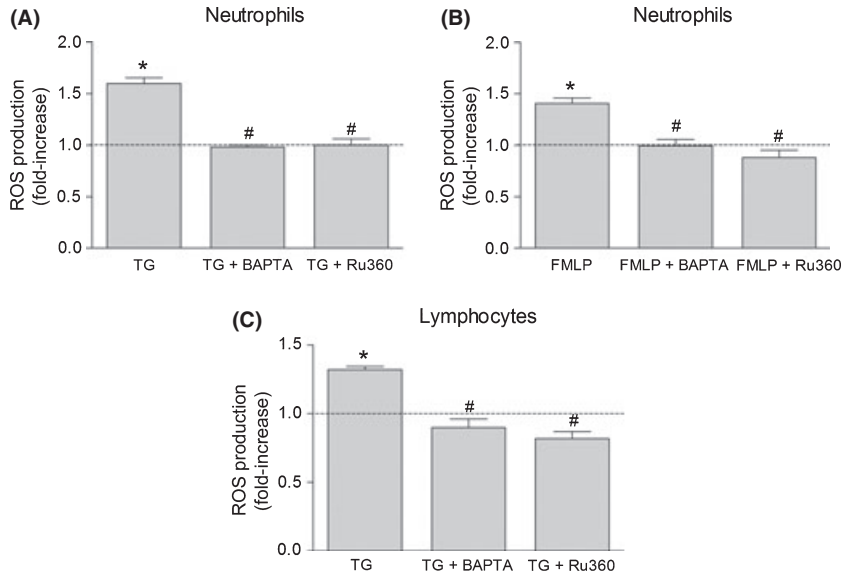

Fig. 2. Blockade of calcium signaling affects intracellular reactive oxygen species (ROS) production. Human leukocytes were preincubated with the intracellular calcium chelator dimethyl 1,2-bis(o-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) (10 μM) or the specific blocker of calcium 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

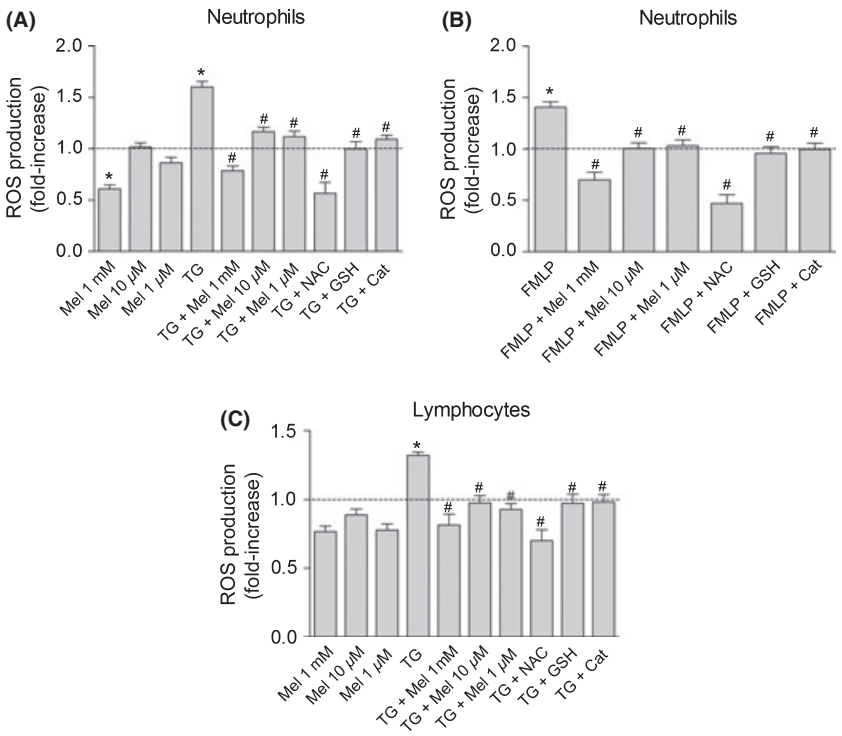

Fig. 3. Melatonin and other antioxidants antagonize intracellular reactive oxygen species (ROS) production induced by calcium signaling. Human leukocytes were alternatively preincubated with different doses of melatonin (Mel), 15 mM *N*-acetyl-L-cysteine, 5 mM reduced glutathione, 100 u/mL polyethylene 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).

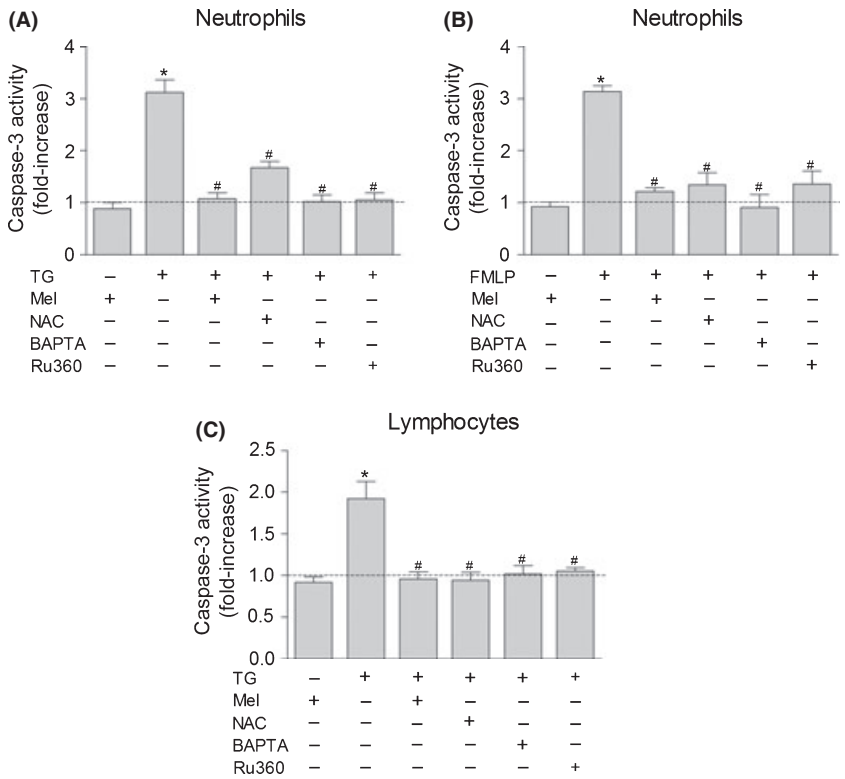

Fig. 4. Melatonin attenuates intracellular calcium overload-induced caspase-3 activation. Human leukocytes were preincubated with 1 μM melatonin (Mel) or 15 mM *N*-acetyl-L-cysteine for 1 hr, 10 μM dimethyl BAPTA or 10 μM Ru360 for 30 min, or the vehicle, and then stimulated with 1 μM thapsigargin (TG) (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,

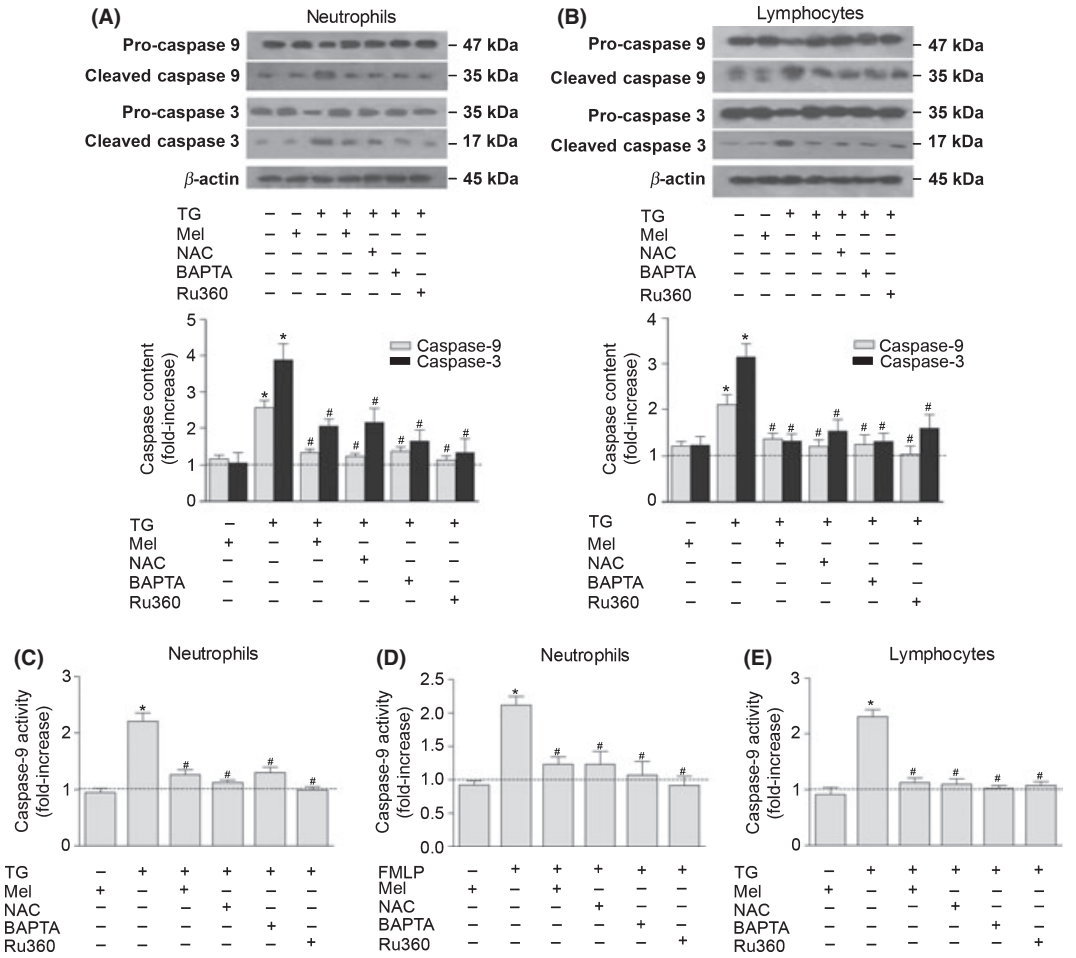

Fig. 5. Melatonin counteracts the stimulatory effect of intracellular calcium overload on the content of active caspases. To determine caspase content (A and B), human neutrophils (A) and lymphocytes (B) were preincubated as described in Fig. 4 and then stimulated with 1 μM thapsigargin (TG) for 1 hr. 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

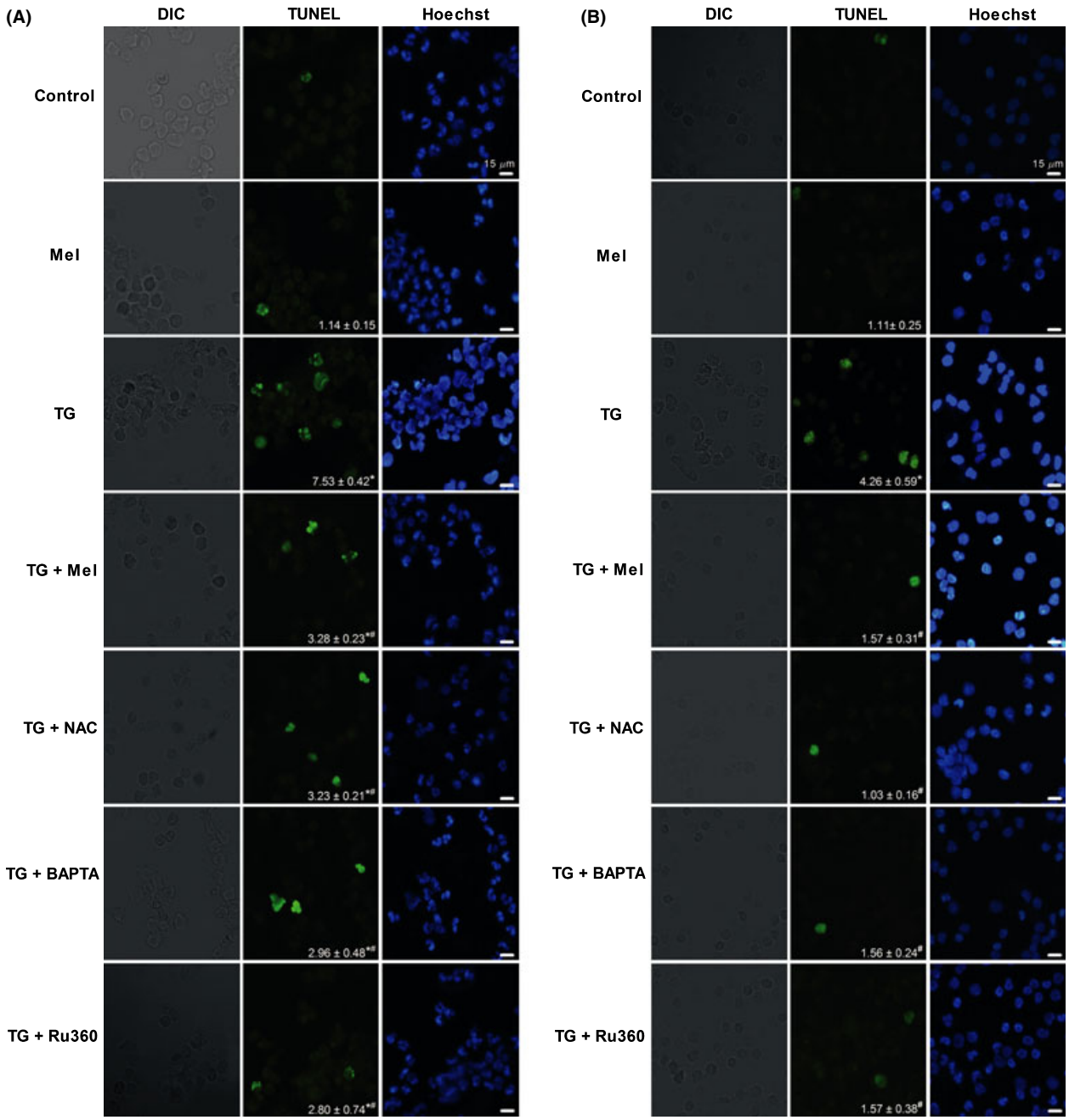

Fig. 6. Melatonin prevents cell death in human leukocytes. Neutrophils (A) and lymphocytes (B) were preincubated with 1 μM melatonin (Mel) or 15 mM *N*-acetyl-L-cysteine for 1 hr, 10 μM dimethyl BAPTA or 10 μM Ru360 for 30 min, or the vehicle, and then stimulated with 1 μM thapsigargin (TG) for 1 hr 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

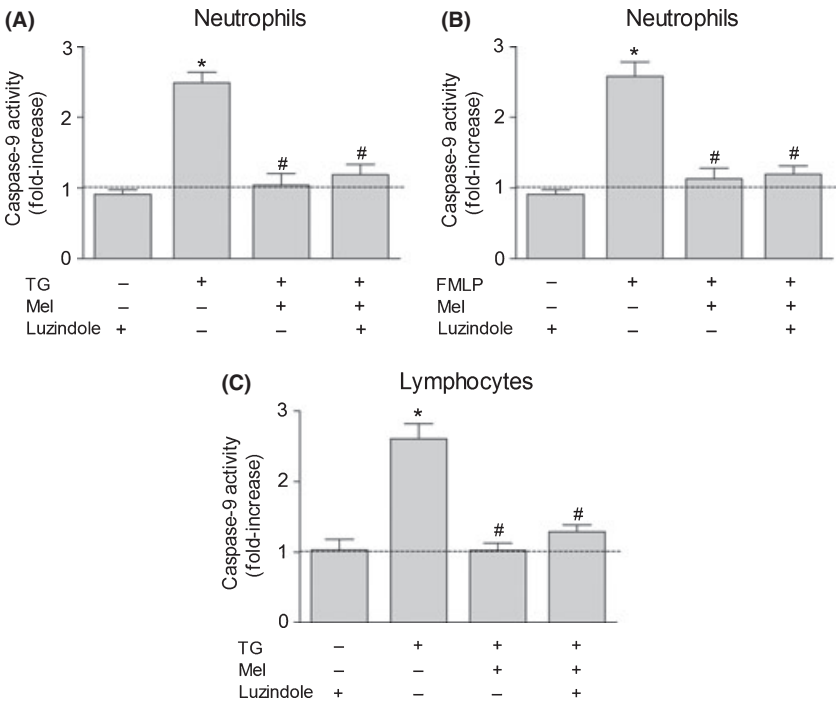

Fig. 7. The protective effect of melatonin on mitochondrial apoptosis is receptor independent. Human leukocytes were preincubated with 1 μM melatonin (Mel) for 1 hr in the absence or the presence of its MT1/MT2 receptor antagonist luzindole and then stimulated with 1 μM thapsigargin (TG) (A and C) or 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.

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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.

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3.3. La inhibición de la apoptosis de leucocitos inducida por TNF-α por acción de la melatonina requiere su interacción con los receptores de membrana MT1/MT2.

3.3. The inhibition of TNF-α–induced leucocyte apoptosis by melatonin involves membrane receptor MT1/MT2 interaction.

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The inhibition of TNF-α-induced leucocyte apoptosis by melatonin involves membrane receptor MT1/MT2 interaction

Abstract: The pro-apoptotic signalling cascades induced by tumour necrosis factor-alpha (TNF-α) have been intensively studied in multiple cellular systems. So far, it is known that TNF-α can simultaneously activate survival and apoptotic cell death responses. The balance between these signals determines the ultimate response of the cell to TNF-α. Moreover, emerging evidence suggests that melatonin may be involved in the protection of different cell types against apoptosis. Thus, the objective of this study was to evaluate the effect of melatonin on TNF-α-induced apoptosis in human leucocytes. Cells were treated with TNF-α alone or in the presence of cycloheximide (CHX), which promotes caspase-8 activation by eliminating the endogenous caspase-8 inhibitor, c-FLIP. Treatment with TNF-α/CHX led to apoptotic cell death, as ascertained by annexin V/propidium iodide (PI) staining. Likewise, in the presence of CHX, TNF-α stimulation produced cFLIP down-regulation and subsequent caspase-8 activation, thus directly triggering caspase-3 activation and causing Bid truncation and subsequent caspase-9 activation. Conversely, pre-incubation of cells with melatonin inhibited TNF-α-/CHX-evoked leucocyte apoptosis. Similarly, pretreatment of leucocytes with melatonin increased cFLIP protein levels, thereby preventing TNF-α-/CHX-mediated caspase processing. Blockade of melatonin membrane receptor MT1/MT2 or extracellular signal-regulated kinase (ERK) pathway with luzindole or PD98059, respectively, abolished the inhibitory effects of melatonin on leucocyte apoptosis evoked by TNF-α/CHX. In conclusion, the model proposed by these findings is that the MT1/MT2 receptors, which are under the positive control of melatonin, trigger an ERK-dependent signalling cascade that interferes with the anti-apoptotic protein cFLIP modulating the cell life/death balance of human leucocytes.

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Key words: cell death, leucocytes, melatonin, MT receptor, protein kinases, tumour necrosis factor-α

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Introduction

Tumour necrosis factor-alpha (TNF-α) is a pleiotropic cytokine produced by macrophages, cytotoxic T lymphocytes and natural killer (NK) cells as an effector molecule in the immune response. TNF-α is involved in multiple cellular responses, ranging from inflammatory cytokine production, cell survival, cell proliferation and, paradoxically, cell death [1]. Unsuitable TNF-α signalling has been implicated in the pathogenesis of many human diseases, such as cancer, sepsis, diabetes and autoimmune diseases. Thus, therapeutic strategies using soluble receptors and neutralising antibodies to inhibit TNF signalling have become frontline therapies in the last decade [2].

Although two different types of transmembrane TNF receptors have been identified, only TNFR1, which is ubiquitously expressed on all eukaryotic cells, is known to mediate apoptosis through its cytoplasmic death domain. This domain sequentially interacts with TNFR1-associated death domain (TRADD), Fas-associated death domain protein (FADD) and procaspase-8 [3], which in turn becomes activated (caspase-8) and mediates characteristic morphological and biochemical changes of death receptor-triggered apoptosis. TRADD, however, can also bind to TNFR-associated factor 2 (TRAF2) and receptor-interaction protein, thereby activating various transcription factors, including nuclear factor-kappa B (NF-κB), which regulates the expression of genes involved in cell survival and inflammation [4].

Interestingly, it has been previously reported that a number of cells are resistant to TNF-α-induced cell-killing when exposed to the cytokine alone [5]. However, when cells are treated with TNF-α in the presence of a protein synthesis inhibitor, such as cycloheximide (CHX) or actinomycin D, most cells become susceptible to TNF-α-stimulated apoptosis [6, 7], thereby suggesting a critical role for protein synthesis. It is now clear that TNF-α activates the transcription factor NF-kB, which controls the expression of anti-apoptotic proteins involved in cell survival, for example, Bcl-x_L, XIAP and cFLIP, among others [8–10]. Consequently, it seems that the suppression of synthesis of these proteins is needed to sensitise most cells to TNF-α-triggered apoptosis [11].

Melatonin (*N*-acetyl-5-methoxytryptamine) is a highly conserved molecule found in organism from unicells to vertebrates [12]. Chemically, melatonin and its metabolites can function as endogenous free radical scavengers and broad-spectrum antioxidants [13–15]. Regarding its therapeutic potential, melatonin modulates the immune response [16, 17], possesses antiproliferative effect on tumour cells [18, 19] and displays cytoprotective properties in normal cells [20–23]. Most beneficial effects resulting from melatonin administration seem to be linked to the protective actions of melatonin on mitochondrial physiology [24–26]. Nevertheless, melatonin may also exert its anti-apoptotic actions by binding specific receptors/interactors, such as the plasma membrane receptors MT1/MT2 [27, 28], thereby eliciting specific signal transduction pathways. Emerging evidence demonstrates that the control of leucocyte apoptosis is one of the most striking immune system-related roles of melatonin [29, 30]. Moreover, melatonin was found to be at extremely high concentrations in bone marrow [31]; this is consistent with the apparent requirement for higher levels of melatonin for apoptosis control than for neuroendocrine functions [32].

The aim of this study was to investigate the intracellular pathways involved in the inhibition provided by melatonin against TNF-α-induced apoptosis in human leucocytes. Because TNF-α activates not only death signals but also survival signals that are mediated by the activation of NF-κB transcription factor, we used CHX to block survival signals dependent on protein synthesis. We found that melatonin, besides preventing TNF-α-evoked oxidative stress most likely due to its antioxidant actions, seemingly required membrane receptor MT1/MT2 interaction and extracellular signal-regulated kinase (ERK) activation so as to counteract TNF-α-stimulated human leucocyte apoptosis.

Materials and methods

Reagents

Melatonin, RPMI-1640 medium, Ficoll-Histopaque separating medium, human recombinant TNF-α, 2-benzyl-*N*-acetyltryptamine (luzindole), PD98059, *N*-acetyl-L-cysteine (NAC), *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methyl coumarin (AC-DEVD-AMC), *N*-acetyl-Leu-Glu-Thr-Asp-7-amino-4-trifluoromethylcoumarin (AC-LETD-AFC), MES hydrate, nonidet-P-40 substitute (NP40), polyethylene glycol (PEG), HEPES, CHAPS and dithiothreitol (DTT) were obtained from Sigma Chemical, Co. (Madrid, Spain). Dihydrorhodamine-123 (DHR 123), Tris–glycine gels and thapsigargin were acquired 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). Propidium iodide (PI) and annexin V-FITC were bought from Immunostep (Salamanca, Spain). Anticaspase-9 (C9), anticaspase-3 (8G10), anticaspase-8 (1C12), anti-Bid, anti-ERK1/2 (137F5), antiphospho-ERK1/2 (D13.14.4E) and anti-β-actin (8H10D10) antibodies were procured from Cell Signaling (Danvers, MA, USA). Anti-cFLIP (H-150) antibody was obtained from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). All others reagents were of analytical grade.

Human leucocyte isolation

Venous blood was drawn from healthy volunteers of both genders and aged between 20 and 45 years under informed consent according to a procedure approved by Local Ethical Committees and in accordance with the Declaration of Helsinki. Human leucocytes 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 h to allow the adherence of monocytes so as to obtain a pure lymphocyte preparation, as previously described [23]. 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 EDTA, pH 7.4) so as to obtain a neutrophil-enriched preparation, as described elsewhere [23]. 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.

Melatonin and other treatments

Melatonin was dissolved in absolute ethanol, further diluted with PBS (final ethanol concentration < 0.2%) and added to the medium at the concentration of 1 mM, as previously described [25]. For the experiments, melatonin was added 60 min prior to apoptosis induction (TNF-α/CHX treatment).

Melatonin action on MT1/MT2 receptors was antagonised by 2-benzyl-N-acetyltryptamine (luzindole), which was added to cell culture media at a final concentration of 50 μM. Experiments of mitogen-activated protein kinases (MAPK) inhibition were carried out using a selective pharmacological inhibitor of ERK pathway, PD98059, which was used at a final concentration of 50 μM. Both inhibitors were added 30 min before the pre-incubation of cells with melatonin.

Assay for caspase activity

The determination of caspase-8, caspase-3 and caspase-9 activities was based on a method previously reported with minor modifications [18]. Stimulated or resting cells were pelleted and washed once with PBS. After centrifugation, cells were resuspended in PBS at a concentration of 10^7 cells/mL. Fifteen μL of the cell suspension was added to a 96-well 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 100 μM of caspase-8 substrate (AC-LETD-AFC) or 40 μM of

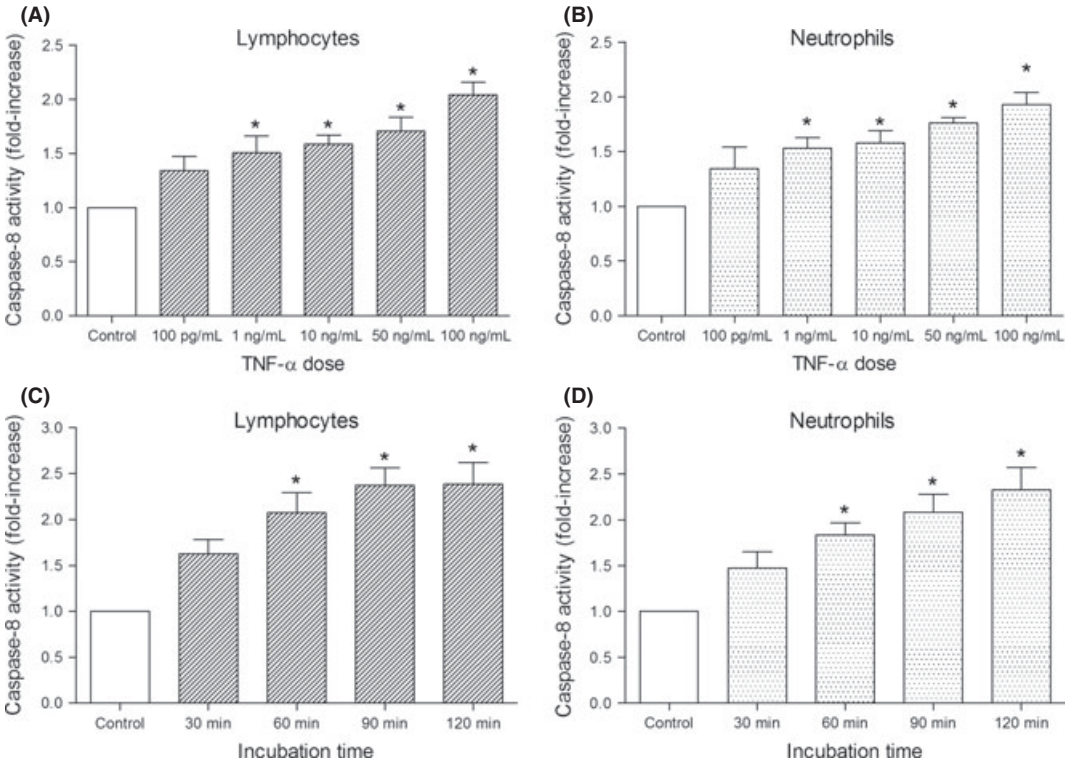

Fig. 1. Effect of TNF-α on caspase-8 activity in human leucocytes. Lymphocytes (A) and neutrophils (B) were incubated with different concentrations of TNF-α (100 pg/mL–100 ng/mL) or left unstimulated (control) for 60 min. On the other hand, lymphocytes (C) and neutrophils (D) were incubated with TNF-α (100 ng/mL) or left unstimulated (control) for 30, 60, 90 and 120 min. Caspase-8 was estimated as described under Materials and methods. 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 with control values.

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, Tecan Austria GmbH, Groedig, Austria) with excitation wavelength of 360 nm and emission at 460 nm for AMC fluorochrome or with excitation wavelength of 400 nm and emission at 505 nm for AFC fluorochrome. Data were calculated as fluorescence units/mg protein and presented as fold increase over the pretreatment level (experimental/control).

Intracellular reactive oxygen species measurement

Dihydrorhodamine-123 (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 reactive oxygen species (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) [33]. Briefly, leucocytes (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 [23]. Cells were then washed in PBS. The fluorescence intensity of Rh-123 was measured in an automatic microplate reader (Infinite M200). Excitation was set at 488 nm and emission at 543 nm. Data were presented as fold increase over the pretreatment level (experimental/control).

Determination of apoptosis

Apoptosis was measured by redistribution of phosphatidylserine (PS) in the presence of propidium iodide (PI) [18]. Once treated, cells were harvested and washed once in ice-cold HEPES medium with additional Ca^{2+} (2.5 mM). The cells (10^5–10^6 cells/mL) were then incubated with annexin V-FITC (1:200), which specifically binds PS residues on the cell membrane of apoptotic cells. After 45 min, cells were washed once and stained with PI, a fluorescent dye that will bind to DNA once the cell membrane has become permeable. Cells were then immediately analysed with a flow cytometer Cytomics FC500 (Beckman Coulter, Vienna, Austria). Ten thousand events were analysed using the FL-1 and FL-3 detector filters. Each sample was tested three to five times in independent experiments. Under all conditions tested, percentages of annexin V-positive (Annexin V^{pos}, PI^{neg}) cells were compared.

Western blot analysis

One-dimensional sodium dodecyl sulphate (SDS) electrophoresis was performed with a 4–12% gradient Tris–glycine gel, and separated proteins were then electrophoretically transferred, for 2 h at 0.8 mA/cm^2, in a semi-dry blotter

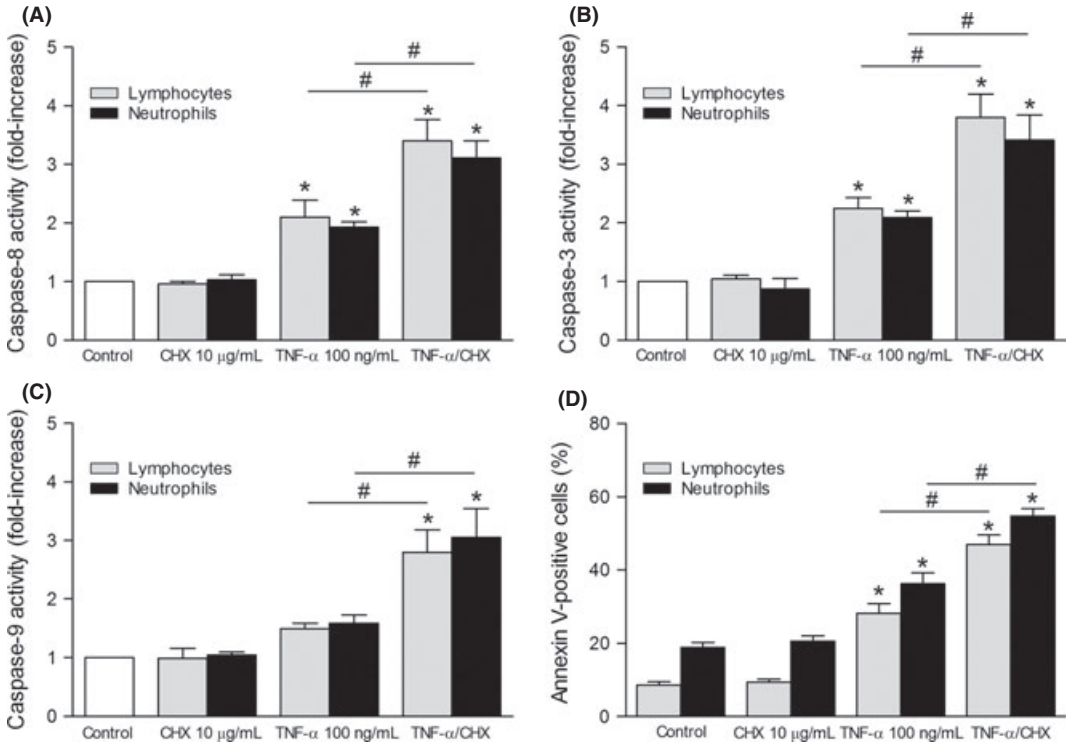

Fig. 2. Effect of TNF-α on human leucocyte apoptosis. Cells were incubated with TNF-α (100 ng/mL) in the absence or in the presence of cycloheximide (CHX; 10 mg/mL) or left unstimulated (control) for 120 min. Caspase-8 (A), caspase-3 (B) and caspase-9 (C) activity were estimated in both lymphocytes and neutrophils as described under Materials and methods. Values are presented as means ± S.E.M. of six separate experiments and expressed as fold increase over the pretreatment level (experimental/control). Apoptosis rates (D) in both lymphocytes and neutrophils are presented as mean ± S.E.M. of four separate experiments and expressed as percentage of annexin V-positive cells. $^{*}P < 0.05$ compared with control values. $^{\#}P < 0.05$ compared with TNF-α alone.

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 h with anticaspase-3 (8G10), anticaspase-9 (C9), anticaspase-8 (1C12), anti-cFLIP (H-150), anti-Bid, anti-ERK1/2 (137F5), antiphospho-ERK1/2 (D13.14.4E) or anti-β-actin (8H10D10) antibodies diluted 1:1000 in TBST. The primary antibody was removed, and blots were 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.

Statistical analysis

Data were 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

Stimulation of freshly isolated human blood leucocytes with increasing concentrations of TNF-α (100 pg/mL–100 ng/mL) for 60 min resulted in a dose-dependent increase in caspase-8 activity compared with control cells (Figs 1A,B), as ascertained by increases in protease activity capable of digesting the fluorogenic caspase substrate LETD-AFC, which is preferentially cleaved by caspase-8. Thus, leucocyte stimulation with 1 ng TNF-α/mL caused a detectable activation of caspase-8, the maximum effect being obtained with the dose of 100 ng TNF-α/mL in both lymphocytes ($P < 0.05$ versus control cells; Fig. 1A) and neutrophils ($P < 0.05$ versus control cells; Fig. 1B). To analyse the time course of caspase-8 activation, human leucocytes were treated with TNF-α (100 ng/mL) for 30, 60, 90 and 120 min and then assayed for caspase-8 activity. Caspase-8 activation could be significantly seen as early as 60 min and reached maximum activation after 120 min of TNF-α stimulation in both lymphocytes ($P < 0.05$ versus control cells; Fig. 1C) and neutrophils ($P < 0.05$ versus control cells; Fig. 1D). It was also shown that blockade of TNF-α-triggered survival signals by the addition of CHX (10 μg/mL) markedly potentiated TNF-α-induced caspase-8 activation in both lymphocytes ($P < 0.05$ versus TNF-α alone; Fig. 2A) and neutrophils

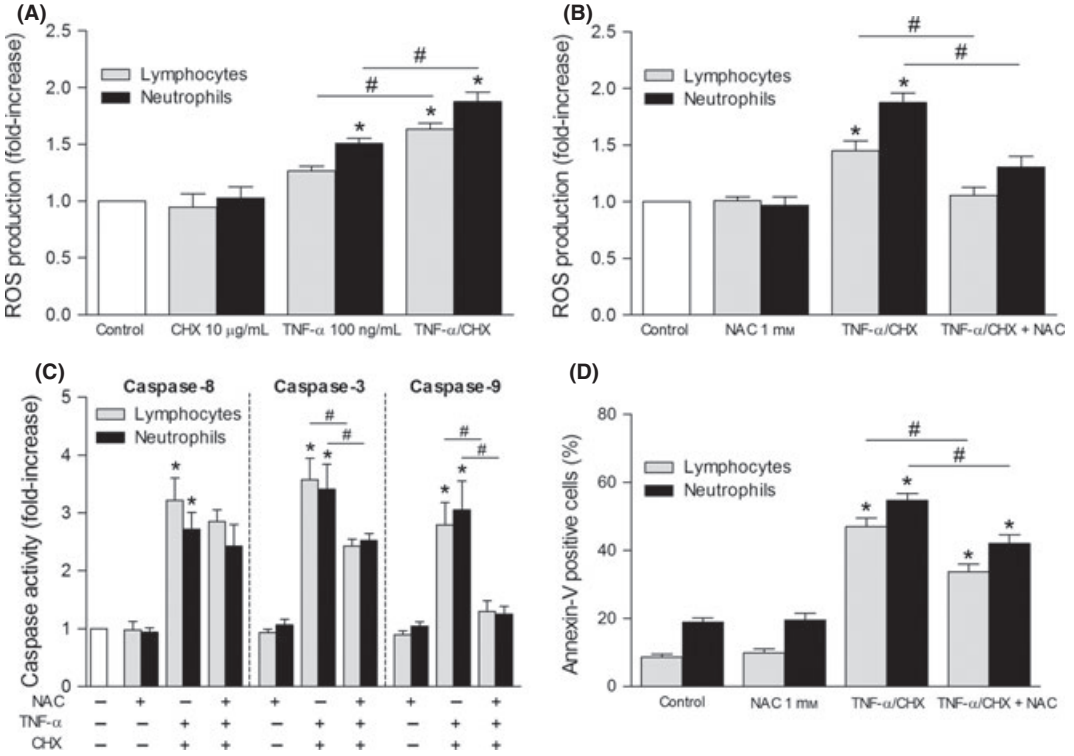

Fig. 3. *N*-acetyl-L-cysteine partially prevents TNF-α-/CHX-induced apoptosis in human leucocytes. Cells were incubated with TNF-α (100 ng/mL) in the absence or in the presence of cycloheximide (CHX; 10 mg/mL) or left unstimulated (control) for 120 min. When indicated, cells were pre-incubated with 1 mM *N*-acetyl-L-cysteine (NAC) or the vehicle for 60 min. ROS production (A and B) and caspase activity (C) were estimated in both lymphocytes and neutrophils as described under Materials and Methods. Values are presented as means ± S.E.M. of six separate experiments and expressed as fold increase over the pretreatment level (experimental/control). Apoptosis rates (D) in both lymphocytes and neutrophils are presented as mean ± S.E.M. of four separate experiments and expressed as percentage of annexin V-positive cells. $^{*}P < 0.05$ compared with control values. $^{\#}P < 0.05$ compared with TNF-α-/CHX-stimulated cells in the presence of NAC.

($P < 0.05$ versus TNF-α alone; Fig. 2A). Moreover, CHX alone was unable to trigger caspase-8 activation (Fig. 2A).

To examine whether caspase-8 activation evoked by TNF-α/CHX stimulation led to caspase-3 (a key downstream effector of apoptosis) activation, protease activity capable of degrading DEVD-AMC, a fluorogenic caspase substrate that is relatively specific for caspase-3, was analysed in both treated and untreated (control) leucocytes. Thus, in the presence of CHX, caspase-3 activity induced by TNF-α was substantially increased in both lymphocytes and neutrophils ($P < 0.05$ versus TNF-α alone; Fig. 2B). Likewise, because caspase-9 is an initiator caspase that is involved in the initial steps of mitochondrial apoptosis [34], protease activity capable of cleaving the fluorogenic caspase substrate LEHD-AMC, which is preferentially degraded by caspase-9, was also checked in TNF-α-treated leucocytes in the absence and presence of CHX to establish whether the mitochondrial pathway may be involved, at least in part, in TNF-α-mediated leucocyte apoptosis. Although TNF-α alone did not succeed in activating caspase-9, the combination of TNF-α/CHX substantially enhanced the activation of caspase-9 in human leucocytes ($P < 0.05$ versus TNF-α alone; Fig. 2C). Furthermore, flow cytometric analysis using fluorescein-labelled annexin V, which specifically binds to PS on the cell surface, showed that the treatment for 120 min with TNF-α/CHX largely augmented the proportion of cells depicting PS exposed on their surface ($P < 0.05$ versus TNF-α alone; Fig. 2D), thereby proving that human leucocytes exhibited greater susceptibility towards TNF-α-mediated cell death, when used in combination of CHX. In general, CHX alone induced negligible apoptotic changes (Fig. 2).

As some studies have reported that extrinsic apoptosis may be linked to intracellular ROS production [35], we also investigated the relationship between TNF-α-induced leucocyte apoptosis and oxidative stress. To this end, human leucocytes were treated with TNF-α alone or in combination with CHX, and then, intracellular ROS generation was measured. Treatment with TNF-α alone did stimulate ROS production in human neutrophils ($P < 0.05$ versus control; Fig. 3A), whereas lymphocytes were unable to significantly generate ROS upon TNF-α stimulation (Fig. 3A). Nevertheless, in the presence of CHX, the effect of TNF-α on ROS production was further increased in both neutrophils and lymphocytes ($P < 0.05$ versus TNF-α alone; Fig. 3A). To further analyse the involvement of ROS on TNF-α-induced leucocyte apoptosis, ROS generation was blocked by adding the well-known antioxidant, *N*-acetyl-L-cysteine (NAC), 60 min before the treatment with TNF-α/CHX. As expected, pre-incubation with 1 mM NAC largely forestalled ROS production evoked by TNF-α/CHX ($P < 0.05$; Fig. 3B). Moreover, pre-incubation

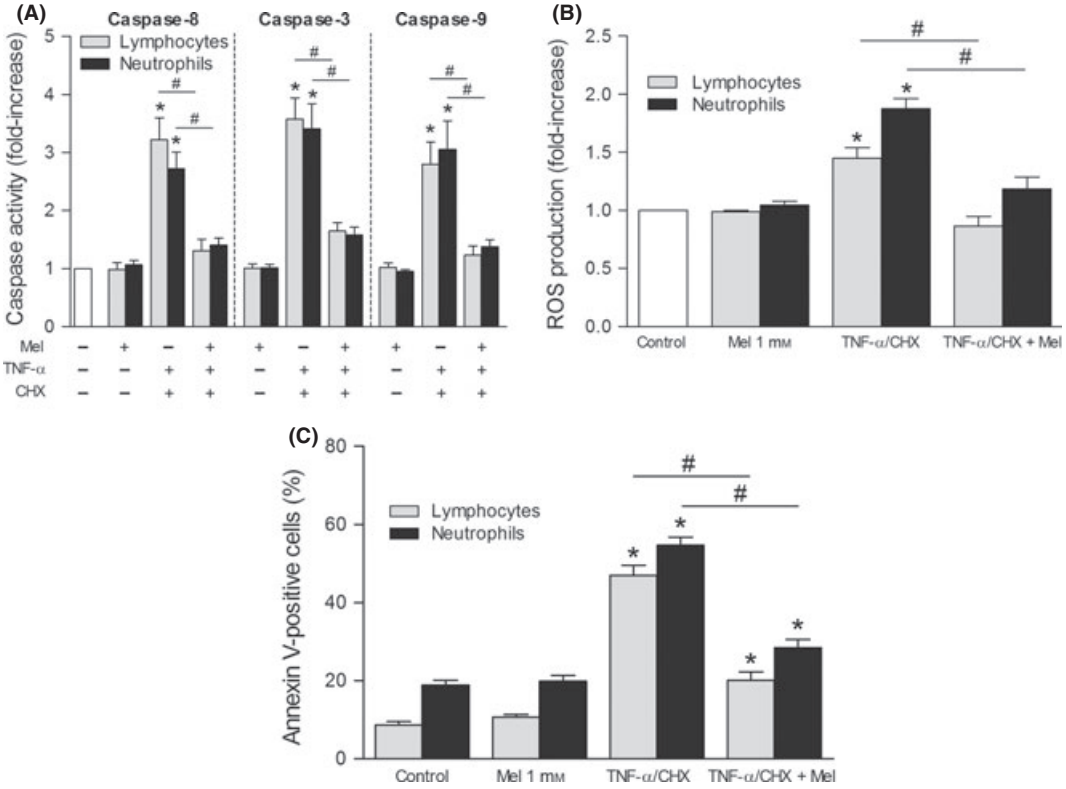

Fig. 4. Melatonin inhibits TNF-α-/CHX-mediated apoptotic cell death in human leucocytes. Cells were pre-incubated with 1 mM melatonin (Mel) or the vehicle for 60 min and then stimulated with TNF-α (100 ng/mL) plus CHX (10 mg/mL) or left untreated (control) for 120 min. Caspase activity (A) and ROS production (B) were estimated in both lymphocytes and neutrophils as described under Materials and methods. Values are presented as means ± S.E.M. of six separate experiments and expressed as fold increase over the pretreatment level (experimental/control). Apoptosis rates (C) in both lymphocytes and neutrophils are presented as mean ± S.E.M. of four separate experiments and expressed as percentage of annexin V-positive cells. $^{*}P < 0.05$ compared with control values. $^{\#}P < 0.05$ compared with TNF-α-/CHX-stimulated cells in the presence of Mel.

with 1 mM NAC was able to significantly reduce TNF-α-/CHX-induced caspase-9 and caspase-3 activities ($P < 0.05$; Fig. 3C), but had a negligible effect on caspase-8 activity, thereby suggesting that caspase-8 activation is independent of endogenous ROS production. Similarly, pre-incubation of leucocytes with 1 mM NAC partially prevented apoptosis caused by TNF-α/CHX ($P < 0.05$; Fig. 3D), as assessed by annexin V/PI staining. Generally, NAC alone produced marginal apoptotic changes (Fig. 3).

Because the anti-apoptotic actions as well as free radical scavenging properties of melatonin are well documented [13, 14, 30], we looked at the effect of melatonin on TNF-α-triggered leucocyte apoptosis. Unlike NAC treatment, pre-incubation of cells with 1 mM melatonin for 60 min not only extensively inhibited caspase-3 and caspase-9 activities evoked by TNF-α/CHX stimulation, but also clearly prevented caspase-8 activation ($P < 0.05$; Fig. 4A). As expected, pretreatment of cells with melatonin substantially lessened ROS production ($P < 0.05$ versus TNF-α/CHX; Fig. 4B) and largely diminished rates of apoptosis in both neutrophils and lymphocytes ($P < 0.05$ versus TNF-α/CHX; Fig. 4C). Melatonin, *per se*, neither modified caspase activity nor affected rates of apoptosis compared with control conditions. Likewise, melatonin alone had no impact on resting intracellular ROS generation (Fig. 4).

To confirm previous findings on caspase activity, caspase content of leucocytes treated with TNF-α/CHX for 120 min was determined by immunoblotting. Thus, in the presence of CHX, TNF-α treatment induced cFLIP down-regulation and subsequent caspase-8 activation (Fig. 5). Specific activation of caspase-8 was investigated by immunoblotting of Bid, a known target of caspase-8. Degradation of Bid occurred upon TNF-α/CHX stimulation (Fig. 5), suggesting that such treatment induces leucocyte apoptosis dependently, at least in part, on the mitochondrial amplification loop. Moreover, treatment of human leucocytes with TNF-α/CHX resulted in a significant activation of caspase-9 and caspase-3 as revealed by the increase in the amount of the corresponding active form and the decrease in the respective inactive procaspase form (Fig. 5). Pre-incubation of cells with 1 mM NAC was unable to prevent cFLIP and Bid degradation or forestall caspase-8 processing; nevertheless, it completely abolished caspase-9 activation and only reduced the amount of caspase-3 active form moderately (Fig. 5), highlighting that TNF-α/CHX stimulation could lead to apoptosis irrespective of mitochondrial death pathway. Interestingly, pre-incubation of cells with 1 mM melatonin for 60 min increased cFLIP protein levels, thereby blocking caspase-8 activation and subsequent Bid truncation (Fig. 5). Likewise, melatonin succeeded in

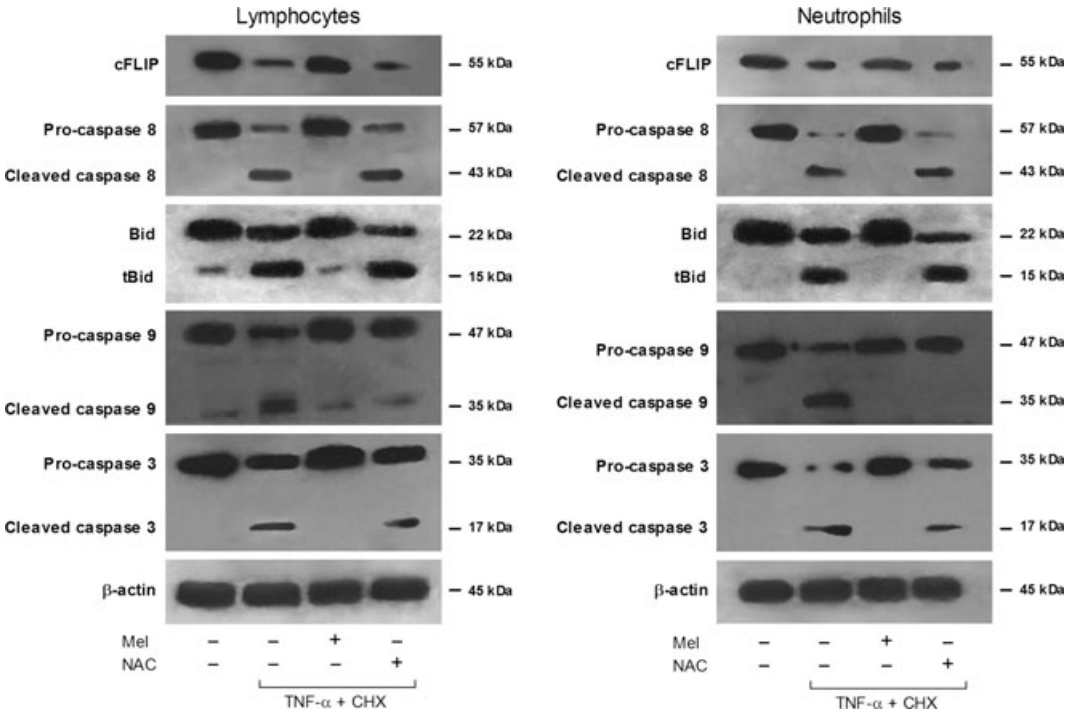

Fig. 5. Melatonin restores cFLIP levels and avoids subsequent caspase-8 processing and Bid degradation. To determine caspase content, human neutrophils and lymphocytes were pre-incubated with 1 mM melatonin (Mel) or 1 mM *N*-acetyl-L-cysteine (NAC) for 60 min and subsequently stimulated with TNF-α (100 ng/mL) plus CHX (10 mg/mL) or left untreated (control) for 120 min. Samples were lysed and then subjected to gradient Tris–glycine isolation and subsequent Western blotting with a specific anticaspase-8 (1C12), anti-cFLIP (H-150), anti-Bid, anticaspase-9 (C9) or anticaspase 3 (8G10) antibodies and reprobed with anti-β-actin (8H10D10) antibody for protein loading control. Blots are representative of four independent experiments.

inhibiting both caspase-9 and caspase-3 processing. Taken together, these findings indicated that the inhibiting effects of melatonin on TNF-α-mediated leucocyte apoptosis seemed to be both dependent on and independent of its antioxidant properties.

As melatonin may also control apoptosis by binding plasma membrane receptors [27, 28], we evaluated the effect of antagonising melatonin binding and activation of the MT1/MT2 receptors in TNF-α-/CHX-treated leucocytes. For this purpose, neutrophils and lymphocytes were exposed to the MT1/MT2 receptor antagonist luzindole (50 μM), 30 min before melatonin incubation. As shown in Fig. 6A, in the presence of luzindole, melatonin was no longer able to counteract TNF-α-/CHX-induced caspase-3 activation ($P < 0.05$ versus TNF-α/CHX in the presence of melatonin), suggesting that the anti-apoptotic actions displayed by melatonin may be membrane receptors-mediated. Luzindole, neither alone nor in combination (data not shown) with TNF-α/CHX treatment, had any effect on caspase-3 activity.

To better establish melatonin's anti-apoptosis mechanism, we assayed different signalling molecules classically involved in survival pathways. Thus, pharmacological inhibition of phosphoinositide 3-kinases (PI3Ks) with 10 μM LY294002 was unable to affect the inhibitory effects of melatonin on TNF-α-/CHX-evoked caspase-3 activation (data not shown). Importantly, blockade of the survival-promoting pathway MAPK/ERK with 50 μM PD98059, a small-molecule inhibitor of MAPK/ERK kinase 1 (MEK-1), almost completely abolished the inhibitory effects of melatonin on TNF-α-/CHX-triggered caspase-3 activation ($P < 0.05$ versus TNF-α/CHX in the presence of melatonin; Fig. 6B). PD98059, neither alone nor in combination (data not shown) with TNF-α/CHX treatment, had any effect on caspase-3 activity. Taken together, these results emphasised the potential implication of ERK activation on the protective actions of melatonin in human leucocytes, whereas PI3Ks seem to lack a role in this system.

We next analysed activation/phosphorylation of ERK following melatonin stimulation in the absence and the presence of luzindole. Phosphorylation of ERK1/2 was partly diminished upon TNF-α/CHX stimulation of leucocytes (Fig. 6C). Nevertheless, pre-incubation of cells with melatonin substantially enhanced p-ERK1/2 levels, which was clearly counteracted by the exposure to luzindole (Fig. 6C), suggesting that this event is MT1/MT2 receptor-dependent. The requirement of MT1/MT2 interaction and ERK activation for melatonin to inhibit TNF-α-/CHX-elicited leucocyte apoptosis was further confirmed by immunoblotting. In this sense, in the presence of luzindole or PD98059, melatonin was no longer able neither to restore cFLIP protein levels nor to inhibit caspase-3 activation (Fig. 6D), thereby underlining the fact that cFLIP is likely to play a pivotal role on the anti-apoptosis actions of melatonin in human leucocytes. Neither luzindole alone nor PD98059 alone affected protein content (data not shown).

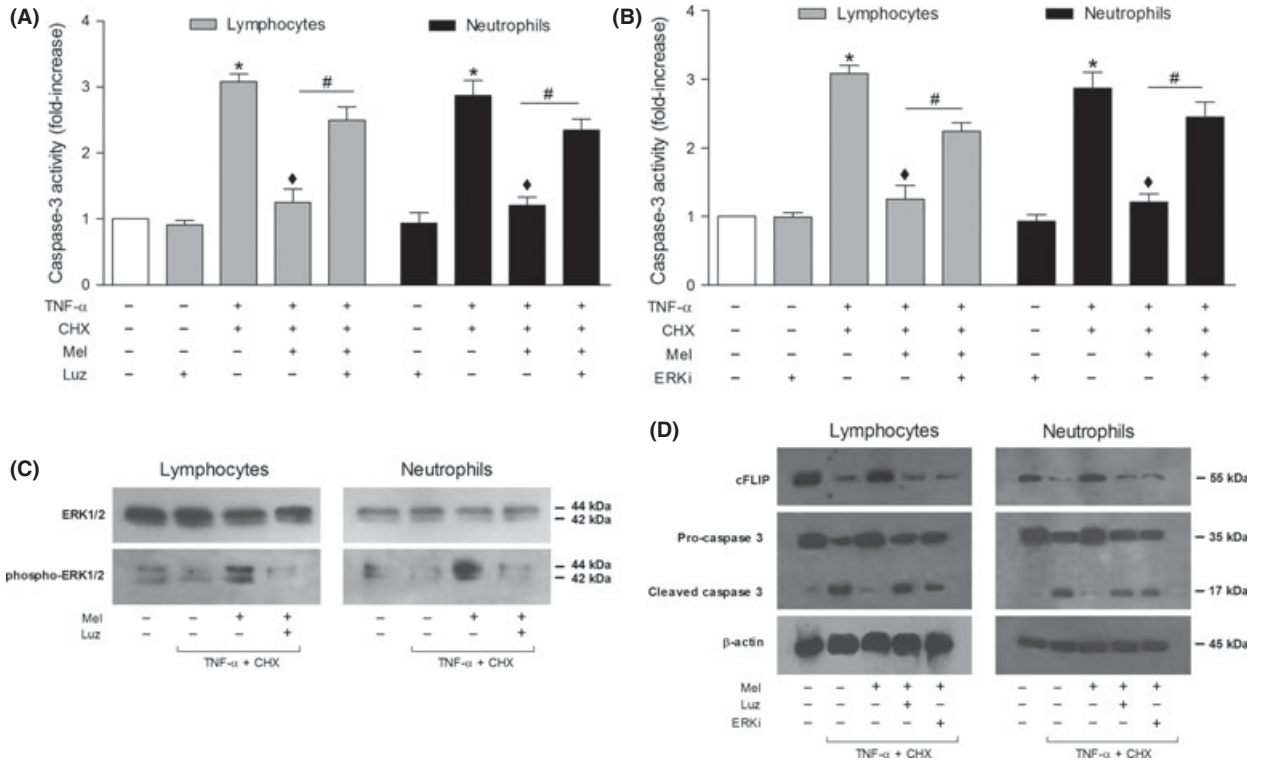

Fig. 6. Melatonin receptors and ERK signalling mediate the inhibiting effects of melatonin on TNF-α-/CHX-stimulated leucocyte apoptosis. Cells were pre-incubated with 1 mM melatonin (Mel) for 60 min in the absence or the presence of its MT1/MT2 receptor antagonist luzindole (Luz) (A) or ERK signalling inhibitor PD98059 (ERKi) (B) and then stimulated with TNF-α (100 ng/mL) plus CHX (10 mg/mL) or left untreated (control) for 120 min. Luzindole and PD98059 were added at the concentration of 50 μM, 30 min before other treatments. Caspase-3 activity was estimated as described under Materials and methods. 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 with control values. $^{\blacklozenge}P < 0.05$ compared with TNF-α-/CHX-treated cells. $^{\#}P < 0.05$ compared with TNF-α-/CHX-stimulated cells in the presence of Mel. (C) Phosphorylative activation of ERK1/2 was assessed by immunoblot analysis. Leucocytes were pre-incubated with melatonin (Mel) in the absence or the presence of luzindole (Luz) and subsequently stimulated with TNF-α plus CHX or left untreated (control) as indicated above. Blots are representative of three independent experiments. (D) Blockade of MT1/MT2 receptor or ERK signalling interferes with the anti-apoptosis mechanism of melatonin. Cells were treated as indicated in panels (A) and (B). Blots are representative of three independent experiments.

Discussion

Despite the fact that TNF-α was discovered more than two decades ago, the mechanisms by which TNF-α induces proliferation, survival and apoptosis are still incompletely understood. Even though almost all eukaryotic cells are known to express TNFR1, not all cells are sensitive to TNF-α-induced growth-modulating effects. The reason why some cells are sensitive while others are resistant to TNF-α is less clear [5]. So far, it is known that TNF-α can simultaneously activate survival and apoptotic responses, the former being NF-κB signalling-mediated. The balance between both signals determines the ultimate response of the cell to TNF-α [3].

Herein, we investigated the pro-apoptotic events initiated by TNF-α stimulation of human leucocytes, when used in combination with CHX to block TNF-α-elicited survival signals. Thus, we reported that human leucocytes became more susceptible to TNF-α, when treated in the presence of CHX. These results are consistent with previous studies showing that the suppression of protein synthesis is needed to sensitise most cells to TNF-α-induced apoptosis [6, 11]. Moreover, our experiments suggested that TNF-α/CHX stimulation of human leucocytes led to cFLIP degradation and subsequent caspase cascade initiation, that is, caspase-8 processing and activation, as it occurs in other cell types [7, 36]. Likewise, TNF-α-/CHX-mediated leucocyte apoptosis was dependent on Bid degradation and therefore involved, at least partly, the mitochondrial amplification loop. At this respect, intracellular ROS generation was likely required for the activation of caspase-9 (Fig. 7), but none of these steps seemed to be crucial for TNF-α-/CHX-evoked leucocyte apoptosis as pharmacological inhibition of ROS production delayed TNF-α-/CHX-stimulated apoptosis only partially (Fig. 3D). Although extrinsic apoptosis of neutrophils has been linked to intracellular ROS production [35], our findings fit into previous research demonstrating that TNF-α-/CHX-induced leucocyte apoptosis does not involve the generation of ROS [37]. Nevertheless, as TNF-α signalling in vivo occurs in the context of complex networks of inflammatory mediators and important cell-to-cell interactions and the present study employed

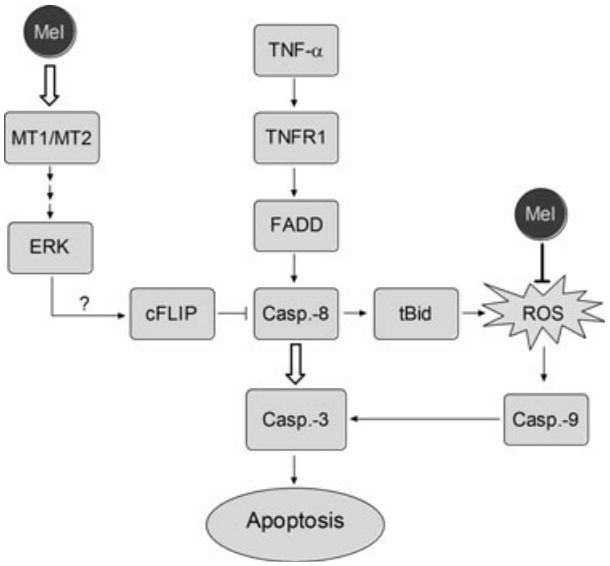

Fig. 7. Working model of melatonin anti-apoptotic mechanism in TNF-α-/CHX-challenged human leucocytes. Melatonin (Mel) prevents TNF-α-/CHX-evoked oxidative stress and subsequent caspase-9 activation most likely due to its antioxidant actions. However, melatonin requires membrane receptor MT1/MT2 interaction and ERK activation to modulate the anti-apoptotic protein cFLIP, thus inhibiting caspase-8 processing and downstream events, controlling in turn the cell life/death balance of leucocytes.

a simplified model system using CHX to focus on the TNF-α death pathway, these results must be interpreted cautiously.

Melatonin is an indoleamine engaged in a number of important physiological functions. Concerning immune function, the role of melatonin as an immunomodulator is well known [38–40]. However, in recent years, interest in melatonin has increased considerably owing to its influence on the process of apoptosis. The precise mechanism whereby melatonin modulates apoptosis remains unclear as melatonin has been reported to function as both pro-apoptotic and anti-apoptotic agent (for review, see [21]). Despite the fact that several mechanisms have been proposed to explain anti-apoptotic actions of melatonin in immune cells, none has been definitively proven [21]. Herein, we first demonstrated that melatonin was able to counteract TNF-α-/CHX-induced leucocyte apoptosis, which may be partly ascribable to melatonin's antioxidant capacity (Fig. 7). Melatonin reportedly displays cytoprotective properties in normal cells likely due to its free radical scavenging actions [20, 30]; however, its antioxidant effect seemed insufficient to explain the plethora of anti-apoptotic actions displayed by melatonin in this study, as inhibition of intracellular ROS generation with the well-known antioxidant NAC was unable to prevent caspase-8 activation, cFLIP degradation or Bid truncation, among other apoptotic features (Fig. 5). In fact, our results indicated that melatonin not only succeeded in inhibiting TNF-α-/CHX-triggered caspase-9 and caspase-3 activation, but also managed to restore cFLIP protein levels, thus avoiding caspase-8 processing and subsequent Bid degradation (Fig. 5).

Given that pharmacological inhibition of melatonin binding and activation of the MT1/MT2 receptor substantially blocked the inhibitory effects of melatonin on TNF-α-/CHX-induced caspase-3 activation, melatonin's anti-apoptotic mechanism apparently required membrane receptor MT1/MT2 (Fig. 6A). At this respect, previous reports have described that melatonin antagonises apoptosis via plasma membrane melatonin receptors in several cell types. Thus, it has been reported that protection rendered by melatonin to motoneurons [27] and spermatozoa [28] is mediated by activation of MT1 and/or MT2 receptors. Similarly, Radogna et al. provided evidence on how melatonin, through interaction with the MT1/MT2 receptor, may elicit a pathway that interferes with Bcl-2, thus modulating the cell life/death balance [41].

On the other hand, the growing interest on MAPK regulation has provided huge evidence indicating that MAPK represent a key node to process survival and death-promoting signals that originate from different endogenous and exogenous stimuli [42, 43]. In this context, the evolutionarily conserved signalling cascade MAPK/ERK that includes the upstream members Ras, Raf and MEK is constitutively expressed in immune cells [44, 45], and its activation is thought to play a major role to sustain the expression of genes that prevent the activation of the apoptotic programme. Accordingly, recent evidence has demonstrated that the activation of ERK pathway represents a prerequisite to suppress apoptosis [42] induced by a variety of stimuli. In this study, we proved that melatonin antagonised TNF-α-/CHX-triggered apoptosis in human leucocytes likely through the ERK survival pathway, as pharmacological inhibition of ERK signalling cascade showed that the phosphorylative activation of ERK was required for melatonin to inhibit TNF-α-/CHX-stimulated leucocyte apoptosis (Fig. 6B). Importantly, melatonin-induced activation of ERK was observed both dependently on (Fig. 6C) and independently of (data not shown) the presence of the stress stimulus, this event being clearly mediated by melatonin binding and activation of MT1/MT2 receptor. Besides, we found that full manifestation of melatonin effects on TNF-α-/CHX-caused leucocyte apoptosis required an intact crosstalk between membrane receptor MT1/MT2 and MAPK/ERK, as blockade of MT1/MT2 receptor or inhibition of ERK signalling cascade interfered with the anti-apoptotic mechanism of melatonin (Fig. 6D). Overall, the model proposed by these findings is that the MT1/MT2 receptor, which is under the positive control of melatonin, triggers an ERK-dependent signalling cascade that modulates the anti-apoptotic protein cFLIP, thereby regulating the cell life/death balance of human leucocytes (Fig. 7). In this sense, previous studies have suggested that melatonin may exert its cytoprotective effects via different MAPK-dependent pathways. Thus, it has been previously reported that the activation of the survival-promoting pathway MAPK/ERK is required for melatonin to antagonise UVB-induced apoptosis of U937 cells [46]. Likewise, despite the fact that we have ruled out the involvement of PI3Ks on the anti-apoptotic mechanism of melatonin, recent studies have noticed that neuroprotective properties of melatonin in astrocytes are mediated through the activation of

plasma membrane receptors and the PI3K-Akt signalling pathway [47].

In summary, melatonin inhibited TNF-α-/CHX-evoked oxidative burst possibly owing to its free radical scavenging actions, on the one hand, and required membrane receptor MT1/MT2 interaction and ERK phosphorylative activation in order to forestall TNF-α-/CHX-induced leucocyte apoptosis, on the other hand. These findings along with the growing number of evidence focusing on the central role that MAPK/ERK signalling plays in the control of survival-promoting and anti-inflammatory signals argue in favour of the view that this product of the pineal gland may find promising application in the therapy of immune and inflammatory diseases linked to accelerated apoptosis.

Acknowledgements

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Author contributions

J.E. carried out the experiments and drafted the manuscript. A.B.R. and J.A.P. designed the experiments and critically revised the manuscript. All authors approved the article before submission.

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3.4. La melatonina es capaz de retrasar la apoptosis y prevenir el estrés oxidativo inducidos por estrés del retículo en leucocitos procedentes de individuos mayores de 65 años.

3.4. Melatonin is able to delay apoptosis and prevent oxidative stress induced by endoplasmic reticulum stress in leukocytes from elderly humans.

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Melatonin is able to delay endoplasmic reticulum stress-induced apoptosis in leukocytes from elderly humans

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Abstract The mechanisms regulating neutrophil apoptosis are basically unaffected by the aging process. However, a significant impairment of cell survival occurs in elderly individuals following neutrophil challenge with pro-inflammatory stimuli, such as granulocyte-macrophage colony-stimulating factor (GM-CSF). The goal of the present study was to prove the effects of melatonin supplementation on apoptosis induced by calcium signaling in human leukocytes from elderly volunteers. Treatments with the specific inhibitor of cytosolic calcium re-uptake, thapsigargin, and/or the calcium mobilizing agonist, *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), induced mitochondrial membrane depolarization, caspase activation, phosphatidylserine (PS) externalization, and DNA fragmentation in leukocytes from both young and elderly volunteers, although such effects were much more evident in aged leukocytes. Importantly, melatonin treatment substantially preserved mitochondrial membrane potential, reversed caspase activation, reduced PS exposure and forestalled DNA fragmentation in leukocytes from both age groups. In conclusion, melatonin is able to delay endoplasmic reticulum stress-induced apoptosis in aged leukocytes and may counteract, at the cellular level, age-related degenerative phenomena linked to oxidative stress.

Keywords Melatonin · Aging · Leukocytes · Apoptosis

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Introduction

Aging is characterized by a general decline in physiological functions that leads to increased morbidity and mortality (Miyoshi et al. 2006). Although aging is an extremely complex, multifactorial process that has been the subject of considerable speculation (Semsei 2000), accumulated evidence identifies oxidative stress as a source of damage to cellular structure and function. Oxidative stress is a condition in which the redox balance between oxidants and antioxidants is disrupted, thereby tilting the equilibrium towards an oxidized state (Sies 1985). The free radical theory of aging proposes that the organismal deterioration that occurs as a result of increasing longevity is specially a consequence of the persistent accumulation of free radical-mediated damage to essential molecules, which gradually compromises the function of cells, of tissues, and, eventually, of the organism itself (Reiter et al. 2008). Consequently,

aging may be viewed as a process of irreversible injuries associated with accumulated oxidative debris.

Severe alterations of the immune system function occur during aging, related to a decrease of B cells that may be responsible of a decreased response to exogenous antigens, and to an increase of activated T cells, that may be associated with an increased frequency of autoimmune phenomena, related to cell-mediated immune response (Gavazzi and Krauze 2002; Larbi et al. 2005; Mazzoccoli et al. 2010). A growing body of evidence suggests that a key role is played by impaired neutrophil activity, and the neutrophil functions most strongly altered with aging are chemotaxis, phagocytosis, respiratory burst, and killing (Antonaci et al. 1984; Wenisch et al. 2000; Butcher et al. 2001). Despite the strict analogies between these alterations and the impaired functional capacities displayed by apoptotic neutrophils (Whyte et al. 1993), some studies have recently reported that advanced age does not affect either spontaneous or Fas-induced apoptotic events (Larbi et al. 2005; Tortorella et al. 1998). Nevertheless, neutrophils from aged individuals show a diminished rescue capacity when challenged with pro-inflammatory stimuli, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), lipopolysaccharides (LPS), or interleukin-2 (IL-2) (Tortorella et al. 1998, 2006).

Apoptosis (or programmed cell death) is characterized by morphological changes such as DNA fragmentation and disintegration of the cell into apoptotic bodies that can be removed by phagocytosis (Zhang et al. 2003). Two major pathways have been described regulating apoptosis: the extrinsic pathway, in which cell plasma membrane receptors act as the starting point of the apoptotic process, and the intrinsic pathway, in which mitochondria play a central role. Numerous reports suggest that aging is accompanied by alterations in the apoptotic behavior of a variety of cell types and tissues (Zhang et al. 2003). Nonetheless, it remains to be established whether enhanced levels of apoptosis serve as a self-protective mechanism to remove increased numbers of dysfunctional cells as a result of aging, or whether they play a destructive role, causing excessive cell death and the decline of organ function (Zhang et al. 2002).

The indole melatonin (*N*-acetyl-5-methoxytryptamine) is mainly secreted in the pineal gland, although it has been also detected in many other tissues. It is a highly lipophilic molecule that crosses cell membranes to easily reach subcellular compartments including mitochondria, where it seems to accumulate in high concentrations (Reiter et al. 2001). Melatonin is able to prevent oxidative stress both through its free radical scavenging effect and by directly increasing antioxidant activity (Reiter and Tan 2003), and different studies have demonstrated its protective role against oxidative damage induced by drugs, toxins, and different diseases (León et al. 2004; Espino et al. 2010a, b). It is known that endogenous melatonin production diminishes in elderly persons (Reiter 1992) and that the total antioxidative capacity of serum correlates well with its melatonin levels in humans (Benot et al. 1999). Moreover, melatonin shows beneficial anti-aging effects in rats, preventing lipid peroxidation and other mechanisms related to oxidative stress (Poeggeler 2005; Paredes et al. 2009). Therefore, the age-related decrease of melatonin secretion may play a role in the elevated oxidative damage observed in the elderly population (Reiter et al. 2002).

Since several pro-inflammatory mediators failed to rescue apoptotic leukocytes from aged individuals and no information exists on whether melatonin may influence the mechanism of programmed cell death in aged leukocytes, the aim of the present study was to prove the effects of melatonin supplementation on apoptosis evoked by endoplasmic reticulum stress in human leukocytes from both young and elderly volunteers.

Materials and methods

Chemicals

Melatonin, RPMI-1640 medium, Ficoll-Histopaque separating medium, bovine serum albumin, Triton-X-100 and *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) were obtained from Sigma Chemical (St. Louis, MO, USA). Thapsigargin and JC-1 were obtained from Molecular Probes (Eugene, OR, USA). Annexin V-fluorescein isothiocyanate conjugate was from Immunostep (Salamanca, Spain). In situ cell death detection kit was obtained from Roche Diagnostics GmbH (Mannheim, Germany). Hoechst 33342 was purchased from Calbiochem (San Diego, CA, USA). All others reagents were of analytical grade.

Human leukocytes isolation

After informed consent was obtained venous blood was drawn from healthy young (20–30 years old) and elderly (65–75 years old) volunteers of both genders 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 h to allow the 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 EDTA, pH 7.4) so as to get 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 phosphate-buffered saline (PBS) and centrifuged at 480 g for 15 min. The supernatant was then discarded and the cell pellet was gently resuspended in RPMI-1640 medium.

Melatonin treatment

Melatonin was dissolved in absolute ethanol, further diluted with PBS (final ethanol concentration <0.2%) and added to the medium at the concentration to be tested, as previously described (Luchetti et al. 2009).

Where indicated, human leukocytes were pre-incubated with 1 μM melatonin or the vehicle (control) for 1 h, and then stimulated with 10 nM fMLP and/or 1 μM thapsigargin for 1 h.

Measurement of mitochondrial membrane potential

Human leukocytes (10^5–10^6 cells/mL) were loaded with the cationic dye JC-1 (10 μg/mL, 15 min, 37°C). After dye loading, cells were centrifuged and resuspended in fresh RPMI medium. JC-1 accumulates in mitochondria forming red fluorescent aggregates at high membrane potentials. However, at low membrane potential, JC-1 exits mainly in the green fluorescent monomeric form. Fluorescence was recorded using an automatic plate reader (Infinite M200, Tecan Austria GmbH, Groedig, Austria). JC-1-loaded cells were excited at 488 nm and emission was detected at 590 nm (JC-1 aggregates) and 525 nm (JC-1 monomers). Data are presented as JC-1 emission ratios (590/525) and expressed as fold-change over the pre-treatment level (experimental/control). Since no significant differences were found between young and aged cells in control conditions, just one bar was displayed to represent control conditions in both groups.

Assay for caspase activity

The determination of caspase-3 and -9 activities was based on a method previously published (Bejarano et al. 2009) 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 which is composed by 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 -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/milligram protein and presented as fold-increase over the pre-treatment level (experimental/control). Since no significant differences were found between young and aged cells in control conditions, just one bar was displayed to represent control conditions in both groups.

Determination of phosphatidylserine (PS) externalization

The PS exposure of resting and stimulated leukocytes (1×10^6 cells/mL) was determined according to a procedure published elsewhere (Bejarano et al. 2008). Briefly, cells were stimulated in HEPES-buffered saline, and cell suspensions (500 μL) were transferred to 500 μL of ice-cold 1% (w/v) glutaraldehyde in PBS for 10 min. Cells were then incubated for 10 min with annexin V-fluorescein isothiocyanate conjugate (0.6 μg/mL) in PBS supplemented with 0.5% (w/v) bovine serum albumin and 2 mM $CaCl_2$. Cell staining was measured by using an automatic plate reader (Infinite M200). Samples were excited at 488 nm and emission was recorded at 516 nm. Data were calculated as fluorescence per milligram of protein and expressed as fold-increase over the pre-treatment level (experimental/control). Since no significant differences were found between young and aged cells in control conditions, just one bar was displayed to represent control conditions in both groups.

In situ detection of DNA fragmentation by TUNEL and Hoechst staining

At the end of the treatments, 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 h, RT) and air-dried on slides for 24 h. Afterwards, 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 TUNEL reaction mixture (50 μL) was added and the cells were incubated (1 h, 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 percent of apoptotic cells, which was expressed as fold-increase over the pre-treatment level (experimental/control).

Statistical analysis

Data are expressed as means±standard error of mean (SEM) 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.

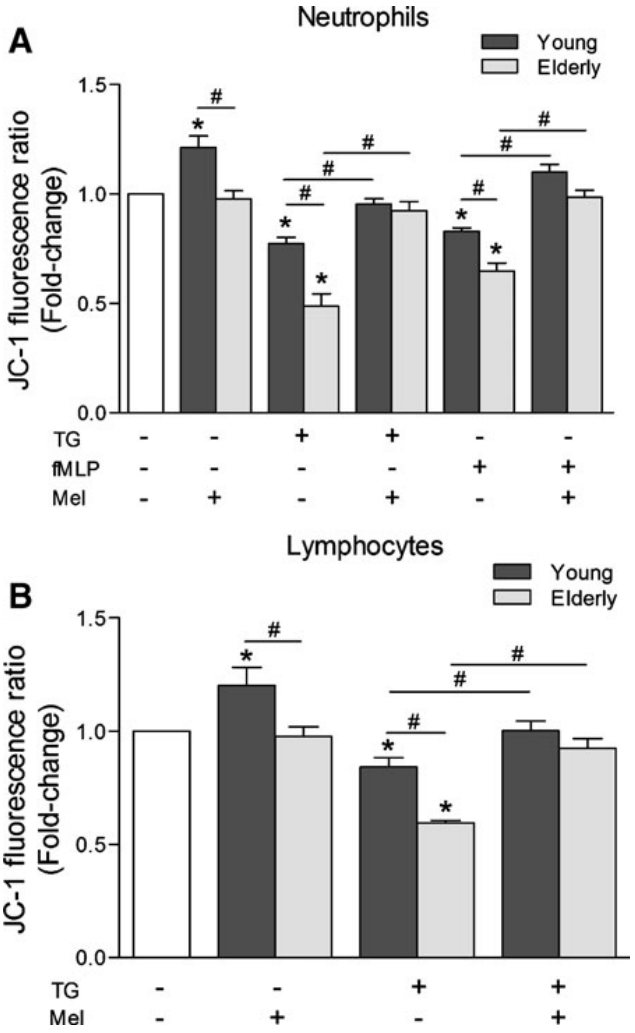

Fig. 1 Melatonin counteracts the mitochondrial membrane depolarization induced by calcium mobilizing agents in aged leukocytes. Neutrophils (**a**) and lymphocytes (**b**) from both young (*black bars*) and elderly (*grey bars*) individuals were pre-incubated with 1 μM melatonin (*Mel*) or the vehicle for 1 h, and then stimulated with 1 μM thapsigargin (*TG*) or 10 nM fMLP for 1 h to check the mitochondrial membrane potential. Mitochondrial membrane potential was estimated as described under the "Materials and methods" section. Values are presented as means±SEM of six separate experiments and expressed as fold-change over the pre-treatment level (experimental/control). *Asterisks* $P<0.05$ compared to control values. *Sharp signs* $P<0.05$

Results

Melatonin inhibits the intracellular calcium overload-induced mitochondrial disruption in leukocytes from elderly subjects

Intracellular calcium has been suggested to play an important role in the induction of apoptosis in response to a number of pathological conditions (Orrenius et al. 2003) and our previous data strongly indicate that increases in intracellular calcium are required for thapsigargin and/or fMLP-induced apoptosis in human leukocytes (Espino et al. 2010b). To evaluate the effect of intracellular calcium overload on mitochondrial membrane potential, human leukocytes were treated with both the specific inhibitor of cytosolic calcium re-uptake, thapsigargin, and the calcium mobilizing agonist, fMLP. The treatment of human neutrophils with 1 μM thapsigargin or 10 nM fMLP for 1 h induced a significant mitochondrial membrane depolarization in both young and elderly subjects as detected by the decrease in JC-1 fluorescence ratio ($P<0.05$; Fig. 1a). Similar results were found when human lymphocytes were treated with 1 μM thapsigargin for 1 h ($P<0.05$; Fig. 1b). Interestingly, the mitochondrial membrane depolarization in thapsigargin- and/or fMLP-

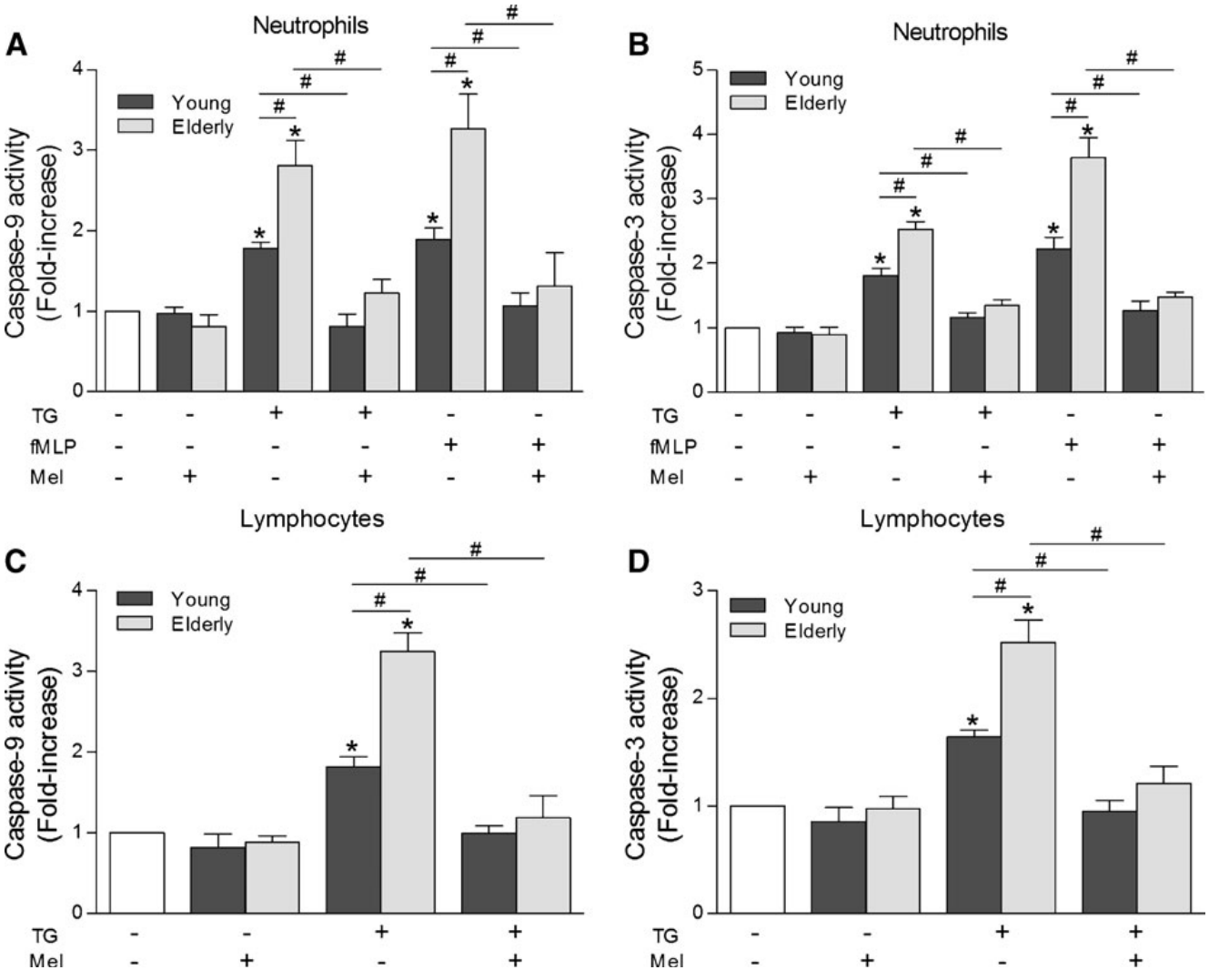

Fig. 2 Caspase activation is reversed by melatonin in aged leukocytes. Cells from both young (*black bars*) and elderly (*grey bars*) individuals were pre-incubated with 1 μM melatonin (*Mel*) or the vehicle for 1 h, and then stimulated with 1 μM thapsigargin (*TG*) or 10 nM fMLP for 1 h to check caspase-9 (**a** and **c**) and caspase-3 (**b** and **d**) activities. Caspase activity was estimated as described under the "Materials and methods" section. Values are presented as means±SEM of six separate experiments and expressed as fold-increase over the pre-treatment level (experimental/control). *Asterisks* $P<0.05$ compared to control values. *Sharp signs* $P<0.05$

treated leukocytes from aged individuals was significantly higher compared to those in young subjects ($P<0.05$; Fig. 1). However, when human leukocytes were pre-incubated with 1 μM melatonin for 1 h, both thapsigargin and fMLP exerted a negligible effect on mitochondrial membrane potential (Fig. 1), thereby indicating that melatonin is able to reverse endoplasmic reticulum stress-induced mitochondrial membrane depolarization in both young and elderly leukocytes. Moreover, in control cells, melatonin was also able to ameliorate mitochondrial membrane potential in human leukocytes from young volunteers (Fig. 1).

Caspase activation is delayed by melatonin pre-incubation in aged leukocytes

Since caspase-9 is an initiator caspase that is involved in the initial steps of mitochondrial apoptosis (Li et al. 1997), we checked the caspase-9 activity in the presence of thapsigargin or fMLP so as to prove whether the mitochondrial membrane depolarization induced by thapsigargin or fMLP is related to mitochondrial apoptosis. As shown in Fig. 2a, the treatment of neutrophils with 1 μM thapsigargin or 10 nM fMLP for 1 h produced a substantial increase of caspase-9 activity ($P<0.05$). Likewise, 1 μM thapsigargin was also able to stimulate the activation of caspase-9 in human lymphocytes ($P<0.05$; Fig. 2c). Moreover, the caspase-9 activity in thapsigargin- and/or fMLP-treated leukocytes from elderly subjects was substantially higher compared to those in young individuals ($P<0.05$; Fig. 2a, c). Intriguingly, the pre-incubation of leukocytes with 1 μM melatonin for 1 h was able to completely counteract the stimulatory effect of thapsigargin and fMLP on caspase-9 activity not only in leukocytes from young volunteers, but also in those from aged subjects ($P<0.05$; Fig. 2a, c). However, melatonin alone was unable to modify the spontaneous caspase-9 activation.

To examine the effect of intracellular calcium overload on caspase-3 activation, 1 μM thapsigargin and/or 10 nM fMLP were again administered to human leukocytes for 1 h. Our results showed that both thapsigargin and fMLP were able to increase the caspase-3 activity in human neutrophils ($P<0.05$; Fig. 2b). Similar findings were obtained when human lymphocytes were treated with 1 μM thapsigargin for 1 h ($P<0.05$; Fig. 2d). Curiously, thapsigargin and/or fMLP caused much higher caspase-3 activation in leukocytes from aged subjects than in those from young individuals ($P<0.05$; Fig. 2b, d). Furthermore, it is worth noting that the pre-incubation of leukocytes with 1 μM melatonin for 1 h almost entirely forestalls the stimulatory effect of thapsigargin and fMLP on caspase-3 activity in both young and elderly subjects ($P<0.05$; Fig. 2b, d). Nevertheless, melatonin alone was not able to alter the spontaneous caspase-3 activation in leukocytes from both young and aged individuals.

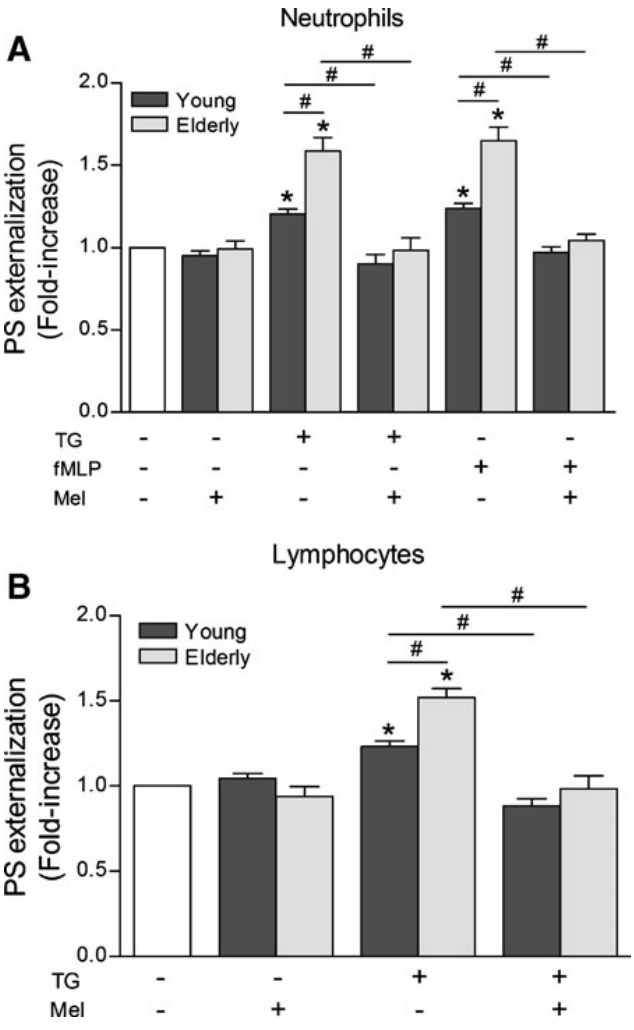

Fig. 3 Effects of melatonin on PS exposure in aged leukocytes. Neutrophils (**a**) and lymphocytes (**b**) from both young (*black bars*) and elderly (*grey bars*) individuals were pre-incubated with 1 μM melatonin (*Mel*) or the vehicle for 1 h, and then stimulated with 1 μM thapsigargin (*TG*) or 10 nM fMLP for 1 h to check PS externalization. PS exposure was estimated as described under the "Materials and methods" section. Values are presented as means± SEM of six separate experiments and expressed as fold-increase over the pre-treatment level (experimental/control). *Asterisks* $P<0.05$ compared to control values. *Sharp signs* $P<0.05$

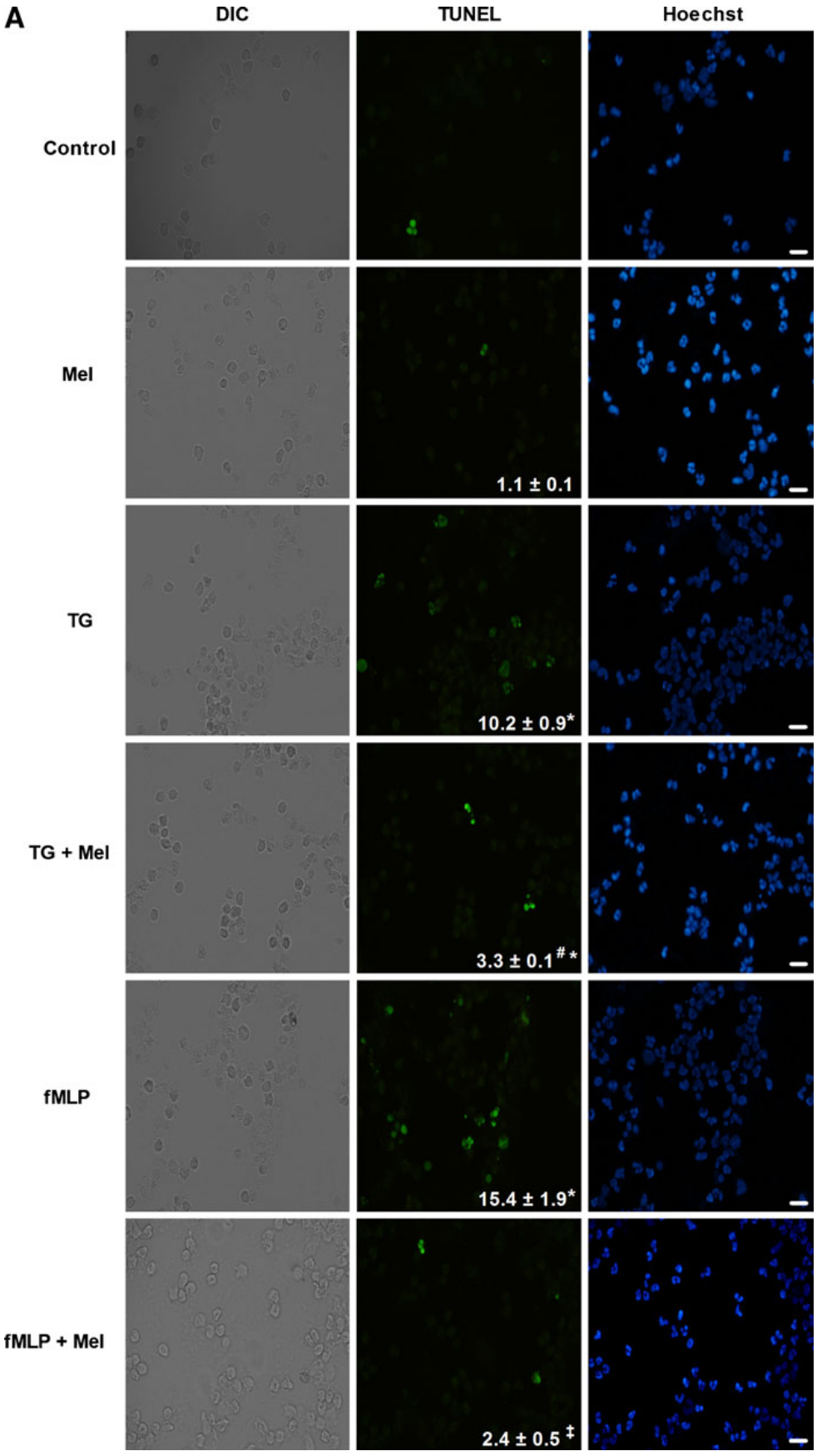

Fig. 4 Melatonin delays cell death in aged leukocytes. Neutrophils (**a**) and lymphocytes (**b**) from elderly subjects were pre-incubated with 1 μM melatonin (*Mel*) or the vehicle for 1 h, and then stimulated with 1 μM thapsigargin (*TG*) or 10 nM fMLP for 1 h to check the proportion of cells depicting DNA fragmentation. DNA fragmentation was estimated as described under the "Materials and methods" section. *Right* and *central panels*: pictures of Hoechst 33342 nuclear stain (*blue*) and TUNEL-positive cells (*green*), respectively. *Left panel* differential interference contrast (*DIC*) images of cultured cells using a 40× objective. *Scale bars* 15 μm. Values inside the central panel are presented as means±SEM of three separate experiments and expressed as fold-increase over the pre-treatment level (experimental/control). *Asterisks* $P<0.05$ compared to control values. *Sharp signs* $P<0.05$ regarding TG alone. *Double crosses* $P<0.05$ regarding fMLP alone

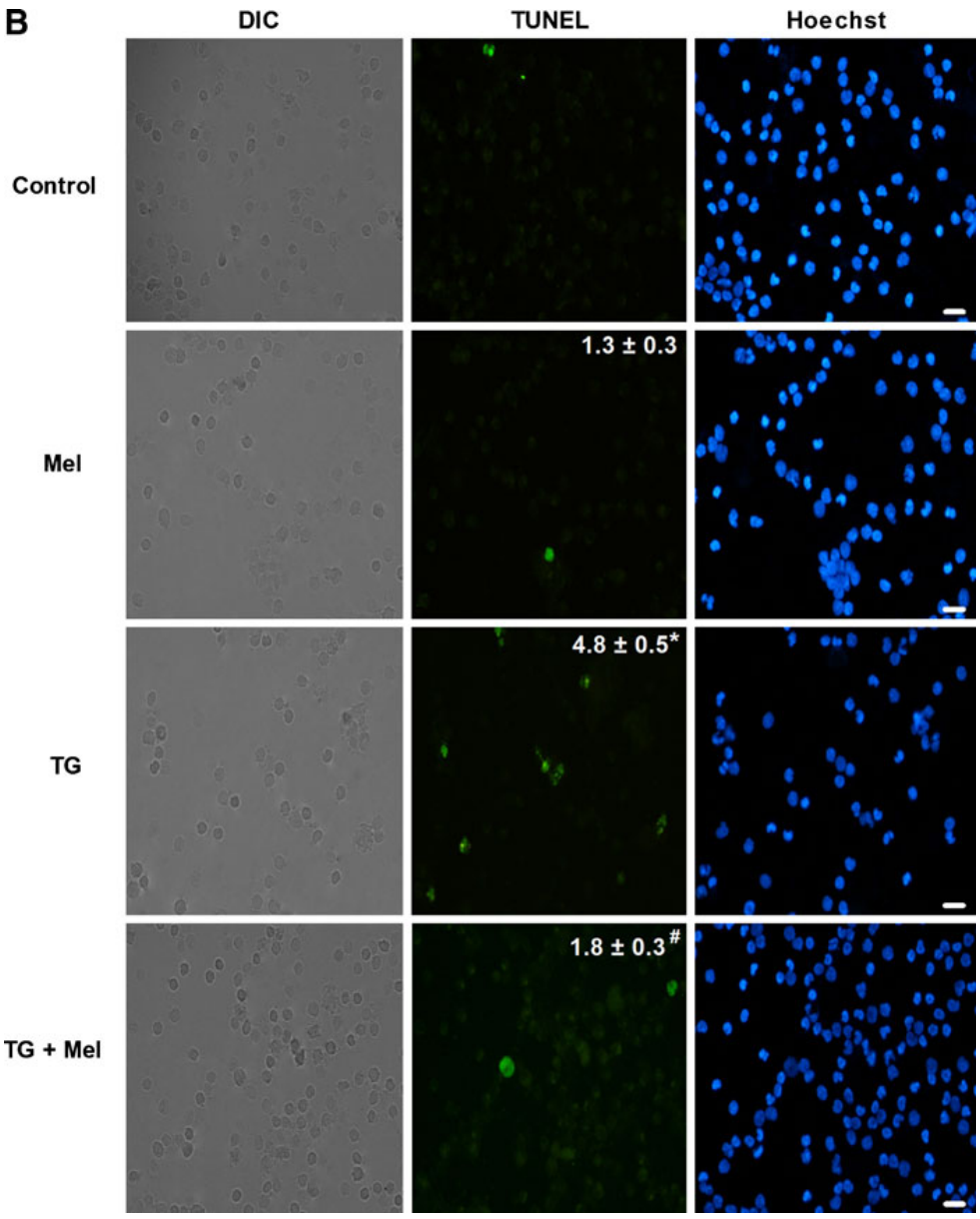

Fig. 4 (Continued)

Melatonin reverses the induction of both early and late apoptosis markers in leukocytes from elderly volunteers

To further investigate the effect of intracellular calcium overload on apoptosis, it was also evaluated the plasma membrane translocation of PS residues, which reflects a relatively early apoptotic stage. As shown in Fig. 3a, the treatment of human neutrophils with 1 μM thapsigargin or 10 nM fMLP for 1 h caused a substantial rise in PS externalization ($P<0.05$). Additionally, when human lymphocytes were treated with 1 μM thapsigargin for 1 h, a statistically significant increase was found in the percentage of annexin V-positive cells ($P<0.05$; Fig. 3b). Moreover, the proportion of thapsigargin- and/or fMLP-treated leukocytes depicting membrane phospholipids externalization was substantially higher in elderly subjects compared to those in young individuals ($P<0.05$; Fig. 3). Remarkably, the pre-incubation of leukocytes with 1 μM melatonin for 1 h was able to prevent the stimulatory effect of thapsigargin and fMLP on PS externalization not only in leukocytes from young volunteers, but also in those from aged individuals ($P<0.05$; Fig. 3). Again, melatonin alone was unable to vary the spontaneous PS exposure.

Lastly, as TUNEL assay is a well-established method for detection of DNA cleavage, a relatively

late apoptotic marker (Heatwole 1999), we assessed the amount of DNA fragmentation in the presence of thapsigargin or fMLP in order to verify whether the mitochondrial membrane depolarization induced by both calcium mobilizing agents, in turn, leads to cell death. Treatment of human neutrophils with 1 μM thapsigargin or 10 nM fMLP for 1 h produced a substantial increase in the proportion of cells depicting DNA fragmentation in elderly subjects ($P<0.05$; Fig. 4a). Similar results were found when aged lymphocytes were treated with 1 μM thapsigargin for 1 h ($P<0.05$; Fig. 4b). Importantly, in all cases, fMLP and/or thapsigargin induced much higher proportion of cells depicting DNA fragmentation in leukocytes from aged subjects than in those from young individuals ($P<0.05$; Table 1), thereby reflecting that aged leukocytes are much more vulnerable to apoptosis induced by endoplasmic reticulum stress. Besides, it is worth noting that the pre-incubation of leukocytes with 1 μM melatonin for 1 h significantly weakens the stimulatory effect of thapsigargin and fMLP on DNA fragmentation in both young and elderly volunteers ($P<0.05$; Table 1 and Fig. 4, respectively), thus substantiating that melatonin is able to delay endoplasmic reticulum stress-induced cell death in leukocytes from elderly humans.

Discussion

The free radical theory of aging proposes that aging and some related diseases are, at least in part, a consequence of oxidative stress (Harman 1992). Actually, several observations suggest a possible implication of ROS as signaling molecules in apoptosis and although a direct connection between apoptosis and aging has not been established, some data suggest that oxidative stress may elicit its effects on aging via regulation of apoptosis (Zhang and Herman 2002; Pollack and Leeuwenburgh 2001).

Melatonin and its metabolites are potent scavengers of damaging free radicals (Tan et al. 1993; Terrón et al. 2001; Reiter et al. 2009) able to counteract apoptosis (Juknat et al. 2005; Mayo et al. 1998) and we have previously demonstrated that melatonin anti-apoptotic actions in human leukocytes are likely related to its free-radical scavenging properties (Espino et al. 2010a, b). In the current work, we first showed that an excessive intracellular calcium load results in a less efficient capacity of leukocytes from old subjects to escape from apoptosis, whilst melatonin supplementation provides a cell survival advantage against intracellular calcium overload.

Melatonin exposure in the elderly delays endoplasmic reticulum stress-induced leukocyte apoptosis, thus suggesting that the beneficial consequences resulting from melatonin administration likely depend on its effect on mitochondrial physiology. On the contrary, the age-related failure to rescue neutrophils from apoptotic cell death induced by pro-inflammatory mediators, such as GM-CSF, seems to be strictly related to an impairment of GM-CSF-dependent PI3-K/Akt and ERK1/2 activation (Tortorella et al. 2006). In fact, we showed that melatonin is able to reverse the loss of mitochondrial membrane potential, as well as the subsequent caspase activation, evoked by intracellular calcium overload. In this regard, both *in vitro* and *in*

Table 1 Effect of melatonin on DNA fragmentation

	Neutrophils		Lymphocytes	
	Young	Elderly	Young	Elderly
Mel	1.1±0.2	1.1±0.1	0.9±0.1	1.3±0.3
TG	7.3±0.3* **	10.2±0.9*	3.4±0.0* **	4.8±0.5*
TG+Mel	3.2±0.3* ***	3.3±0.1* ***	1.2±0.1***	1.8±0.3***
fMLP	7.2±0.5* **	15.4±1.9*	–	–
fMLP+Mel	3.2±0.4* ****	2.4±0.5****	–	–

Proportion of cells depicting DNA fragmentation is presented as mean±SEM of three separate experiments and expressed as fold-increase over the pre-treatment level (experimental/control)

*$P<0.05$ regarding its corresponding control value; **$P<0.05$ regarding its corresponding value in aged cells; ***$P<0.05$ regarding its corresponding TG alone value; ****$P<0.05$ regarding its corresponding fMLP alone value

vivo experiments have shown that melatonin can influence mitochondrial homeostasis. Thus, melatonin stabilizes mitochondrial inner membrane (García et al. 1999) thereby improving electron transport chain activity. As a matter of fact, melatonin increases the activities of the brain and liver mitochondrial respiratory complexes I and IV in a time-dependent manner (Martín et al. 2000). Moreover, melatonin maintains the efficiency of oxidative phosphorylation and stimulates ATP synthesis while protecting the mitochondria from oxidative damage (León et al. 2005; Carretero et al. 2009). Finally, recent finding indicates that melatonin is able to prevent mitochondrial cardiolipin oxidation/depletion, which controls several processes involved in mitochondrial bioenergetics, in mitochondrial steps of cell death, as well as in mitochondrial membrane stability and dynamics (Paradies et al. 2010). Nevertheless, we cannot rule out that the delay of leukocyte apoptosis exerted by melatonin is produced via melatonin receptors, since melatonin treatment in murine senescence models has been reported to favor the pro-survival pathways, such as sirtuins or PI3K/Akt signalling, as well as modulate the intrinsic apoptotic pathway, thus increasing pro-survival factors and reducing pro-death proteins or enzymes (Gutierrez-Cuesta et al. 2008; Tajes Orduña et al. 2009).

In conclusion, melatonin is able to delay calcium overload-induced leukocyte apoptosis in advanced age likely due to its antioxidant properties. Therefore, owing to its well-documented protective effects along with its low toxicity to humans, molecular mechanisms involved in melatonin protection should be further investigated to clarify its potential therapeutic use for ageing and age-related disorders.

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Melatonin Counteracts Alterations in Oxidative Metabolism and Cell Viability Induced by Intracellular Calcium Overload in Human Leucocytes: Changes with Age

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Abstract: Ageing is associated with an increased production of free radicals and alterations in the mechanisms of adaptation to oxidative stress. In fact, the free radical theory of ageing proposes that deleterious actions of free radicals are responsible for the functional deterioration associated with ageing. Moreover, a close relationship exists between calcium homeostasis and oxidative stress. The current work was aimed at proving that intracellular calcium overload induced by *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) and/or thapsigargin leads to oxidative stress. We additionally examined the effect of melatonin on the levels of reactive oxygen species (ROS) and cell viability in human leucocytes collected from young (20–30-year-old) and elderly (65–75-year-old) individuals under both basal and oxidative stress-induced conditions. Treatments with 10 nM FMLP and/or 1 μM thapsigargin induced a transient increase in cytosolic free-calcium concentration ($[Ca^{2+}]_c$) in human leucocytes due to calcium release from internal stores, and led in turn to oxidative stress, as assessed by intracellular ROS measurement. Non-treated leucocytes from aged individuals exhibited higher ROS levels and lower rates of cell survival when compared to leucocytes from young individuals. Similar results were obtained in FMLP and/or thapsigargin-treated leucocytes from elderly individuals when compared to those from the young individuals. Melatonin treatment significantly reduced both hydrogen peroxide (H_2O_2) and superoxide anion levels, likely due to its free-radical scavenging properties, and enhanced leucocyte viability in both age groups. Therefore, melatonin may be a useful tool for the treatment of disease states and processes where an excessive production of oxidative damage occurs.

Ageing is associated with an increased production of free radicals and alterations in the mechanisms of adaptation to oxidative stress. In fact, the free radical theory of ageing states that the organismal deterioration that occurs as a result of increasing longevity is specially a consequence of the persistent accumulation of free radical-mediated damage to essential molecules, which gradually compromises the function of cells, of tissues and, eventually, of the organism itself [1]. Consequently, ageing may be viewed as a process of irreversible injuries associated with accumulated oxidative debris.

Oxidative stress occurs when the production of intracellular reactive oxygen species (ROS), e.g. superoxide anion (O_2^-), hydroxyl radical (HO) and hydrogen peroxide (H_2O_2), exceeds their removal by antioxidant defence mechanisms. To counteract the harmful actions of ROS, aerobic cells are equipped with a series of antioxidant enzymes that metabolize toxic reactants to less reactive or totally innocuous molecules. Superoxide dismutases (SODs), glutathione peroxidase and catalase are among these antioxidative enzymes. However, this protective machinery seems to be impaired with ageing. In particular, SOD activity has been shown to decrease in aged individuals [2–4], although this finding remains disputed [5]. Conversely, catalase and glutathione peroxidase activities have been reported to be augmented with ageing, which might reflect a compensatory response to extremely elevated basal levels of ROS in cells from aged individuals [3].

On the other hand, ageing also affects the immune system [6]. Age-related declines in immune function render the aged more susceptible to infectious diseases resulting in increased morbidity and mortality. This is a well-known phenomenon referred to as immunosenescence, which is defined as the set of immunological changes associated with age that lead to deterioration in the functional capacity of the immune system. In particular, functional impairment has been reported in neutrophils from aged individuals, which exhibit diminished chemotactic and phagocytic activities, as well as disturbances in the degree of oxidative stress [7,8]. Likewise, T cell functions in the elderly decrease because of the accumulation of age-related defects, thereby limiting effective immune responses [9,10].

The evidence is strong that melatonin may possess anti-ageing properties, since it exhibits both direct scavenging actions against free radicals and related products [11–14], as well as indirect antioxidative actions via its ability to stimulate antioxidant enzymes, including SOD [4], to promote the synthesis of another important intracellular antioxidant, i.e. as gluthatione [15], and to diminish free radical formation at the mitochondrial level by reducing the leakage of

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electrons from the electron transport chain [16]. Additionally, melatonin synergizes with other antioxidants to protect against oxidative stress. This combination of actions makes melatonin an important agent in combating some signs of ageing and/or the initiation of age-related diseases.

Calcium homeostasis and oxidative stress seem to be closely related. In fact, calcium may play a role in mitochondrial ROS generation, since the accumulation of calcium in mitochondria increases ROS production [17,18]. In addition, mitochondria are known to buffer cytosolic calcium by sequestering excessive calcium from the cytosol [19]; this might increase mitochondrial calcium content and thus trigger ROS production.

To date, no study has been carried out to evaluate alterations in oxidative metabolism and cell viability induced by intracellular calcium overload in human leucocytes. Moreover, since exogenous administration of melatonin mitigates oxidative stress-induced cellular damage, and considering that ageing may also be a consequence of the loss of resistance or adaptability to stress, the aim of the present work was to prove that endoplasmic reticulum (ER) stress induced by the specific inhibitor of calcium re-uptake, thapsigargin, and the calcium mobilizing agonist, *N*-formyl-methionyl-leucyl-phenylalanine (FMLP), lead to oxidative stress, as well as to examine the effect of melatonin on oxidative stress in human leucocytes from young and elderly individuals under both basal and oxidative stress-induced conditions.

Materials and Methods

Chemicals. Melatonin, Roswell Park Memorial Institute-1640 medium (RPMI-1640), Ficoll-Histopaque separating medium, *N*-formyl-methionyl-leucyl-phenylalanine (FMLP), methylthiazolyldiphenyl-tetrazolium bromide (MTT), dimethylsulfoxide (DMSO), *p*-nitroblue tetrazolium chloride (NBT) and ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Fura-2 acetoxymethyl ester (fura-2/AM), dihydrorhodamine 123 (DHR 123) and thapsigargin were obtained from Molecular Probes (Eugene, OR, USA). All others reagents were of analytical grade.

Human leucocytes isolation. Venous blood was drawn from healthy young (20–30-year-old) and elderly (65–75-year-old) volunteers of both genders under informed consent according to a procedure approved by Local Ethical Committees and in accordance with the Declaration of Helsinki. Human leucocytes 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 hr to allow the adherence of monocytes so as to obtain a pure lymphocyte preparation, as previously described [20]. 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 EDTA, pH 7.4) so as to get a neutrophil-enriched preparation, as described elsewhere [21]. 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 and centrifuged at $480 \times g$ for 15 min. The supernatant was then discarded and the cell pellet was 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).

Where it is indicated, human leucocytes were pre-incubated with 1 mM melatonin or the vehicle (control cells) for 60 min., and then stimulated with 10 nM FMLP and/or 1 μM thapsigargin for 60 min. Data were expressed as percentages of basal conditions, where basal levels were set at 100% and obtained from those cells harvested at the beginning of the experiment (time = 0).

Measurement of cytosolic free-calcium concentration ($[Ca^{2+}]_c$). Leucocytes 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 [22]. 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 fluorescence spectrophotometer (Shimadzu RF-5301PC series; Shimadzu Corporation, 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.* [23]. 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.

Detection of intracellular H_2O_2 concentration. Dihydrorhodamine 123 (DHR 123) is a non-fluorescent, non-charged dye that easily penetrates cell membrane. Once inside the cell, DHR 123 reacts with hydrogen peroxide (H_2O_2) to yield rhodamine 123 (Rh 123), a highly fluorescent compound, which subsequently accumulates in the mitochondria [24]. Briefly, leucocytes (1×10^6 cells/ml) were washed with serum-free RPMI 1640 medium and incubated with 20 μM DHR 123 (this concentration is relatively in excess for intracellular H_2O_2, and will not harm the cell activity) at 37°C for 25 min. Cells were then washed three times in phosphate-buffered saline (PBS). The fluorescence intensity of Rh 123 in cell suspensions was analysed by a fluorescence spectrophotometer (Shimadzu RF-5301 PC series). Excitation was set at 488 nm and emission was at 543 nm.

Superoxide generations determination: nitroblue tetrazolium assay. Superoxide generation was detected by nitroblue tetrazolium (NBT) assay [25], but with slight modifications. NBT (a water-soluble, yellow tetrazolium salt) was reduced by superoxide to formazan-NBT (a water-insoluble, dark blue product). Neutrophils (1.5×10^6 cells/ml) and lymphocytes (3×10^6 cells/ml) were exposed to the appropriate treatment at 37°C, and then incubated with 0.2% NBT in PBS for 60 min. Cells were centrifuged ($5000 \times g$ for 2 min. at 4°C), the supernatant was removed, and formazan (NBT reduced-insoluble) was extracted from leucocytes by 2 M KOH (120 μl) and dimethylsulfoxide (DMSO; 140 μl). The absorbance of formazan was determined at 560 nm in a microtitre plate reader (Infinite M200, Tecan Austria GmbH, Groedig, Austria).

Cell viability assay. Cell viability was evaluated using the MTT assay, which is based on the ability of viable cells to convert a water-soluble, yellow tetrazolium salt into a water-insoluble, purple formazan product. The enzymatic reduction of the tetrazolium salt happens only in living, metabolically active cells but not in dead cells. Cells were seeded in 96-well plates at a density of 5×10^5 cells per well and were subsequently exposed to the appropriate treatment at 37°C. After the treatments, the medium was removed and MTT was added into each well, and then incubated for 60 min. at 37°C in a shaking water bath. The supernatant was discarded and DMSO was added to dissolve the formazan crystals. Treatments were carried out in triplicate. Optical density was measured in an automatic plate reader (Infinite M200) at 490 nm and 650 nm.

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

Calcium signalling induced by agonists.

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), also reflecting the release of calcium from internal pools. In addition, when 1 μM thapsigargin was administered 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 were elevated in comparison to those obtained in the absence of extracellular calcium. Furthermore, significant differences due to ageing were not observed in typical transient calcium increase induced by both treatments (data not shown).

Melatonin reverses H_2O_2 production evoked by calcium signalling.

To evaluate the effect of intracellular calcium overload on oxidative metabolism, human leucocytes were treated with both the calcium mobilizing agonist, FMLP and the specific inhibitor of calcium re-uptake, thapsigargin. In control cells, the mitochondrial H_2O_2 levels reached were significantly higher than those obtained in basal conditions in both neutrophils and lymphocytes from elderly individuals ($p < 0.05$; fig. 2B and D). However, when control leucocytes from aged individuals were incubated with 1 mM melatonin for 60 min., mitochondrial H_2O_2 production was significantly reduced ($p < 0.05$; fig. 2B,D). Interestingly, control leucocytes from elderly individuals exhibited significantly elevated mitochondrial H_2O_2 levels regarding those found in control

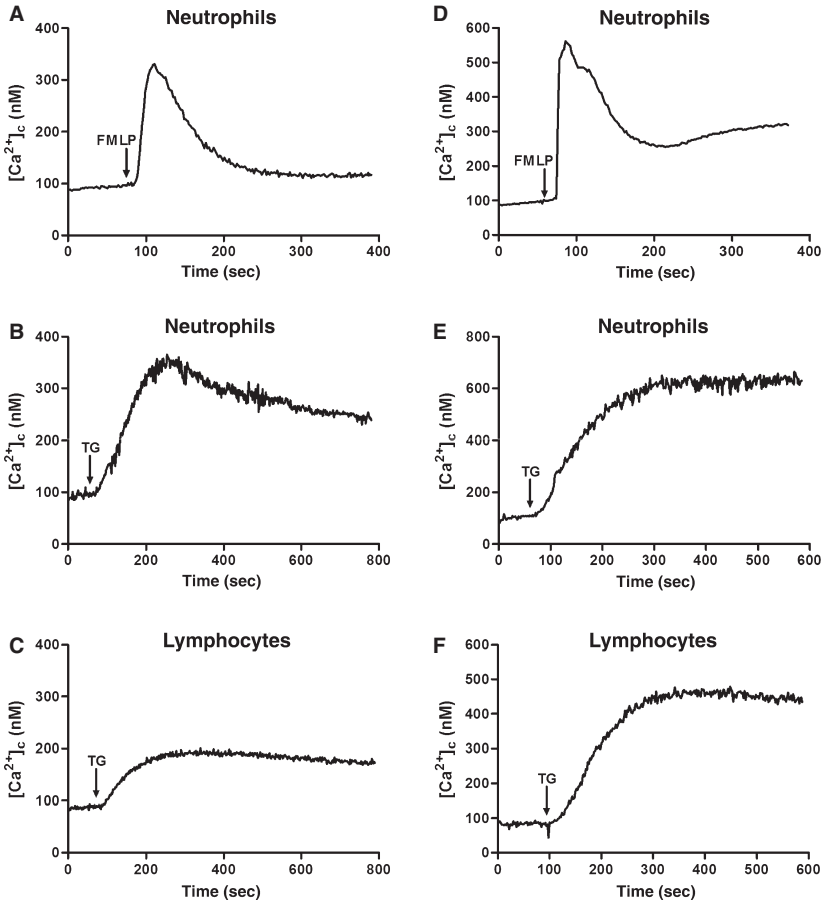

Fig. 1. Calcium mobilization in human leucocytes. Fura-2-loaded human leucocytes were stimulated with 10 nM *N*-formyl-methionyl-leucyl-phenylalanine (FMLP; panels A and D) or 1 μM thapsigargin (TG; panels B, C, E and F), as indicated, in a calcium-free medium ($[Ca^{2+}]_o = 0$ mM + 1 mM EGTA was added;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

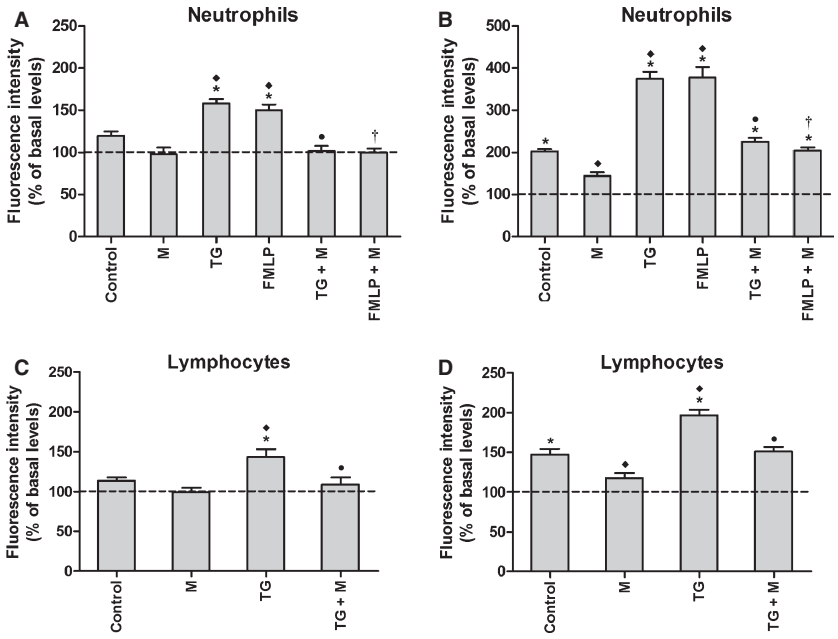

Fig. 2. Mitochondrial H_2O_2 production induced by calcium mobilizing agents in human leucocytes. Cells from both young (panels A and C) and elderly (panels B and D) individuals were pre-incubated with 1 mM melatonin (M) or the vehicle (control cells) for 60 min., and then stimulated with 10 nM FMLP and/or 1 μM thapsigargin (TG) for 60 min. to check the mitochondrial H_2O_2 production. Mitochondrial H_2O_2 production was estimated as described under Materials and Methods section. Values are presented as means ± S.E.M. of six separate experiments, which were carried out by duplicate and expressed as percentage of basal levels (time = 0). $^{*}p < 0.05$ with regard to basal levels. $^{\blacklozenge}p < 0.05$ with regard to control cells. $^{\bullet}p < 0.05$ with regard to TG alone-treated cells. $^{\dagger}p < 0.05$ with regard to FMLP alone-treated cells.

leucocytes from young individuals ($p < 0.05$; table 1). Moreover, treatments with 10 nM FMLP or 1 μM thapsigargin for 60 min. caused a significant increase of mitochondrial H_2O_2 production in both neutrophils and lymphocytes from young and elderly individuals ($p < 0.05$; fig. 2), indicating that the intracellular calcium overload induced by both agents seems in turn lead to oxidative stress. Likewise, the rise of mitochondrial H_2O_2 levels in FMLP- and thapsigargin-treated neutrophils from aged individuals was higher compared to those measured in neutrophils from young individuals ($p < 0.05$; table 1). Conversely, melatonin (1 mM, 60 min.) obviously counter-acted the augmented mitochondrial H_2O_2 levels provoked by the treatment with FMLP and thapsigargin in leucocytes from both young and aged individuals ($p < 0.05$; fig. 2), underlining the powerful antioxidative effect (direct or indirect) of melatonin against free radicals.

Prevention of the calcium signalling-induced superoxide anion production by melatonin.

We further investigated the effect of intracellular calcium overload on oxidative metabolism by checking superoxide anion generation. In control leucocytes, there was a significant increase in superoxide generation with respect to the values obtained in basal conditions in cells from both young and elderly individuals, as measured by NBT reduction assay ($p < 0.05$; fig. 3). Nevertheless, when control leucocytes from both young and aged individuals were incubated with 1 mM melatonin for 60 min., superoxide production was significantly suppressed ($p < 0.05$; fig. 3). Curiously, control leucocytes from elderly individuals exhibited significantly raised superoxide anion levels again regarding those found in control leucocytes from young individuals ($p < 0.05$; table 1). Furthermore, treatments with 10 nM FMLP or 1 μM thapsigargin for 60 min. provoked a significant rise of NBT reduction in both neutrophils and lymphocytes from young and elderly individuals ($p < 0.05$; fig. 3), indicating that both agents induced oxidative stress by elevating superoxide anion generation. Also, the augment of the superoxide anion levels in thapsigargin-treated lymphocytes from aged individuals was higher compared to those recorded in lymphocytes from young individuals ($p < 0.05$; table 1). Contrarily, melatonin (1 mM, 60 min.) significantly neutralized the elevated superoxide anion levels provoked by the treatment with FMLP and thapsigargin in human leucocytes from both young and aged individuals ($p < 0.05$; fig. 3), again highlighting the scavenger actions of melatonin against free radicals.

Effect of calcium signalling on cell viability.

Finally, we tested whether oxidative stress induced by intracellular calcium overload contributes to the fate of the cell. To that end, we examined the effect of FMLP and thapsigargin on cell viability and compared the results with those obtained after the exogenous administration of H_2O_2, a commonly used tool to induce oxidative stress in experimental models. In control leucocytes, a significant reduction in cell survival was found when compared with the values obtained under basal conditions in cells from both age

Table 1.

H_2O_2 production, nitroblue tetrazolium (NBT) reduction and cell viability, assessed as percentage (%) of basal levels (time = 0), in human leucocytes from young and elderly individuals, either control and after 60 min. of treatment with 1 lM thapsigargin (TG) or 10 nM N-formyl-methionyl-leucyl-phenylalanine (FMLP).

	H_2O_2 production (%)				NBT reduction (%)				Cell viability (%)			
	Neutrophils		Lymphocytes		Neutrophils		Lymphocytes		Neutrophils		Lymphocytes	
	Young	Elderly	Young	Elderly	Young	Elderly	Young	Elderly	Young	Elderly	Young	Elderly
Control	119.5 ± 3.5	203.2 ± 5.1[1]	113.5 ± 4.1	147.3 ± 7.1[1]	118.05 ± 4.4	139.7 ± 2.5[1]	116.5 ± 4.4	133.7 ± 6.3[1]	87.9 ± 3.7	77.4 ± 2.7[1]	90.1 ± 2.2	79.7 ± 6.3[1]
TG-treated	158.2 ± 5.1[2]	374.2 ± 16.7[1,2]	143.5 ± 9.8[2]	196.7 ± 7.2[2]	149.9 ± 5.5[2]	162.9 ± 6.6[2]	143.7 ± 4.2[2]	163.3 ± 3.7[1,2]	78.4 ± 5.2[2]	58.6 ± 2.1[1,2]	77.7 ± 1.7[2]	63.8 ± 3.4[2]
FMLP-treated	150.0 ± 6.7[2]	377.9 ± 24.7[1,2]	–	–	158.6 ± 6.8[2]	161.9 ± 8.3[2]	–	–	74.6 ± 3.1[2]	56.0 ± 5.3[1,2]	–	–

Each value represents the mean ± S.E.M. of six separate experiments.
[1] $p < 0.05$ compared to their respective values in young individuals.
[2] $p < 0.05$ compared to their respective values in control cells.

groups ($p < 0.05$; fig. 4). Nonetheless, when control leucocytes from both young and aged individuals were incubated with 1 mM melatonin for 60 min., the reduction in cell viability was mitigated ($p < 0.05$; fig. 4). Again, control leucocytes from elderly individuals exhibited significantly lower levels of cell viability compared with those obtained in control leucocytes from young individuals ($p < 0.05$; table 1). In FMLP- and thapsigargin-treated leucocytes from both age groups, there was a significant diminution in cell survival with respect to the values obtained under both basal and control (non-treated cells) conditions ($p < 0.05$; fig. 4). Additionally, FMLP and thapsigargin produced comparable levels of cell death compared with those obtained after the treatment with 100 μM H_2O_2 for 60 min. (fig. 4), verifying that FMLP- and thapsigargin-induced oxidative stress effectively leads to cell death. However, the decrease in cell viability in FMLP-treated neutrophils from aged individuals was higher regarding those recorded in neutrophils from young individuals ($p < 0.05$; table 1). Intriguingly, melatonin (1 mM, 60 min.) forestalled cell death provoked by all of the treatments in each experimental group ($p < 0.05$; fig. 4), emphasizing the protective role of melatonin against cell death.

Discussion

Cytosolic calcium has been proposed as a key regulator of cell survival and this ion may also induce cell death in response to a number of pathological conditions [26]. In addition, mitochondria are known to buffer cytosolic calcium by sequestering excessive calcium from the cytosol, playing a pivotal role in calcium signalling [19]. Thus, sustained increases in mitochondrial calcium levels may induce oxidative stress due to respiratory chain damage, and cell death owing to the release of apoptosis promoting factors [27,28]. In the present study, we report the effect of the calcium mobilizing agonist FMLP on intracellular ROS generation in human leucocytes, which evoked a significant increase in mitochondrial free radicals and superoxide anion levels, and ultimately led to cell death. Similarly, the specific inhibitor of calcium re-uptake, thapsigargin, which totally depleted the intracellular calcium stores within several minutes, also produced elevated intracellular ROS levels and subsequently caused cell death. Moreover, rates of cell death induced by the treatments with FMLP and/or thapsigargin are comparable to those obtained in H_2O_2-treated leucocytes, emphasizing the noxious effect of sustained calcium elevations. Our findings are in agreement with previous reports where it was proven that intracellular calcium overload induced by different calcium mobilizing agents contributes to oxidative damage and cell death [29,30].

Although no differences due to ageing were observed in transient calcium response induced by FMLP and thapsigargin, healthy ageing is accompanied by subtle alterations in calcium homeostasis and signalling, including alterations in the ER calcium load and release. Thus, the expression and/or function of ryanodine receptors, IP_3 receptors,

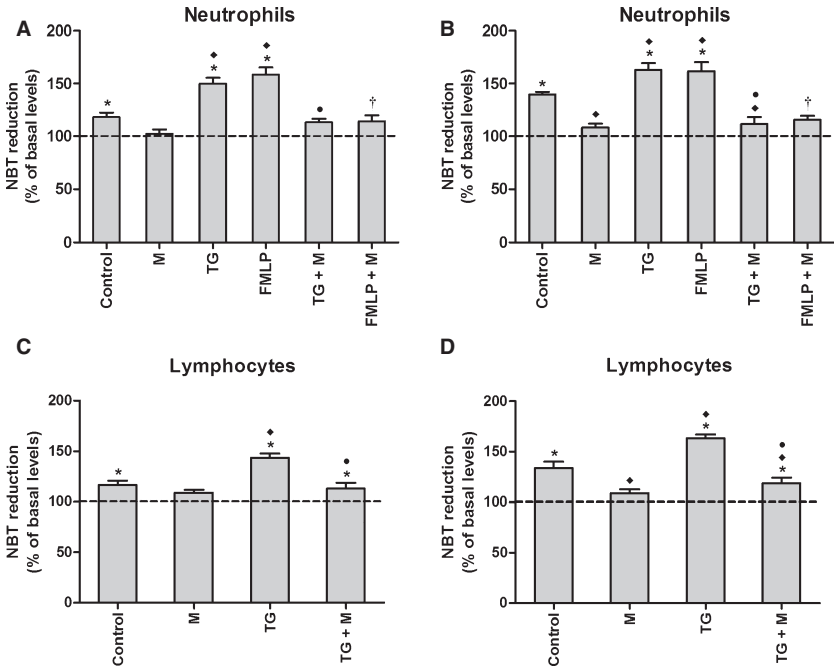

Fig. 3. Superoxide anion production induced by calcium mobilizing agents in human leucocytes. Cells from both young (panels A and C) and elderly (panels B and D) individuals were pre-incubated with 1 mM melatonin (M) or the vehicle (control cells) for 60 min., and then stimulated with 10 nM FMLP and/or 1 μM thapsigargin (TG) for 60 min. to check the superoxide anion production, as assessed by NBT reduction assay. NBT reduction was analysed as described under the Materials and Methods section. Values are presented as means ± S.E.M. of six separate experiments, which were carried out by duplicate, and expressed as percentage of basal levels (time = 0). $^{*}p < 0.05$ compared to basal levels. $^{\blacklozenge}p < 0.05$ compared to control cells. $^{\bullet}p < 0.05$ compared to TG alone-treated cells. $^{\dagger}p < 0.05$ compared to FMLP alone-treated cells.

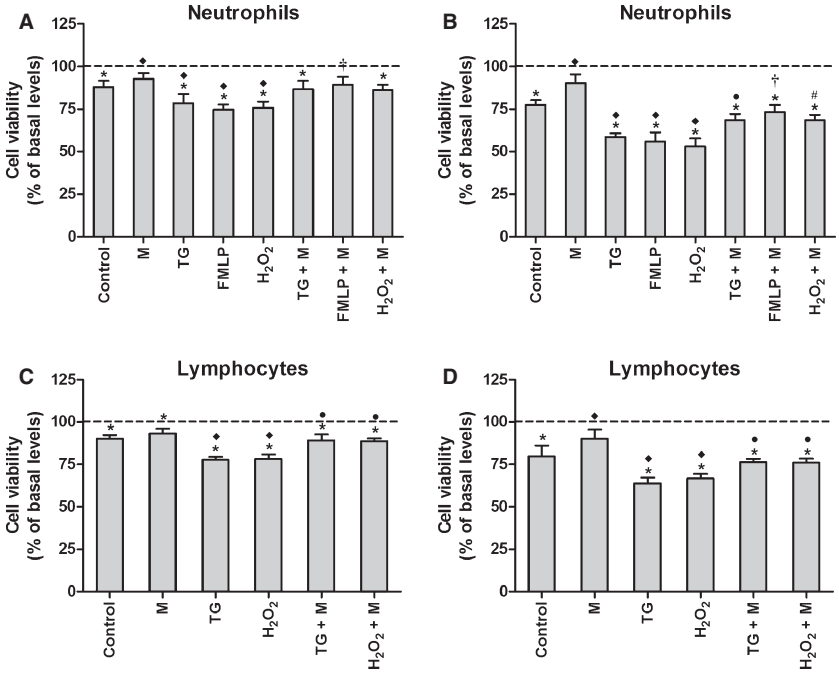

Fig. 4. Effect of calcium mobilizing agents on cell viability. Human leucocytes from both young (panels A and C) and elderly (panels B and D) individuals were preincubated with 1 mM melatonin (M) or the vehicle (control cells) for 60 min., and then stimulated with 10 nM FMLP, 100 μM hydrogen peroxide (H_2O_2) and/or 1 μM thapsigargin (TG) for 60 min. to check the effect of these treatments on cell viability. Cell viability was assessed as described under the Materials and Methods section. Values are presented as means ± S.E.M. of eight separate experiments, which were carried out by duplicate, and expressed as percentage of basal levels (time = 0). $^{*}p < 0.05$ regarding basal levels. $^{\blacklozenge}p < 0.05$ regarding control cells. $^{\bullet}p < 0.05$ regarding TG alone-treated cells. $^{\dagger}p < 0.05$ regarding FMLP alone-treated cells. $^{\#}p < 0.05$ regarding H_2O_2 alone-treated cells.

sarco-endoplasmic reticulum calcium-ATPase (SERCA) calcium pumps located in the ER membrane, and calcium-binding proteins within ER lumen all seem to be affected in aged cells [for review see (31)]. However, variations owing to ageing were found in intracellular ROS production in control conditions, since higher H_2O_2 and superoxide anion (as assessed by NBT reduction) levels were measured in both human neutrophils and lymphocytes from elderly individuals compared to those obtained in leucocytes from young individuals. Similar differences were observed in oxidative stress-induced (FMLP- and/or thapsigargin-treated cells) conditions; thus, there were elevated H_2O_2 and superoxide anion levels in 'aged' neutrophils and lymphocytes, respectively, compared to the values measured in 'young' cells. Since healthy cells continually produce low levels of ROS, which are buffered by multiple antioxidant systems, one likely reason for the augmented ROS levels may be the reduction in the activity of major antioxidant enzymes, such as SOD, observed in aged individuals [2–4,32], although other causes are not precluded. Likewise, age-related differences in the resistance of leucocytes to oxidative stress observed in the present study, as shown by increases in cell death in elderly individuals with respect to young individuals, are consistent with previous reports in ringdove heterophils [33,34].

Melatonin and its metabolites are potent scavengers of damaging free radicals [14,35,36]. *In vitro* and *in vivo* studies have reported that, at both physiological and pharmacological concentrations, the pineal indole and its byproducts protect against free radical damage [37]. In the present study, melatonin administration enhanced the reduction in intracellular ROS levels observed in FMLP- and thapsigargin-treated leucocytes from both young and elderly individuals, as well as in control cells from aged individuals. The results confirm that the observed protective effects of melatonin may be at least partially based on its radical scavenging properties. As already mentioned, large variety of studies have reported antioxidative properties of melatonin and its precursor, the amino acid tryptophan, at delaying free radical damage and oxidative stress [11–13,33,38,39] and at potentiating the activity of antioxidant enzymes, such as SOD [4,32]. Likewise, melatonin proved to be effective at enhancing cell survival, since when control, as well as FMLP- and/or thapsigargin-treated leucocytes from both young and elderly individuals were incubated with melatonin for 60 min., the decline in viability was very much lower in treated cells. These findings agree again with previous reports where both melatonin and its precursor, the amino acid tryptophan, counter-acted oxidative stress-induced cell death [33,38].

Taking into account that a decrease in oxidative stress has beneficial effects on the immune system [40], these results are especially important in the case of aged individuals, since it is known that immune function, as well as many hormones associated with maintenance of immune function (such as melatonin), decline with advancing ageing, at the same time that the levels of oxidative stress increase. In addition, the inter-relationship between the endocrine system and the immune system is considered of great importance in normal immune physiology and in mediating age-associated degenerative diseases [41]. As leucocytes constitute important components of the human immune system, the stimulatory action of melatonin shown in the present study reflects a significant immuno-enhancing property, highlighting the likely application of melatonin as a 'replacement therapy' to limit or reverse some of the effects of the changes that occur during immunosenescence.

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4. Discusión General

El calcio citosólico se considera como un regulador clave de la supervivencia celular, pero también puede inducir apoptosis en respuesta a diferentes condiciones patológicas (Hajnoczky y cols., 2003; Orrenius y cols., 2003). Por otro lado, las mitocondrias actúan como un sistema tamponante de calcio, secuestrando el exceso de calcio en el citoplasma. Este hecho es relevante en el contexto de la muerte celular programada puesto que la sobrecarga de calcio en la mitocondria puede desencadenar un programa apoptótico mediante la estimulación de la liberación de distintos factores promotores de la apoptosis, tales como el citocromo *c*, así como mediante la generación de ERO debido a daños en la cadena respiratoria (Brookes y cols., 2004; Hajnoczky y cols., 2006). Además, se ha demostrado que las mitocondrias desempeñan un papel esencial en la homeostasis del calcio intracelular (Hajnoczky y cols., 2003). En la presente Tesis Doctoral se ha puesto de manifiesto que las sobrecargas de calcio intracelular inducidas por un agonista movilizador de calcio, como el fMLP, o por un inhibidor específico de la recaptación de calcio por el RE, como la tapsigargina, desencadenan un programa apoptótico en leucocitos humanos caracterizado por una activación dosis-dependiente de caspasas tanto iniciadoras (caspasa-9) como efectoras (caspasa-3) (Espino y cols., 2010b). En este sentido, nuestro grupo de investigación había demostrado previamente que el vaciamiento de los almacenes de calcio no mitocondriales va acompañado por un incremento en la activación de caspasas, tanto en plaquetas (Rosado y cols., 2006) como en espermatozoides (Bejarano y cols., 2008) humanos.

Puesto que la activación de caspasas en nuestro modelo apoptótico está íntimamente relacionada con el calcio, también se comprobó si dicha activación podía deberse a la inducción del PPT de la mitocondria. Para estudiar la inducción del PPT se recurrió a la metodología propuesta por Petronilli y colaboradores (1999), basada en el uso de calceína en presencia de cloruro de cobalto, el cual apaga la fluorescencia de la calceína en todos los compartimentos intracelulares excepto en las mitocondrias intactas. En efecto, los resultados obtenidos en esta Tesis mostraron que el vaciamiento de los depósitos intracelulares de calcio por acción del fMLP y/o la tapsigargina produjo una pérdida significativa de la señal fluorescente de la calceína, la cual es indicativa de la apertura de un poro lo suficientemente grande como para permitir la salida de la

calceína de las mitocondrias o la entrada en las mismas del cobalto. Además, estos resultados se correlacionan con la activación de la proteína pro-apoptótica Bax y la consiguiente liberación de citocromo *c* desde las mitocondrias debidas a los tratamientos con tapsigargina (Espino y cols., 2010b). De manera interesante, se ha demostrado previamente que la trombina, un agonista movilizador de calcio en plaquetas, induce la activación y translocación mitocondrial de Bax (López y cols., 2008). Asimismo, es bien conocido que los agentes inductores de estrés del RE, como la tunicamicina y la brefeldina A, provocan cambios conformacionales y oligomerización de Bax en el RE y en la mitocondria (Zong y cols., 2003).

Aparte de las sobrecargas de calcio, la excesiva producción de ERO también puede estimular la apoptosis, tal y como se ha demostrado en varios tipos celulares (Tan y cols., 1998; Bejarano y cols., 2008). Se ha probado que algunas ERO, como el H_2O_2, incrementan la $[Ca^{2+}]_c$ en ausencia de calcio extracelular, sugiriendo que el H_2O_2 moviliza calcio desde los almacenes intracelulares (Pariente y cols., 2001). En este sentido, en esta Tesis Doctoral se ha demostrado que las sobrecargas de calcio intracelular inducidas por fMLP y/o tapsigargina provocan un aumento en los niveles intracelulares de ERO (Espino y cols., 2011b). Además, para profundizar en el papel del calcio en nuestro modelo apoptótico, se diseñó un experimento para evitar la sobrecarga de calcio mitocondrial, según el cual, se pre-incubaron los leucocitos humanos con el quelante de calcio intracelular dimetil BAPTA o con rojo de rutenio, el inhibidor específico del MCU. Los resultados de la presente Tesis mostraron que tanto la inhibición de los incrementos en la $[Ca^{2+}]_c$, por acción del dimetil BAPTA, como el bloqueo de la recaptación de calcio por las mitocondrias, por acción del rojo de rutenio, impiden el aumento en los niveles de ERO y la consiguiente activación del proceso apoptótico (medido como activación de caspasas y fragmentación de ADN) (Espino y cols., 2011b). Dichos resultados, a su vez, concuerdan con estudios previos realizados en diferentes tipos celulares (Bejarano y cols., 2008; Fang y cols., 2008; Lin y cols., 2011).

La melatonina es una indolamina que está involucrada en muchas funciones fisiológicas. En términos de función inmune, el papel de la melatonina como inmunomodulador es ampliamente conocido (Rodríguez y cols., 1999; 2005; Barriga y

cols., 2005). Sin embargo, en los últimos años, el interés por la melatonina ha crecido enormemente debido a su influencia sobre el proceso apoptótico. El mecanismo exacto por el cual la indolamina regula la apoptosis aún se desconoce, ya que se ha descrito que la melatonina presenta acciones pro- y anti-apoptóticas (Sainz y cols., 2003b). Aunque se han propuesto varios mecanismos para explicar los efectos anti-apoptóticos de la melatonina en células inmunes, ninguno de ellos ha sido probado (Sainz y cols., 2003b). En la presente Tesis se ha observado que la melatonina es capaz de reducir tanto la fragmentación de ADN como la activación de caspasa-3 y -9 inducidas por sobrecargas de calcio intracelular con fMLP y/o tapsigargina, tanto en neutrófilos como linfocitos humanos (Espino y cols., 2010b; 2011b). Esta inhibición de la apoptosis leucocitaria por parte de la melatonina no podría ser consecuencia de los efectos antagónicos de la indolamina sobre la señal de calcio, puesto que la melatonina fue incapaz de modificar la señal de calcio evocada por el fMLP o la tapsigargina (Espino y cols., 2010b; 2011b). No obstante, los resultados encontrados en esta Tesis ponen de manifiesto que los efectos inhibitorios de la indolamina se deben probablemente al bloqueo de la apertura del PPT. De hecho, la pre-incubación de los leucocitos con melatonina redujo significativamente la pérdida de la señal fluorescente de la calceína mitocondrial inducida por el vaciamiento de los depósitos intracelulares de calcio, algo similar a lo ocurrido tras la pre-incubación con ciclosporina A, un inhibidor del PPT (Espino y cols., 2010b).

Para confirmar estos resultados, en esta Tesis Doctoral se trató de comprobar el efecto de la melatonina sobre la liberación del citocromo *c* inducida por el tratamiento con tapsigargina. Los resultados obtenidos indican que la melatonina fue capaz de reducir la liberación de citocromo *c* inducida por la tapsigargina (Espino y cols., 2010b), lo cual concuerda con estudios previos realizados en mitocondrias aisladas de tejido cardíaco donde se demostró que la melatonina revertía la apertura del PPT y la liberación de citocromo *c* inducidas por sobrecargas de calcio mitocondrial (Petrosillo y cols., 2009a). Resultados similares fueron observados en un estudio *in vivo* donde la disfunción mitocondrial debida al envejecimiento fue prevenida por acción de la melatonina (Petrosillo y cols., 2008). Estos efectos de la indolamina parecen deberse a su habilidad de inhibir la peroxidación de la cardiolipina (Luchetti y cols., 2007;

Petrosillo y cols., 2009b). Adicionalmente, esta Tesis ha demostrado que la melatonina inhibe la activación de la proteína pro-apoptótica Bax inducida por el vaciamiento de los almacenes intracelulares de calcio, tanto en neutrófilos como linfocitos humanos (Espino y cols., 2010b). En este sentido, Radogna y colaboradores (2008) demostraron recientemente que la indolamina reduce la apoptosis en monocitos tumorales humanos ya que promueve la relocalización de la proteína Bcl-2 que, a su vez, impide la activación de Bax. Por tanto, este conjunto de resultados, correspondientes a los objetivos nº 1 y 2 de la presente Tesis, parece indicar que los efectos protectores de la melatonina en leucocitos humanos se producen probablemente a través de la inhibición de la apertura del PPT mitocondrial y la activación de Bax (Espino y cols., 2010b). No obstante, no podemos descartar la posibilidad de que los efectos protectores de la melatonina frente a la apoptosis mitocondrial estén relacionados con sus bien conocidas acciones como antioxidante y secuestrador de radicales libres (Tan y cols., 1993). De hecho, esta Tesis Doctoral demuestra que la melatonina es capaz de revertir los elevados niveles intracelulares de ERO al tiempo que inhibe la fragmentación de ADN y la activación de caspasa-3 y -9 inducidas por sobrecargas de calcio intracelular. Adicionalmente, la protección proporcionada por la melatonina es comparable a la aportada por el conocido antioxidante N-acetilcisteína (Espino y cols., 2011b).

A pesar de que el TNF-α se descubrió hace más de dos décadas, los mecanismos a través de los cuales induce supervivencia o apoptosis no se han identificado por completo. Hasta el momento, se sabe que el balance entre las señales apoptóticas y de supervivencia determina la respuesta definitiva de la célula al TNF-α (Aggarwal, 2003). En esta Tesis Doctoral se investigaron también los procesos apoptóticos que se activan en respuesta a la estimulación con TNF-α en leucocitos humanos, especialmente cuando la citoquina se usa en combinación de cicloheximida (CHX), un inhibidor de la síntesis proteica que bloquea la vía de supervivencia activada por el TNF-α. Así, los resultados obtenidos indican que los leucocitos humanos se vuelven más susceptibles a TNF-α cuando son tratados en presencia de CHX (Espino y cols., 2013), lo cual concuerda con estudios previos que demuestran que es necesario suprimir la síntesis de proteínas para sensibilizar a la mayoría de células a la apoptosis inducida por TNF-α (Wang y cols., 1998; Yamashita y cols., 1999). Además, los resultados que hemos encontrado sugieren

que la estimulación de los leucocitos con TNF-α/CHX provoca la degradación de cFLIP, el procesamiento y activación de la caspasa-8, y la consiguiente iniciación de la cascada de caspasas (Espino y cols., 2013), tal y como se ha demostrado para otros tipos celulares (Muzio y cols., 1998; Wang y cols., 2008). Asimismo, resultados de esta Tesis han puesto de manifiesto que el proceso apoptótico activado por el tratamiento con TNF-α/CHX es dependiente de la degradación de Bid y por tanto implica, al menos en parte, el bucle de amplificación mitocondrial (Espino y cols., 2013). En este aspecto, la generación intracelular de ERO parece ser necesaria para la activación de la caspasa-9, aunque ninguno de estos dos eventos pareció ser imprescindible para la apoptosis inducida por TNF-α/CHX, ya que la inhibición farmacológica de la producción de ERO tan sólo bloqueó parcialmente la apoptosis inducida por TNF-α/CHX (Espino y cols., 2013). La implicación de las ERO en la apoptosis extrínseca de leucocitos es un tema controvertido ya que recientes investigaciones han descrito que la apoptosis extrínseca de neutrófilos depende de la producción intracelular de ERO (Geering y cols., 2011), mientras que otros estudios han demostrado que la apoptosis de leucocitos inducida por la combinación de TNF-α/CHX no implica la generación de ERO (Cowburn y cols., 2005).

Por otro lado, en esta Tesis hemos demostrado que la melatonina es capaz de contrarrestar la apoptosis de leucocitos inducida por TNF-α/CHX, lo cual puede ser parcialmente atribuible a la capacidad antioxidante de la indolamina (Espino y cols., 2013). Es bien conocido que la melatonina presenta propiedades citoprotectoras en células normales, probablemente, debido a sus habilidades como secuestrador de radicales libres (Martín y cols., 2002b); sin embargo, los efectos antioxidantes de la indolamina parecen insuficientes para explicar la gran cantidad de acciones anti-apoptóticas mostradas por la melatonina en este conjunto de experimentos relativos al objetivo nº 3. De hecho, la inhibición de la producción intracelular de ERO con el antioxidante N-acetilcisteína fue incapaz de revertir la activación de la caspasa-8, la degradación de cFLIP o el procesamiento de Bid, entre otros eventos apoptóticos. No obstante, la melatonina no sólo fue efectiva bloqueando la activación de la caspasa-3 y -9 provocada por el tratamiento con TNF-α/CHX, sino que también consiguió

restablecer los niveles de proteína cFLIP, evitando de ese modo el procesamiento de la caspasa-8 y la consecuente degradación de Bid (Espino y cols., 2013).

A la luz de los experimentos llevados a cabo en esta Tesis, podría decirse que las acciones anti-apoptóticas de la melatonina requieren probablemente la participación de los receptores de membrana MT1/MT2. De hecho, la inhibición farmacológica de la interacción de la melatonina con los receptores MT1/MT2 mediante el antagonista luzindol bloqueó de manera sustancial los efectos inhibitorios de la melatonina sobre la activación de caspasa-3 estimulada por el tratamiento con TNF-α/CHX, (Espino y cols., 2013). En este sentido, estudios previos han descrito que la indolamina antagoniza la apoptosis a través de sus receptores de membrana en varios tipos celulares. Así, se ha sugerido que la protección proporcionada por la melatonina a las motoneuronas (Das y cols., 2010) o los espermatozoides humanos (Espino y cols., 2011a) está mediada por la activación de los receptores MT1 y/o MT2. De modo similar, Radogna y colaboradores (2008) aportaron evidencias experimentales sobre cómo la melatonina, a través de su interacción con los receptores MT1/MT2, puede activar una vía de señalización que depende de Bcl-2 y, de esta manera, puede modular la apoptosis y la supervivencia de la célula.

En relación a las MAPK, estas proteínas representan un centro de integración clave para el procesamiento de las señales de muerte y supervivencia que se originan a partir de distintos estímulos tanto endógenos como exógenos (Xia y cols., 1995; Junttila y cols., 2008). En este contexto, la cascada de señalización MAPK/ERK se expresa de manera constitutiva en células inmunes y se piensa que su activación juega un papel fundamental en el mantenimiento de la expresión de genes que previenen la activación del programa apoptótico (Kurland y cols., 2003; Monick y cols., 2008). En consecuencia, estudios recientes han demostrado que la activación de la vía de señalización MAPK/ERK representa un prerrequisito para suprimir la apoptosis inducida por distintos estímulos (Junttila y cols., 2008). En esta Tesis, hemos demostrado que la melatonina antagoniza la apoptosis estimulada por el tratamiento con TNF-α/CHX probablemente mediante la vía de supervivencia dependiente de ERK, ya que la inhibición farmacológica de la cascada de señalización de ERK con PD-98059 bloqueó los efectos anti-apoptóticos de la melatonina (Espino y cols., 2013). De esta

manera, el análisis del contenido proteico mediante western blotting reveló que la melatonina indujo la activación fosforilativa de ERK, tanto en presencia como en ausencia del estímulo apoptótico (Espino y cols., 2013). Además, la activación de ERK por acción de la melatonina fue bloqueada mediante la inhibición con luzindol de la interacción de la melatonina con los receptores MT1/MT2 (Espino y cols., 2013). Asimismo, nuestros resultados han indicado que la manifestación plena de los efectos de la melatonina sobre la apoptosis de los leucocitos inducida por TNF-α/CHX requería de una comunicación intacta entre los receptores de membrana MT1/MT2 y la cascada de señalización MAPK/ERK, puesto que el bloqueo de los receptores MT1/MT2 con luzindol o la inhibición de la vía de señalización de ERK con PD-98059 interfería con el mecanismo anti-apoptótico de la melatonina (Espino y cols., 2013).

En general, el modelo propuesto por este conjunto de resultados correspondientes al objetivo nº 3 de la presente Tesis es que los receptores MT1/MT2, los cuales son activados por la melatonina, desencadenan una cascada de señalización dependiente de ERK que modula los niveles de la proteína anti-apoptótica cFLIP, regulando de este modo la apoptosis y la supervivencia de los leucocitos humanos (Espino y cols., 2013). En este sentido, estudios previos han sugerido que la vía de supervivencia MAPK/ERK es necesaria para las acciones protectoras de la melatonina frente a la apoptosis inducida por luz ultravioleta en células U937 (Luchetti y cols., 2009). Igualmente, a pesar de que nosotros hemos descartado la implicación de la ruta de PI3K en los mecanismos anti-apoptóticos de la melatonina, investigaciones recientes han destacado que las propiedades neuroprotectoras de la melatonina en astrocitos están mediadas a través de la activación de los receptores de membrana MT1/MT2 y de la vía de señalización de PI3K/Akt (Kong y cols., 2008).

Por último, entre las diferentes teorías del envejecimiento, destaca la teoría de los radicales libres, la cual propone que el envejecimiento y algunas enfermedades relacionadas con la edad son, al menos en parte, una consecuencia del estrés oxidativo (Harman, 1992). En realidad, aunque no se ha establecido una relación directa entre envejecimiento y apoptosis, algunas observaciones sugieren que los efectos del estrés oxidativo sobre el envejecimiento podrían estar mediados a través de la regulación que las ERO ejercen sobre la apoptosis (Pollack y Leeuwenburgh, 2001; Zhang y

Herman, 2002). Como ya se ha comentado, la melatonina y sus metabolitos son potentes secuestradores de radicales libres (Tan y cols., 1993; Reiter y cols., 2009b) capaces de contrarrestar la apoptosis (Juknat y cols., 2005; Mayo y cols., 1998b). En relación al objetivo nº 4, resultados de esta Tesis han probado que las sobrecargas de calcio intracelular, provocadas por fMLP y/o tapsigargina, resultan en una capacidad menos eficiente de los leucocitos de individuos de edad avanzada para escapar de la apoptosis, mientras que la administración de melatonina proporciona a la célula una mayor probabilidad de supervivencia frente a la apoptosis (Espino y cols., 2011c). En concreto, nuestros resultados muestran que la melatonina es capaz de revertir, de manera eficiente, la pérdida del potencial de membrana de la mitocondria y la consiguiente activación de caspasas en leucocitos procedentes de personas mayores de 65 años, lo cual sugiere que las consecuencias beneficiosas derivadas de la administración de melatonina dependen probablemente de sus efectos sobre la fisiología mitocondrial (Espino y cols., 2011c). A este respecto, se ha demostrado mediante experimentos tanto *in vitro* como *in vivo* que la melatonina puede influir sobre la homeostasis mitocondrial. Así, la melatonina estabiliza la MMI mejorando, de este modo, la actividad de la cadena de transporte de electrones (García y cols., 1999). Además, la indolamina incrementa la actividad de los complejos I y IV de la cadena respiratoria mitocondrial tanto en tejido cerebral como en hígado (Martín y cols., 2000). La melatonina también mantiene la eficiencia de la fosforilación oxidativa y estimula la síntesis de ATP (León y cols., 2005; Carretero y cols., 2009). Finalmente, resultados recientes indican que la indolamina es capaz de prevenir la oxidación de la cardiolipina mitocondrial, la cual está implicada en la regulación de la bioenergética mitocondrial, de la apoptosis mitocondrial, y de la dinámica mitocondrial (Paradies y cols., 2010).

No obstante, no podemos descartar que los efectos inhibitorios de la melatonina sobre la apoptosis de leucocitos procedentes de individuos mayores de 65 años sean debidos a la capacidad antioxidante de la indolamina. De hecho, hemos demostrado que la administración de melatonina reduce los niveles de ERO tanto en condicionas basales (sin estímulo apoptótico) como tras la inducción de apoptosis mediante sobrecargas de calcio intracelular, lo cual se correlaciona con menores porcentajes de células apoptóticas, tal y como se observa por la disminución de células positivas para la

tinción de TUNEL tras la estimulación con melatonina (Espino y cols., 2010c; 2011c). Otro posible mecanismo implicado en las acciones anti-apoptóticas de la indolamina durante el envejecimiento estaría mediado por sus receptores de membrana, puesto que se ha descrito que el tratamiento con melatonina en modelos de senescencia de ratón activa las vías de supervivencia celular, como la señalización mediada por sirtuínas o la vía PI3K/Akt, al mismo tiempo que inhibe la apoptosis intrínseca (Gutiérrez-Cuesta y cols., 2008; Tajes Orduña y cols., 2009).

5. Conclusiones

De los resultados obtenidos en la presente Tesis Doctoral en la que nos propusimos estudiar los mecanismos implicados en las propiedades anti-apoptóticas de la melatonina en células inmunocompetentes no tumorales, como son los leucocitos humanos, valorando la participación de los receptores de membrana de la melatonina en dichos mecanismos, así como la acción directa de la indolamina a través de su capacidad antioxidante, podemos concluir que:

1. La melatonina es capaz de revertir la activación de la caspasa-3 y -9 inducida por sobrecargas de calcio intracelular, lo cual se debe aparentemente a la modulación tanto de la apertura del PPT de la mitocondria como de la activación de Bax por acción de la indolamina.

2. Los efectos protectores resultantes de la administración de melatonina sobre la apoptosis mitocondrial de leucocitos humanos inducida por aumentos sostenidos en la $[Ca^{2+}]_c$ parecen ser dependientes de las propiedades antioxidantes de la indolamina.

3. La melatonina reduce la apoptosis inducida por TNF-α mediante la interacción con los receptores MT1/MT2 y la activación fosforilativa de ERK, al mismo tiempo que inhibe el estallido oxidativo inducido por el tratamiento con TNF-α posiblemente debido a sus acciones como secuestrador de radicales libres.

4. La melatonina puede limitar o revertir algunos de los cambios que ocurren durante la inmunosenescencia, ya que es capaz de retrasar la apoptosis y previene el estrés oxidativo inducidos por sobrecargas de calcio en leucocitos procedentes de individuos mayores de 65 años.

CONCLUSIÓN GENERAL:

La melatonina proporciona efectos protectores frente a la apoptosis mitocondrial de leucocitos humanos al mismo tiempo que previene algunos cambios que acontecen durante la inmunosenescencia, aparentemente debido a sus propiedades antioxidantes y a la regulación que ejerce sobre la homeostasis mitocondrial. Asimismo, la indolamina es capaz de modular la apoptosis extrínseca de leucocitos humanos mediante la interacción con sus receptores de membrana y la activación de la vía de señalización MAPK/ERK.

5. Conclusions

From the results obtained in the present PhD Thesis, in which we intended to study the mechanisms involved in the anti-apoptotic properties of melatonin in immunocompetent non-tumor cells, evaluating the involvement of melatonin membrane receptors in these mechanisms and the direct action of the indoleamine through its antioxidant capacity, we can conclude that:

1. Melatonin is able to reverse caspase-3 and -9 activation triggered by intracellular calcium overload, which is likely due to modulation of mitochondrial permeability transition pore opening and Bax activation.

2. The protective effects resulting from melatonin administration on mitochondrial apoptosis of human leukocytes seem to be dependent on melatonin´s antioxidant properties.

3. Melatonin requires membrane receptor MT1/MT2 interaction and ERK phosphorylative activation in order to forestall TNF-α–induced leukocyte apoptosis, on the one hand, and counteracts TNF-α–evoked oxidative burst possibly owing to its free radical scavenging actions, on the other hand.

4. Melatonin may limit or reverse some of the changes that occur during immunosenescence, since it is able to delay apoptosis and prevent oxidative burst induced by intracellular calcium overload in leukocytes from elderly individuals.

GENERAL CONCLUSION:

Melatonin provides protective effects against mitochondrial apoptosis of human leukocytes while preventing some changes that occur during immunosenescence, apparently due to its antioxidant properties and the regulation exerted on mitochondrial homeostasis. Also, this indoleamine may modulate extrinsic apoptosis of human leukocytes through interaction with membrane receptors and the activation of MAPK/ERK signaling.

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7. Anexo

En este apartado se incluye un review publicado que engloba resultados de la presente Tesis Doctoral.

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Review Article

Oxidative Stress and Immunosenescence: Therapeutic Effects of Melatonin

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Age-associated deterioration in the immune system, which is referred to as immunosenescence, contributes to an increased susceptibility to infectious diseases, autoimmunity, and cancer in the elderly. A summary of major changes associated with aging in immune system is described in this paper. In general, immunosenescence is characterized by reduced levels of peripheral naïve T cells derived from thymus and the loss of immature B lineage cells in the bone marrow. As for macrophages and granulocytes, they show functional decline with advancing age as evidenced by their diminished phagocytic activity and impairment of superoxide generation. The indole melatonin is mainly secreted in the pineal gland although it has been also detected in many other tissues. As circulating melatonin decreases with age coinciding with the age-related decline of the immune system, much interest has been focused on melatonin's immunomodulatory effect in recent years. Here, we underlie the antioxidant and immunoenhancing actions displayed by melatonin, thereby providing evidence for the potential application of this indoleamine as a "replacement therapy" to limit or reverse some of the effects of the changes that occur during immunosenescence.

1. Introduction

All organisms experience the inevitable biological process referred to as aging. In general, aging is characterized by a time-dependent functional decline that leads to increased morbidity and mortality as a consequence of the cell's incapacity to face external and internal challenges. Although aging is an extremely complex, a multifactorial process that has been the subject of considerable speculation, accumulated evidence identifies free radicals as a source of damage to cellular structure and function [1].

Among the countless theories proposed for aging, the free radical theory of aging (also known as oxidative stress theory) put forward by Harman in 1956 [2] has received extensive support. This theory proposes that organismal deterioration that occurs as a result of increasing longevity is specially a consequence of the persistent accumulation of free radical-mediated damage to essential molecules, which gradually compromises the function of cells, of tissues, and eventually of the organism itself [3]. Consequently, aging may be viewed as a process of irreversible injuries associated with accumulated oxidative debris.

Since it was posed, the oxidative stress theory of aging has been continuously studied and modified [4, 5], giving a central involvement of mitochondria in determining the timing of senescence, that is, lifespan, as these organelles generate a disproportionately large amount of oxygen-based free radicals and related nonradical species in cells [6]. Nevertheless, despite the fact that the mitochondrial oxidative stress theory of aging is one of the most plausible theories for explaining aging, it has also received some criticisms in the last few years since some groups have proven that knockout mice for antioxidant enzymes did not show any sign of accelerated aging, thus suggesting that mitochondrial oxidative stress may not be causal for age-related degenerative phenomena [7].

Traditionally, oxygen-based free radicals are designated as reactive oxygen species (ROS), whereas nitrogen-based toxic reactants are generally referred to as reactive nitrogen species (RNS). Both ROS and RNS arbitrarily mutilate

macromolecules in the area of where they are produced, this mutilation leading, in many cases, to death of the cell via programmed cell death or apoptosis [8, 9]. Oxidative stress is a condition in which the redox balance between oxidants and antioxidants is disrupted, thereby tilting the equilibrium towards an oxidized state [10]. To counteract the harmful actions of ROS, aerobic cells are equipped with a series of antioxidant enzymes that metabolize toxic reactants to less reactive or totally innocuous molecules. Superoxide dismutases (SODs), glutathione peroxidase (GPx), and catalase are among these antioxidative enzymes. However, this protective machinery seems to be impaired with aging. In particular, SOD activity has been shown to decrease in aged individuals [11–13] although this finding remains disputed [14]. Conversely, catalase and glutathione peroxidase activities have been reported to be augmented with aging, which might reflect a compensatory response to extremely elevated basal levels of ROS/RNS in cells from aged individuals [13].

Furthermore, melatonin is a powerful antioxidant produced naturally by the pineal gland that exhibits relevant antiaging properties [15–18]. Obviously, the use of therapeutic drugs that are intended to improve the quality of life in the elderly implies the identification of molecules that have both antioxidant and immunoenhancing capabilities. In this sense, some of the evidence suggesting that melatonin is efficient to combat age-related deterioration in immune function will be summarized and discussed in this paper with the objective of fostering melatonin as a potential therapeutic agent for enhancing overall quality of life in the elderly.

2. Aging and the Immune System

During aging, the immune system loses functionality and responsiveness. This deterioration is closely linked to a decreased capacity of the immune system to respond to antigenic stimulation and contributes to the increased susceptibility to infectious diseases and cancer in the elderly [19]. This age-associated decline in immune function, which is known as immunosenescence, results in altered cytokine microenvironment and impairment of both innate and adaptive immunity [20].

In general, all immune cells are affected by aging, thereby contributing to the high vulnerability to infections and increased mortality observed in the elderly [21]. Concerning the macrophage, it has been suggested that the existence of a direct relation between age and macrophage activation seems to be responsible for the presence of a subclinical chronic inflammatory process in the elderly. This increase in proinflammatory status at an organismal level, caused by chronic age-related stimulation of the macrophage, is referred to as "inflamm-aging" [22]. Thus, enhanced macrophage ability to produce proinflammatory mediators such as interleukin (IL)-1, IL-6, and IL-8 occurs in both healthy aged subjects and individuals showing pathological aging [22, 23]. Nonetheless, this phenomenon is only a part of the whole spectrum of change characteristic of immunosenescence, and indeed the macrophage is not the only cell involved in the aging process. The progressive functional T and B lymphocyte deficits have been also suggested as the main responsible factors for age-associated disorders [24]. Certainly, lymphocytes are also largely affected during immunosenescence, and the continuous age-related antigenic stress provokes a variety of changes even in the most evolutionarily recent immune system. These alterations include the expansion of memory B cells, the decrease and even the exhaustion of naïve T cells, and the shrinkage of the T-cell repertoire [25]. Likewise, the reduction both in the number of naïve T cells and in their responsiveness with increased age causes the decline of specific immunization response in aged individuals [26].

As for granulocytes, a functional impairment of these cells has been found in elderly individuals, including diminished intracellular phagocytic capacity, decreased chemotactic activity, degranulation in response to Gram-positive bacteria, and reduced ability to respond to survival factors such as granulocyte macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), and lipopolysaccharides (LPS) [27, 28]. In this vein, the attenuation of Fc-mediated phagocytosis in the elderly has been suggested as the major factor for the age-related decline in neutrophil function [27, 28]. Moreover, a reduction in superoxide production of granulocytes has been reported in centenarians, irrespective of subject's health conditions [29].

Furthermore, the activity of natural killer (NK) cells during aging has been extensively studied, and different results have been reported. Strikingly, the most consistent data indicate an increase in cells with high NK activity with advancing age [29, 30]. In fact, cells from healthy centenarians can efficiently kill target cells [31]. This age-associated increase in NK cell number has been interpreted as a compensatory response to overcome the generally decreased immune function that could otherwise trigger neoplastic growth [32]. However, it has been found that aging may severely affect cytokine production of NK cells. Indeed, NK cells of elderly subjects exhibited a diminished production of cytokines in response to IL-2 [33]. Similarly, it has been shown a substantial impairment in the production of mRNA transcripts encoding several cytokines in NK/LAK (lymphokine-activated killer) cells of aged mouse [34].

3. Oxidative Stress and Immunosenescence

Although aging is not considered as a disease by itself, it makes the organism more vulnerable to many of them, including diabetes, obesity, atherosclerosis, cardiovascular diseases, and neurodegenerative diseases [35]. Two essential biochemical mechanisms link immunosenescence to oxidative stress: a reduction in cellular functions owing to oxidative damage of proteins, lipids, and carbohydrates and apoptotic cell death triggered by the accumulation of oxidative debris. The increased amount of free radicals observed in many aged cells has been reported in cells of the immune system as well [36]. In addition, the levels of MnSOD, which is an antioxidant enzyme located in

the mitochondria and protects macrophages from apoptosis induced by oxidized low-density lipoprotein (LDL), are also decreased in aging macrophages [37] thereby contributing to the increased cellular oxidative stress [36, 38].

The observed oxidative modifications occurring on different macromolecules have been shown to compromise the functionality of subcellular organelles, compartments, and membranes [39]. In this sense, the alterations in membrane lipids composition and function due to an increase in the amount of polysaturated and oxidized fatty acids affect activation of T cells and then contribute to human immunosenescence [40, 41]. Moreover, change and damage to the membrane composition also influence receptor-mediated functions of dendritic cells, including the phagocytic clearance of pathogens [42]. Likewise, a consistent decline in the proteolytic activity of the proteasome has been demonstrated with advancing age, implicating an important role for the proteasome in immunosenescence. Besides the inability to clear-damaged proteins, loss in proteasomal proteolysis has far-reaching implications within the immune system. This includes lowered T-cell functional response, reduced antigenic peptide generation for binding to MHC (major histocompatibility complex) class I molecules, decreased maturation of dendritic cells, and, ultimately, dysregulated proliferation because of altered regulation of the cell cycle [43]. Finally, T and B cell plasma membrane receptors, which are directly involved in immune recognition, have also been shown to be affected by oxidative stress. As a matter of fact, it has been reported that many events of T-cell receptor (TCR) signal transduction, such as protein tyrosine kinase (PTK) and mitogen-activated protein kinase (MAPK) activation, are known to be altered with advancing age due to oxidative modification [44]. Additionally, oxidative inactivation of the CD45 protein tyrosine phosphatase was also described to contribute to T-cell dysfunction in the elderly [45].

A second link between oxidative stress and immunosenescence is the induction of cellular apoptosis following the accumulation of oxidized molecular aggregates. Apoptosis is crucially involved in the age-related remodeling of the immune system, which includes thymic involution and alterations in T cells [46, 47]. In this regard, oxidative stress contributes to damage-induced apoptosis by increasing the number of cells undergoing cell death as a result of the accumulation of oxidatively damaged molecules [48], as shown in aged human leukocytes [49, 50]. In fact, we have observed that unstimulated neutrophils and lymphocytes isolated from elderly patients accumulate higher amounts of ROS, present decreased SOD activity, and are less resistant to cell death compared to those cells obtained from young individuals (Table 1). Furthermore, we have determined that aged neutrophils and lymphocytes are more vulnerable to apoptosis triggered by intracellular calcium overload than those cells obtained from young subjects, as ascertained by the activation of different apoptotic hallmarks (Table 2).

Apart from that, the accumulation of proteins modified with advanced glycation end products (AGEs) has been shown to induce T-cell apoptosis in an oxidative stress-associated and caspase-dependent manner with involvement of the mitochondrial pathway [51]. Similarly, studies in cultured macrophages indicated a positive correlation between exposure to oxidized LDL and cell death [37]. Moreover, the stimulation of macrophages with serum glycated proteins, such as pentosidine, a well-characterized AGE found in plasma and tissue of diabetic and uremic subjects, also leads to a loss of cell viability and presumably to cell death [52].

Table 1: Reactive oxygen species (ROS) levels, superoxide dismutase (SOD) activity and rates of cell death in unstimulated leukocytes from young and elderly individuals.

	Neutrophils		Lymphocytes	
	Young	Aged	Young	Aged
ROS levels	+	+++	+	++
SOD activity	++	+	++	+
Rate of cell death	+	++	+	++

The levels of ROS, SOD activity, and rate of cell death were all classified on a scale of + (indicating lowest level, activity or rate) to +++ (indicating highest intensity level, activity or rate). Data were taken from Espino et al. 2010 [49].

Table 2: Activation of different apoptotic events in thapsigargin-treated leukocytes from young and elderly individuals.

	Neutrophils		Lymphocytes	
	Young	Aged	Young	Aged
MMD	+	+++	+	+++
Caspase-9 activity	+	+++	+	+++
Caspase-3 activity	+	++	+	++
PS exposure	+	++	+	++
DNA fragmentation	+	++	+	++

The degree of apoptosis was classified on a scale of + (indicating lowest apoptosis) to +++ (indicating highest apoptosis). MMD: mitochondrial membrane depolarization; PS: phosphatidylserine. Data were taken from Espino et al. 2011 [50].

4. Synthesis and Function of Melatonin

Melatonin, or *N*-acetyl-5-methoxytryptamine, is a widespread physiological mediator. It has been found in most organisms studied from bacteria to humans. The indole melatonin is mainly secreted in the pineal gland of vertebrates, although it is now known to be produced in many other tissues as well [53]. In the pineal gland, melatonin is converted in two steps from the amino acid tryptophan into serotonin (5-hydroxytryptamine), and then acetylated by arylalkylamine *N*-acetyltransferase (AA-NAT), before finally being converted into melatonin by hydroxyindole-*O*-methyltransferase (HIOMT), which represents the rate-limiting step in magnitude of melatonin biosynthesis [54]. The pineal gland synthesizes and releases melatonin primarily during the dark phase. Thus, melatonin levels in the circulation exhibit a distinctive circadian rhythm in which the highest blood concentration is observed at night, while baseline levels are measured during the day [55].

It is well known that endogenous melatonin production wanes in the elderly [56] and that the total antioxidative

capacity of serum correlates well with its melatonin levels in humans [57]. In this regard, the participation of melatonin in slowing the deterioration of tissues and organs due to aging has been proposed many times. Thus, it has been shown that the removal of the pineal gland early in life exaggerates molecular damage in terms of lipid peroxidation, accumulation of 8-hydroxy deoxyguanosine in the DNA, and levels of protein carbonyls, as well as reducing membrane fluidity in old animals [58], whereas exogenous administration of melatonin reduces lipid peroxidation [59]. These results, considered in light of the free radical theory of aging, suggest that the age-associated melatonin reduction may be linked to the increase in oxidative damage observed with age [60].

From a physiological perspective, melatonin has been classically related to the physiological adjustment in circadian rhythms and mediating seasonal reproductive events in photoperiodically dependent species. It also alters the function of other endocrine organs and may be involved in sleep regulation in at least diurnally active species [61]. Moreover, melatonin interacts with the cardiovascular system [62] and has been implicated in metabolic control [63]. From a pharmacological view, the phase-advancing effects of melatonin have been frequently exploited [64, 65], with the indoleamine proven to be effective in the treatment of insomnia [66, 67] and efficient in limiting jet lag when travelling across time zones [68].

Finally, melatonin has a particular ability to neutralize free radicals [69] and prevent tissue damage associated with oxidative stress. Thus, it exhibits both direct scavenging actions against free radicals and related products [70–72], as well as indirect antioxidative actions via its ability to stimulate the cellular antioxidant defense system by increasing mRNA levels and activities of several important antioxidant enzymes, including SOD [12], to promote the synthesis of another important intracellular antioxidant, that is, gluthatione [73], to reduce the activity of the prooxidative enzyme nitric oxide synthase [74], and to diminish free radical formation at the mitochondrial level by reducing the leakage of electrons from the electron transport chain [75]. Additionally, different studies have demonstrated its protective role against oxidative damage induced by drugs, toxins, and different diseases [49, 76–78]. This combination of actions makes melatonin an important agent in combating some signs of aging and/or the initiation of age-related diseases.

Apart from that, melatonin has been recently proven to exert antisenescence actions through the activation of SIRT1, a sirtuin that promotes cell survival by inhibiting apoptosis or cellular senescence in mammalian cells. Thus, it has been reported that melatonin increases SIRT1 expression, which reduces inflammatory and apoptotic signaling related to p53, and diminishes vasoconstriction via increasing nitric oxide bioavailability [79]. Likewise, in a murine model of senescence (SAMP8), melatonin protects neurons against frailty by enhancing SIRT1 expression [80], and subsequently decreasing the amount of the acetylated (active) form of p53 [81].

5. Therapeutic Effects of Melatonin on Immune Function

As the age-related decline of the immune system first appears around 60 years of age coinciding with the reduction of plasma melatonin concentration, much attention has been devoted to the possible interaction between melatonin and the immune system in the last decade [32, 82]. In 1986, Maestroni and collaborators first showed that blockade of melatonin synthesis causes the inhibition of cellular and humoral responses in mice [83]. From that point on, a variety of investigations has revealed several modulating actions of melatonin on immune system.

Exogenous administration of melatonin has been proven to stimulate the production of cells mediating the nonspecific immunity, that is, NK cells and macrophage/monocyte lineage cells, in both the bone marrow and the spleen [84–86]. As both these populations constitute the first line of defense against neoplastically transformed and virus-transfected cells, these findings account for melatonin's ability to halt neoplastic growth and to destroy virus-infected cells. Additionally, the action of melatonin on NK cells has been proposed to reflect, at least in part, the fact that NK cells are exquisitely sensitive to cytokines produced by melatonin-stimulated T helper cells, including IL-2, IL-6, IL12, and interferon (IFN)-γ [86], since the immunostimulatory role of melatonin is exerted mainly on both T helper cells and T-lymphocyte precursors [32]. Likewise, monocyte production stimulated by melatonin has been suggested to be driven either directly [87], because cells of this lineage do possess melatonin receptors [88], or indirectly, in response to the triggered cascade of monocyte-sensitive stimulants, such as IL-3, IL-4, IL-6, and GM-CSF, set in place by melatonin activation of T helper cells [84–86].

Furthermore, melatonin administration has been revealed to upregulate the level of gene expression of transforming growth factor (TGF)-β, macrophage-colony stimulating factor (M-CSF), tumor necrosis factor (TNF)-α and stem cell factor (SCF) in peritoneal exudates cells, and the level of gene expression of IL-1β, M-CSF, TNFα, IFN-γ, and SCF in splenocytes [89]. In addition, an inhibitory influence of melatonin on parameters of the immune function has also been demonstrated. Thus, melatonin has been shown to inhibit the production of proinflammatory cytokines, such as IL-8 and TNFα, in neutrophils [90], suggesting that the indolamine may help to reduce acute and chronic inflammation. Melatonin has been also reported to counteract the inhibitory effect of prostaglandin E2 on IL-2 production in human lymphocytes via its MT1 membrane receptor [91]. In this sense, it has been suggested that melatonin may be involved in the regulation of cytokine production by modulating the activity of T cells and monocytes via nuclear orphan receptor (RZR/ROR)-mediated transcriptional control [92, 93].

A correct modulation of apoptosis may be useful for prolonging the lifespan or at least reducing age-related degenerative, inflammatory, and neoplastic diseases whose incidence increases with age. In this sense, melatonin's immunoenhancing effect not only depends on its ability to

improve the production of cytokines, but also on its anti-apoptotic and antioxidant actions. The first data supporting a role for melatonin in drug-induced apoptosis appeared in 1994, when Maestroni and coworkers demonstrated that melatonin-rescued bone marrow cells from toxicity caused either *in vivo* or *in vitro* by anticancer compounds, with this mechanism involving the endogenous production of GM-CSF [85]. Additionally, it has been indicated that orally administered melatonin can substantially boost the survival of newly formed B cells in mouse bone marrow, thereby providing evidence for a role of melatonin as a checkpoint regulator in early B-cell development [94]. Interestingly, Tan and colleagues found extremely high levels of melatonin in bone marrow cells of rats, with melatonin concentrations in the bone marrow being two orders of magnitude higher than in the circulation [95]. Given the high sensitivity of bone marrow cells to oxidative agents, for example, anticancer agents, the presence of high melatonin concentrations in bone marrow cells could be important to preserve their integrity.

The severe loss of thymocytes with age is the main cause of structural thymic atrophy and thymic weight loss. In this respect, it has been indicated that melatonin administration rejuvenates degenerated thymus and redress peripheral immune dysfunctions in aged mice [96]. This reversal of age-related thymic involution by melatonin is attributable to increments in thymic cellularity caused by both the antiapoptotic and the proliferative-enhancing effects of melatonin [97] although other mechanisms involving glucocorticoid receptor cannot be ruled out [98].

The antioxidant ability of melatonin and its metabolites may also account for its antiapoptotic actions on immune cells [99]. In fact, we have demonstrated that melatonin is able to inhibit intracellular calcium overload-induced leukocyte apoptosis by blocking caspase-9 and caspase-3 processing, which is mainly due to the modulation of both the opening of the mitochondrial permeability transition pore and the activation of the proapoptotic protein Bax [77]. Moreover, we have also proved that the beneficial effects resulting from melatonin administration on leukocyte apoptosis likely depend on melatonin's antioxidant properties, since this protection is unaffected by the MT1/MT2 antagonist luzindole, that is, independent of plasma membrane MT1/MT2 receptor stimulation [78]. More interestingly, melatonin is able to delay damage-induced apoptosis in aged neutrophils and lymphocytes and therefore may counteract, at the cellular level, age-related degenerative phenomena linked to oxidative stress [50]. This fact is especially remarkable as neutrophils from aged individuals show a diminished rescue capacity when challenged with proinflammatory stimuli, such as GM-CSF, GCSF, LPS, or IL-2 [100, 101].

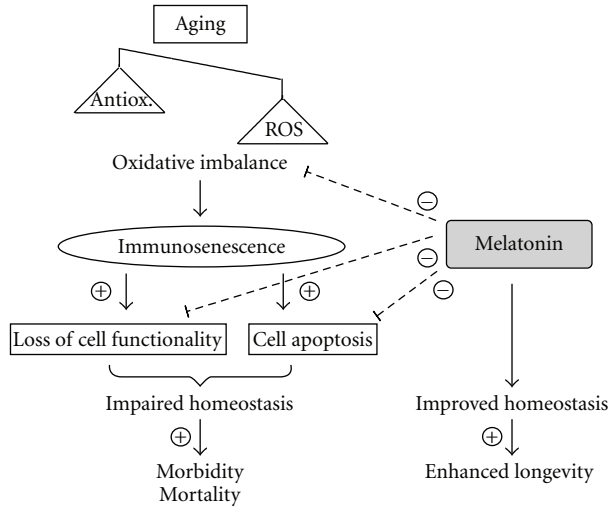

Figure 1: Schematic diagram depicting beneficial effects of melatonin on age-associated deterioration of immune system.

6. Concluding Remarks

The age-associated decline in immune function, known as immunosenescence, is characterized by a decrease in the functional activity of NK cells, granulocytes, and macrophages. In general, these age-associated changes in the immune system render organisms more sensitive to infections, autoimmune diseases, and even to cancer (Figure 1). In the last few years, growing evidence has indicated a tight, cause-effect link between oxidative stress and immunosenescence. Strikingly, several studies have highlighted the reversibility of some of the changes induced by oxidative stress with advancing age, which is particularly important for most cells in the immune system which, having a very short lifespan, are constantly replaced by newly produced elements. Therefore, these cells can potentially benefit from short-term therapies aimed at decreasing oxidative stress. In this sense, it is worth noting the possibility that melatonin supplementation could prevent or delay the functional deterioration of the immune system that accompanies aging and, perhaps, return it to that of the "younger" situation (Figure 1). Indeed, dietary supplementation of melatonin has been shown to ameliorate the attenuated immune responses associated with senescence [102]. Likewise, melatonin-enriched foodstuffs has been proven to modulate serum inflammatory markers in both rats and ringdoves, causing a reduction in proinflammatory markers along with an increase in anti-inflammatory markers which suggests amelioration or reorganization of immunity to a noninflamed state [103, 104]. Based on the experimental data that have accumulated and considering its lack of toxicity, its high lipophilicity, and its large capacity to forestall cell damage, melatonin is one of the most appealing agents to be examined in relation to age-associated deterioration in the immune system and should be considered as a potential agent to improve the quality of life in a rapidly aging population.

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