Melatonin Counteracts Ischemia-Induced Apoptosis in Human Retinal Pigment Epithelial Cells

Neville N. Osborne, Mark S. Nash, and John P. M. Wood

PURPOSE. To investigate whether the neurohormone melatonin can prevent apoptosis caused by deprivation of oxygen, glucose, and serum (experimental ischemia) in cultured human retinal pigment (RPE) cells.

METHODS. Cultures of human RPE cells established from a variety of donors were grown to passage four and then subjected to experimental ischemia, with or without various substances, for up to 72 hours. Cells were examined for morphologic changes and breakdown of DNA, assessed by TdT-dUTP terminalnick-end labeling (TUNEL) and agarose gel electrophoresis. Changes in transcription and translation of various proto-oncogenes (bcl-2, TIAR, ICH-1a, ICH-1b) were assessed by analysis of mRNA and protein levels, respectively. The effect of various substances on the iron-ascorbate-induced formation of reactive oxygen species (ROS) in chick retinal dissociates was also investigated.

RESULTS. Cultured human RPE cells on coverslips that were incubated in serum-free medium, glucose, and oxygen remained viable for up to 40 hours. Thereafter, there was a steady decrease in cell numbers and an increase in the number of cells labeled by the TUNEL method. By 72 hours 65% of cells remained attached to the coverslips, of which approximately 65% were TUNEL positive. Furthermore, most of the experimental ischemia-treated cells exhibited a shrunken appearance typical of apoptosis. Fragmentation of the DNA from cells in which ischemia was induced for 72 hours was also confirmed by agarose gel electrophoresis. Inclusion of 100 μM melatonin significantly decreased the amount of apoptotic cell nuclei after ischemia, but the effect was mild compared with that of fetal calf serum, which almost completely counteracted cell death. The action of melatonin was not prevented by 0.01 mM to 1 mM luzindole, a melatonin receptor antagonist. In addition, 100 μM ascorbate did not counteract ischemia-induced apoptosis. Treatment of RPE cells with 100 μM flupirtine gluconate for 72 hours caused an upregulation of the proto-oncogene protein Bcl-2 and a decrease in TIAR and ICH-1 proteins compared with that in control cells. Melatonin at 100 μM had no such effect. The levels of the mRNA transcripts for ICH-1L relative to those for ICH-1S were significantly decreased in cultures treated with 100 μM flupirtine or 100 μM melatonin when compared with levels in control cells. However, the effect of flupirtine was greater than that of melatonin. Ten micromolar ascorbate and 5 μM iron stimulated the formation of ROS in chick retinal cell dissociates. Ascorbate, melatonin, and flupirtine (all at 100 μM) blunted this response in the order flupirtine > melatonin > ascorbate. Luzindole had no effect, alone or in the presence of melatonin.

CONCLUSIONS. The presented data show that melatonin counteracted ischemia-induced apoptosis in human RPE cells by a process that seemed to be independent of melatonin receptors. Moreover, melatonin and flupirtine counteracted iron-ascorbate-induced ROS formation and decreased the ratio of mRNA for ICH-1L and ICH-1S. However, melatonin was less potent than flupirtine in its action in each case, which suggests that either the two compounds act on different signaling pathways or that they act on the same pathway with differing potency. The failure to detect an effect of melatonin on the levels of Bcl-2, ICH-1L, and TIAR proteins when compared with the effect of flupirtine was probably caused by the sensitivity of the procedures. It is suggested that substances that can prevent ROS formation can potentially nullify apoptotic cell death, but this is difficult to detect experimentally when the substance has only a mild effect, such as in the case of ascorbate. (Invest Ophthalmol Vis Sci. 1998;39:2374-2383)
Apoptosis is one of two major mechanisms by which cells die, the other being necrosis. The preferred form of cell death in aging is apoptosis, in that it does not induce an inflammatory response, and it is under tighter control. Good evidence exists to show that low levels of ROS formation cause apoptosis, whereas nonphysiological levels of oxidants cause necrosis. Treatment of cells with low levels of ROS causes apoptosis. Antioxidants have been found to block apoptosis in several systems, including that mediated by experimental ischemia, tumor necrosis factor-α, and glucocorticoids. It is also significant that the Bcl-2 proto-oncogene protein prevents apoptosis in diverse systems and has also been shown to block oxidative damage.

Recently, melatonin has also been shown to act in a way similar to an antioxidant, scavenging 'OH and ROO' radicals, to stimulate the enzyme glutathione peroxidase and increase the expression of mRNA for manganese and copper-zinc superoxide dismutases. Additional support for the antioxidant characteristics of melatonin comes from a variety of in vitro experiments that have shown that the substance counteracts the formation of ROS. It is these antioxidant characteristics of melatonin that may explain why neurons are protected against kainate-induced apoptosis and necrosis. However, because melatonin receptors are known to be associated with many neuron types, it is possible that they play a part in the neuroprotective action of melatonin.

It is known that melatonin is synthesized in the pineal gland and the retina. Available evidence suggests that melatonin is involved in the physiological release of dopamine in the retina and in retinal pigment epithelial (RPE) cells. We have recently shown that when confluent cultured RPE cells are subjected to experimental ischemia or oxidative stress (serum-free medium, glucose, and oxygen) the cells die by apoptosis. This process may be prevented by the compound flupirtine. Flupirtine has been shown to stimulate glutathione peroxidase and prevent formation of ROS in vitro, characteristics that are also associated with melatonin. The described experiments were therefore undertaken to examine whether melatonin can protect RPE cells from experimental ischemia-induced apoptosis and to determine whether it does so in a way similar to flupirtine.

**METHODS**

**ROS Assay**

The method used was based on that described by LeBel and Bondy. It involves the use of the nonfluorescent probe 2'7-dichlorofluorescein diacetate (DCFH-DA; Molecular Probes, Leiden, The Netherlands) which crosses cell membranes readily and, once present in the cell, is hydrolyzed to the nonfluorescent product 2',7'-dichlorofluorescein, which is oxidized by any ROS to form the highly fluorescent product 2',7'-dichlorofluorescein (DCF).

Freshly dissected 2-4-day-old chick retinas were dissociated into medium at 1 retina/ml (L-15; Gibco Life Technologies, Paisley, UK) as described previously. Chicks were obtained from Park Farm (Northmoor, UK) Aliquots of the suspended cells were incubated alone or with combinations of ascorbate, FeSO₄, flupirtine (flupirtine gluconate), melatonin, or deferoxamine mesylate and left to incubate for 60 minutes at 37°C. A spectrofluorometer (Perkin Elmer, Warrington, UK) was then used to record the fluorescence of the samples using an excitation wave length of 488 nm (bandwidth 10 nm) and an emission wavelength of 525 nm (bandwidth 10 nm). The temperature of the cuvette was maintained at 30°C, and fluorescence measurements were recorded at 15-minute intervals for a period of 60 minutes. The amount of fluorescent DCF formed was quantified by reference to a standard curve produced from known concentrations of DCF (Sigma, Poole, UK) and expressed as nanomolar DCF formed per hour per milligram protein.

**Human RPE Cell Culture**

RPE cell cultures were prepared as described by Nash and Osborne. Eyed were obtained 24 to 48 hours after death from donors 50 to 65 years of age. The culture medium consisted of Hams F-10 supplemented with 10% fetal calf serum (FCS), 0.4% glucose, 2 mM glutamine, 2.5 µg/ml amphotericin B, and 100 µg/ml gentamicin. Unsupplemented Hams F-10 contains approximately 4 mM glucose, and addition of the extra quantity of this sugar results in a final concentration of 20 mM. The primary cultures were grown in 25-cm² tissue culture flasks in an incubator at 35.5°C in 5% CO₂-95% air (moist environment) and passaged in a ratio of 1:3. After the third passage, some of the cells were grown on 13-mm glass coverslips in 24-well plates (seeding density, 10,000 cells/well).

**Experiments on Fourth-Passage Cultures Grown on Coverslips**

The solution was removed from confluent cultures and replaced with serum-free medium for 4 hours. This was replaced with medium that free of serum and supplemented glucose and often contained additional substances (serum, melatonin, or luzindole). The cultures were incubated under normal growth conditions at 35.5°C in 5%CO₂-95% air (moist environment) or in conditions containing reduced oxygen at 35°C in 5%CO₂-95%N₂ (moist environment). After various periods, the coverslips containing the cultures were removed, fixed for 30 minutes in 4% paraformaldehyde, and stained in 1% methylene blue or processed for breakdown of DNA by TdT-dUTP terminal nick-end labeling (TUNEL) in some instances the fixed cells were processed for the localization of Bcl-2 protein immunoreactivity.

For TUNEL, the fixed cells were treated as described previously. Total cell counts (stained with 1% methylene blue) and TUNEL-positive cells on coverslips were determined by means of a hemocytometer (Weber, London, UK). Cells in five fields outlined by the hemocytometer were determined and averaged to give the amount of cells and TUNEL-positive cells present on a coverslip. Some experiments were performed in parallel. The cells in one experiment were treated with 0.1 mg/ml DNase I for 15 minutes at 37°C and stained for TUNEL. The number of cells that had nuclei stained positively for TUNEL was compared with studies performed in parallel in which the total number of cells was determined by staining with 1% methylene blue.

For the localization of Bcl-2 immunoreactivity the fixed cells were incubated overnight at 4°C with 1:10 mouse monoclonal (Dako, High Wycombe, UK) or 1:50 rabbit polyclonal anti-human antibodies (Santa Cruz Biotechnology,
Santa Cruz, CA) and subsequently developed in 1:50 fluorescein isothiocyanate-conjugated rat anti-mouse or rat anti-rabbit IgG, respectively, in 0.1 M sodium phosphate-buffered saline (PBS) containing 0.2% Triton X-100. Detection of DNA fragmentation by agarose gel electrophoresis was also as described previously.\textsuperscript{16}

**Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis and Western Blot Analysis**

Confluent RPE cells grown in flasks were harvested and samples for electrophoresis prepared as already described.\textsuperscript{13} An aliquot was taken for determination of proteins by the method of Bradford.\textsuperscript{40} Electrophoresis of samples was performed by the method of Laemmli\textsuperscript{43} using 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate. Proteins were transferred to nitrocellulose according to the method of Towbin et al.\textsuperscript{44} Before immunostaining was continued, the membranes were stained with a 0.1% solution of Ponceau S to confirm that the total amount of protein loaded for each sample was the same. The blots were then labeled for the localization of Bcl-2 protein with 1:50 polyclonal antibodies (Santa Cruz Biotechnology) and 1:100 monoclonal antibodies (Dako). Monoclonal antibodies were also used to label for other proto-oncogene proteins associated with apoptosis (Bad, Bax, Bcl-x, p53, CPP32, and Fas; Apoptosis Sampler Kit; Signal Transduction Laboratories, Exeter, UK). The procedure used was as described elsewhere.\textsuperscript{45,46}

**ICH-1 mRNA Expression in Human RPE Cells**

Total RNA was isolated from human RPE cells cultured in six-well culture plates using reagent (TriReagent; Sigma) according to the manufacturer’s instructions. Four micrograms RNA was DNase treated by incubation with 1 U RNase-free DNase (RQ1; Promega, Southampton, UK) and 40 units RNasin ribonuclease inhibitor (Promega) in a total volume of 18 μl at 37°C for 10 minutes and then at 65°C for 10 minutes. First-strand synthesis of cDNA was performed by incubation of a 9-μl aliquot of the DNase-treated RNA with 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl\textsubscript{2}, 10 mM dithiothreitol, 5 ng oligodeoxythymidine\textsubscript{30}, 24 U RNasin, 0.6 mM each deoxynucleotide triphosphate, and 200 U M-MLV reverse transcriptase (Promega) at a total volume of 15 μl at 42°C for 1 hour. Control samples were reaction mixtures without reverse transcriptase. The reactions were diluted by addition of 135 μl H\textsubscript{2}O and 10-μl aliquots stored at −20°C until use.

Oligonucleotide primers for amplification of *Ich-1* cDNA were designed using the published sequence\textsuperscript{47} and a software program (Primer 3; available at http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi) and chosen to flank the intron sequence enabling the identification of both splice variants of the *Ich-1* gene on the basis of size. The sense primer was 5'-ATCTATGGTGTGGATGGGAAAC-3' (808-829 bp)\textsuperscript{47} and the anti-sense 5'-AGAAAACACTTGAGCAAGAGCC-3' (1146-1125 bp). The mRNA encoding the truncated version of the *Ich-1* protein, *Ich-1s*, thus includes a 61-bp intron sequence, so that PCR results in amplification of a 400-bp product in contrast with a 339-bp product from the alternatively spliced mRNA encoding *Ich-1*.

Amplification of the cDNA was performed in a reaction volume of 50 μl containing 10 μl cDNA, PCR buffer (10 mM Tris-HCl [pH 8.3] and 50 mM KCl), 5 mM MgCl\textsubscript{2}, 800 μM each deoxynucleotide triphosphate, 4 ng/μl each primer, and 2.5 U Taq (AmpliTaq Gold; Perkin Elmer, Warrington, UK). The reaction mix was then incubated at 94°C for 10 minutes to activate the polymerase and was subjected to 42 cycles of PCR (94°C, 45 seconds; 51°C, 30 seconds; 72°C, 60 seconds) using a thermal cycler (Progene; Techne, Cambridge, UK) followed by a final extension at 72°C for 3 minutes. The PCR products in 10-μl aliquots of the reaction mixes were then separated by 2% agarose gel electrophoresis, visualized using ethidium bromide, and photographed using black and white print film (Polaroid Type 667, Sigma). The abundance of each band was determined by scanning the photographs into a computer-based densitometer scanner (Photoshop 3.0; Adobe, Edinburgh, Scotland, UK) and the average pixel density determined over a fixed area using commercial software (SigmaScan; Jandel Scientific, Erkrath, Germany). The data were then expressed as a ratio of *Ich-1L* to *Ich-1S* and analyzed using Student’s paired t-test.

**RESULTS**

The effect of ischemia (reduced glucose and absence of oxygen) on cultured human pigment epithelial cells with time. In these experiments fetal calf serum was absent from the culture medium. For 40 to 50 hours, most of the cells remained attached to the coverslips (●) with an insignificant number stained positively by TdT-DUTP terminal nick-end labeling (TUNEL), suggesting apoptosis (○). After 70 hours, approximately 40% of the cells dissociated from the coverslips and approximately 60% of the attached cells had TUNEL-positive nuclei. After 120 hours in culture, only 40% of the original cells remained attached to the coverslips, approximately 80% of which were TUNEL positive. Results are means ± SEM for five experiments.

**FIGURE 1.** The effect of ischemia (reduced glucose and absence of oxygen) on cultured human pigment epithelial cells with time. In these experiments fetal calf serum was absent from the culture medium. For 40 to 50 hours, most of the cells remained attached to the coverslips (●) with an insignificant number stained positively by TdT-DUTP terminal nick-end labeling (TUNEL), suggesting apoptosis (○). After 70 hours, approximately 40% of the cells dissociated from the coverslips and approximately 60% of the attached cells had TUNEL-positive nuclei. After 120 hours in culture, only 40% of the original cells remained attached to the coverslips, approximately 80% of which were TUNEL positive. Results are means ± SEM for five experiments.
FIGURE 2. Effect of various treatments on the morphology and stain of the nuclei by TdT-dUTP terminal nick-end labeling (TUNEL). After 72 hours in serum-free medium, the retinal pigment epithelial cells on the coverslips were dense (A). Treatment of these cells with DNase after TUNEL shows that all the nuclei stained darkly for fragmentation of DNA (B). After 72 hours of ischemia (absence of serum and oxygen) the cells had a shrunken appearance and TUNEL-positive nuclei (C). This effect was unchanged when 1 μM melatonin (D), 100 μM ascorbate (E), or 100 μM luzindole (H) was included. Addition of 10 μM melatonin suggested some protection from ischemia-induced apoptosis (E), but this was clearly observed only when 100 μM melatonin (F) or fetal calf serum (G) was present.
The influence of ischemia alone and together with various treatments on the total number of RPE cells in serum-free medium after 72 hours in culture is shown in Figure 4. Ischemia reduced the number of cells on the coverslips by approximately 30% (the number of cells before ischemia averaged 500/mm²; after ischemia the average was 390/mm²). The inclusion of 10% FCS reduced the effect of ischemia significantly whereas melatonin, ascorbate, and luzindole had little or no effect (Fig. 4).

Studies have shown that flupirtine, which protects against ischemia-induced apoptosis in RPE cells, causes an upregulation (Bcl-2) or downregulation (ICH-1, TIAR) of proto-oncogene proteins associated with apoptosis.¹³ It was decided, therefore, to perform similar experiments with melatonin and use flupirtine for comparative purposes in parallel experiments. Human confluent RPE cells in serum-free medium were incubated with melatonin or flupirtine (100 μM in each case) for 72 hours. Immunocytochemistry (Fig. 5) and sodium dodecyl sulfate-western blot analyses (Fig. 6) showed clearly that Bcl-2 protein was upregulated in the flupirtine-treated cells but was unaffected by melatonin. Moreover, TIAR and ICH-1 encoding proteins were downregulated in the flupirtine-treated cells but were unaffected by melatonin (Fig. 7). No positive immunoreactivity was detected with any of the other proteins (Bad, Bax, Bcl-x, p53, CPP32, Fas) from the commercial kit (Apoptosis Sampler Kit; Signal Transduction) that were investigated by immunocytochemistry or immunoblot analysis.

Reverse transcription-PCR using primers specific for the Bcl-1 gene clearly showed amplification of products derived from the two alternately spliced transcripts that encode the ischemia-induced TUNEL-positive cells, but insignificantly. Moreover, 100 μM luzindole did not counteract the effect of 100 μM melatonin.

Confluent human RPE cells on the coverslips placed in serum-free medium for 72 hours seemed healthy (Fig. 2A). Treatment of such cells with DNase followed by TUNEL showed that the nuclei of the cells stained darkly for fragmentation (Fig. 2B). After 72 hours of experimental ischemia, the RPE cells were reduced in number, had a shrunken appearance, and had a large number of TUNEL-positive nuclei (Fig. 2C). Such cultures appeared unchanged when 100 μM luzindole (Fig. 2E) or 100 μM ascorbate (Fig. 2D) was included in the medium. However, inclusion of 100 μM melatonin in the medium during ischemia (Fig. 2F) drastically reduced the number of cells that stained by TUNEL, with the unstained cells appearing less shrunken. This effect was dose dependent with 1 μM melatonin hardly having an effect (Fig. 2D) whereas 10 μM of the substance slightly reduced the amount of TUNEL-positive cells. The cells also appeared healthier (Fig. 2E).

Data shown in Figure 2 from several separate experiments are summarized in Figure 3. Approximately 70% of all cells remaining on the coverslips after 72 hours' ischemia stained positively for DNA fragmentation by TUNEL. The inclusion of 10% FCS almost completely counteracted the effect of ischemia, with less than 8% of cells having TUNEL-positive nuclei. The only other substance tested that significantly counteracted ischemia-induced apoptosis was 100 μM melatonin, with which the number of TUNEL-positive cells was reduced from 70% to 45%. Lower concentrations of melatonin (less than 100 μM) or 100 μM ascorbate also tended to reduce the number of cells stained by TUNEL compared with control samples; Student's t-test.

![Figure 3](image) Results show the percentage of cells with positively stained nuclei after TdT-dUTP terminal nick-end labeling (TUNEL). Cells were exposed for 72 hours to ischemia, with or without specific substances. Inclusion of 10% fetal calf serum (FCS) almost completely prevented the effect of ischemia. One hundred micromolar melatonin (mel) also significantly blunted the effect of ischemia. Addition of 10 μM or 100 μM luzindole (luz) did not counteract the effect of 100 μM melatonin, suggesting that melatonin receptors were not involved in the effect of melatonin. One hundred micromolar ascorbate, 10 μM melatonin, or 10 μM luzindole had no effect on the number of TUNEL-positive cells after ischemia. Luzindole at 100 μM significantly increased the number of TUNEL-positive cells in ischemia. Results are expressed as means ± SEM for six separate experiments. *P < 0.001, †P < 0.05, *P < 0.05 compared with control samples; Student's t-test.

![Figure 4](image) Effect of ischemia and substance on total numbers of cells attached to the coverslips, compared with numbers in control samples. One hundred micromolar or 10 μM luzindole (luz), alone or with 10 μM melatonin (mel), significantly (‡P < 0.05; Student's t-test) potentiated the loss of cells from the coverslips. Only fetal calf serum (FCS) had a significant counter effect (†P < 0.05; Student's t-test). Results are expressed as means ± SEM for six separate experiments. RPE, retinal pigment epithelium.
FIGURE 5. Immunocytochemical analysis of Bcl-2 immunoreactivity in passage-four human retinal pigment epithelial cells (donor age, 60) using a polyclonal antiserum. Cells that were incubated 72 hours in serum-free medium without (A) or with (B) 100 μM melatonin showed no immunoreactivity for Bcl-2 protein, but after treatment for the same time with serum-free medium containing 100 μM flupirtine gluconate, intense immunolabeling was observed throughout all cells (C). Scale bar, 20 μm.

FIGURE 6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blot analyses that show 26 kDa Bcl-2 immunoreactivity in extracts from passage-four human retinal pigmented epithelial cells (donor age, 56 years). There was a greater intensity of immunolabeling in cell extracts after treatment for 72 hours with 100 μM flupirtine gluconate (F) or 10% fetal calf serum (S) than with 100 μM melatonin (M) or serum-free medium alone (C).

FIGURE 7. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blot analyses of passage-four human retinal pigmented cell extracts (donor age, 56) labeled for TIAR (A, 50/42 kDa) and ICH-1, (B, 48 kDa) immunoreactivity. Treatment with melatonin (M) had no observable effect on immunolabeling for TIAR or ICH-1, proteins compared with labeling in cells incubated in serum-free medium (C). Cells that were grown in medium containing 10% fetal calf serum (S) showed less of both TIAR proteins but an equivalent level of ICH-1, protein compared with the level in cells incubated without serum. Cells treated with 100 μM flupirtine (F) had a decreased quantity of immunoreactive protein in both cases compared with the level in the sample taken from cells incubated without serum.
FIGURE 8. Relative levels of expression of the two transcripts of Ich-1 gene in human retinal pigment epithelial cells after 72 hours in serum-free medium. This ratio was significantly (P < 0.05; Student's paired t-test) decreased when cells in serum-free medium were incubated in the presence of 100 μM flupirtine and 100 μM melatonin. The insert shows an example of the detection of two polymerase chain reaction products with ethidium bromide after agarose electrophoresis. The 400-bp product is derived from the mRNA transcript for the ICH-1s protein, and the 339-bp product from the ICH-1L protein. C, control; F, flupirtine-treated; M, melatonin-treated. Results are means ± SEM; n = 4).

ICH-1s and ICH-1L proteins (Fig. 8; 339 bp and 400 bp, respectively). RPE cells treated with flupirtine showed an increase in the levels of the amplicon derived from the transcript encoding the truncated ICH-1s protein relative to that encoding the ICH-1L protein (Fig. 8). This was not visibly apparent on the gels in the case of melatonin-treated samples, but densitometric analysis clearly showed a statistically significant decrease in the ICH-1s-to-ICH-1L ratio, although to a lesser extent than that induced by flupirtine.

The effect of 72 hours’ ischemia on the degradation or laddering of DNA revealed by agarose gel electrophoresis is shown in Figure 9. In the control sample of cells incubated for 72 hours in serum-free medium no fragments of DNA were generated. Ischemia caused some DNA degradation, shown by fragments forming a ladderlike appearance on the gel. This laddering appearance was completely removed by serum and partially removed by melatonin.

A combination of 10 μM ascorbate and 5 μM FeSO4 stimulated the formation of DCF in chick retinal cells (Fig. 10). Formation of DCF is caused by ROS.39 The inclusion of 100 μM deferoxamine or 100 μM flupirtine almost completely blunted this response, but 100 μM luzindole had no effect. The forma-

FIGURE 9. Agarose gel electrophoretic separation of DNA fragments isolated from passage-four human retinal pigment epithelial cells (donor age, 60 years). After experimental ischemia for 72 hours (lane 3), much fragmented DNA was present at the size that is characteristic of the intronucleosomal DNA cleavage that occurs during apoptosis. This effect was not seen in DNA extracted from cells incubated in serum-free medium (lane 1) or in cells subjected to experimental ischemia in the presence of 10% fetal calf serum (lane 4). The addition of 100 μM melatonin to cells during the insult has a discernible alleviating effect on DNA fragmentation (lane 5). Lanes 1 and 6 show the appearance of DNA size markers (123-bp multiples).
Addition of 100 μM ascorbate and 5 μM FeSO₄ (Fe) caused a significant stimulation of ROS formation. One hundred micromolar luzindole (luz) alone had no effect and did not counteract the influence of 100 μM melatonin or 100 μM flupirtine. **P < 0.05 compared with control samples and *P < 0.05 compared with 10 μM ascorbate and 5 μM FeSO₄; Student’s t-test.

DISCUSSION

The present results corroborate those in studies that show that when human RPE cells are subjected to experimental ischemia (reduced glucose and absence of oxygen) in serum-free medium, the cells die by what seems to be apoptosis. It is suggested by the cells’ shrunken appearance and the nuclei stained positively for the breakdown of DNA by TUNEL. Analysis of isolated DNA on gels suggests breakdown into oligonucleosomal-sized fragments to support this view. Although these criteria do not unequivocally indicate apoptosis, they nevertheless strongly support it.

The major finding of this study is that ischemia-induced apoptosis in cultured human RPE cells is significantly reduced by melatonin. This is in agreement with studies on nervous tissue that have shown that induction of apoptosis by kainate, radiation or ischemia is much reduced by melatonin. Luzindole, a melatonin receptor antagonist, did not blunt the protective effect of melatonin in the ischemia-induced apoptosis, suggesting that the melatonin receptors associated with RPE cells were not involved in the process. Present information indicates that apoptosis can be initiated experimentally by a variety of mechanisms. These include the manipulation of developmental signals and activation of cell surface receptors, such as those of tumor necrosis factor, Fas-ligand (FasL) and N-methyl-D-aspartate. 

In addition, apoptosis can be initiated by UV or γ-irradiation, heat shock, oxidative stress, viral infection, ROS, free radicals, and detachment of cells from the extracellular matrix. The mitochondria seem to play an essential role in the effector phase. Moreover, the concentrations of several substances such as ROS and certain proto-oncogene proteins change in a characteristic manner in apoptosis. Although the present study provides no information on the possible trigger for apoptosis in RPE cells in experimental ischemia, it seems to suggest that free radicals/ROS could be involved. This conclusion is based on the finding that melatonin and flupirtine protect against ischemia-induced RPE cell apoptosis along with good available evidence that suggests that melatonin and flupirtine function as antioxidants or free radical scavengers (see the introduction). Furthermore, studies from our laboratory have shown that RPE cells that have depleted glutathione and therefore potentially excessive levels of toxic free radicals/ROS also die in apoptosis. This process can also be counteracted by flupirtine and melatonin.

Generation of ROS in vitro in chick retinal cells with low concentrations (5 μM) of ascorbate and iron confirmed that melatonin and flupirtine antagonized this process. Melatonin receptors seemed to be uninvolved, as in apoptosis, because the melatonin effect was not blunted by luzindole. Comparing the effectiveness of identical concentrations of ascorbate, melatonin, and flupirtine on iron-ascorbate-induced formation of ROS shows that melatonin was significantly weaker than flupirtine, with ascorbate being the weakest. The order of effectiveness of the three substances in combating ROS formation was mirrored in the way they counteracted ischemia-induced apoptosis in RPE cells. One hundred micromolar melatonin blocked apoptosis by no more than 50% with the blunting effect of ascorbate insignificant. It has been shown that 100 μM flupirtine nullifies the same apoptosis by 85%. Therefore, there is a correlation between the effectiveness of ascorbate, melatonin, and flupirtine in reducing the formation of ROS in vitro and that in reducing ischemia-induced apoptosis.

There are now persuasive data to show that melatonin is a potent antioxidant. From the data presented here and elsewhere, it is clear that flupirtine is even more effective than melatonin in functioning as an antioxidant. It seems likely, therefore, that it is the antioxidant characteristics of the two molecules that are important in combating the ischemia-induced apoptosis to RPE cells. Because certain proto-oncogene proteins known to be associated with apoptosis are affected in RPE cells by flupirtine, it was decided to see whether melatonin acts in the same way. We therefore compared the effects of flupirtine and melatonin in parallel experiments to see whether both change the amounts of proto-oncogene proteins Bcl-2, ICH-1, and TIAR in RPE cells. Consistent with previous findings, Bcl-2 protein was upregulated and ICH-1 and TIAR proteins downregulated in cultures treated with flupirtine. In contrast, melatonin seemed to have no effect on any of these proto-oncogene proteins.
proteins, which can be interpreted to suggest that the manner by which flupirtine and melatonin counteracted apoptosis was different. This difference may be expected, because many distinct signaling pathways can cause or block apoptotic cell death. One alternative explanation is that the weaker effect of melatonin in blunting apoptosis and formation of ROS exerted only a slight influence on the proto-oncogene proteins and that the methods used did not have the sensitivity to detect the changes.

Support for the latter view comes from the analysis of the relative levels of expression of the mRNA transcripts for the two forms of the ICH-1 protein, ICH-1C, and ICH-1L. The Icb-1 programmed cell death gene is a homologue of ced-3, interleukin-1β-converting enzyme, and encodes protein products capable of inducing and suppressing apoptosis. 47 Overexpression of Icb-1L induces programmed cell death, whereas increased expression of Icb-1C suppresses apoptosis of Rat-1 cells induced by serum deprivation. 47 Moreover, tissues commonly associated with high levels of apoptosis, such as the thymus, contain only detectable levels of Icb-1C, mRNA expression, whereas other tissues such as adult brain show expression of both mRNA transcripts. 47 Cultured human RPE cells expressed both forms of ICH-1 and so the relative levels of the two transcripts may determine whether cells enter apoptosis or not. The ratio of the mRNA levels for ICH-1C-to-ICH-1L was significantly lower in flupirtine- and melatonin-treated RPE cells than in control cells, and the effect of flupirtine was much greater. These data show a similarity in the action of melatonin and flupirtine in arresting apoptosis and confirm the greater potency of flupirtine. Therefore, melatonin may have influenced proto-oncogene proteins in the same way as flupirtine, but the weaker effect was difficult to detect experimentally. Such a conclusion would be consistent with the data that showed no apparent effect of ascorbate on ischemia-induced apoptosis. Ascorbate is much weaker than melatonin in countering the formation of ROS in vitro, and because melatonin only blocks apoptosis by less than 46% (whereas serum or flupirtine blocks apoptosis by approximately 90%) it is not surprising that the influence of ascorbate on apoptosis is undetectable. A close inspection of the overall data suggests, however, that ascorbate provided weak protection against ischemia-induced apoptosis and loss of cells from coverslips, but the protection was too weak to be clearly or significantly characterized.

The results presented in this study suggest that substances that reduce or counteract the damaging effects of free radicals prevent death of RPE cells by apoptosis. Although we have shown here that only relatively high concentrations of melatonin are required to elicit protection from apoptosis it is probable that less harsh insults, as may occur in situ, would require lower concentrations of melatonin. Moreover, because melatonin is synthesized in photoreceptors and the apical villi of the RPE cells are closely associated with the outer segments, it is possible that melatonin originating from the photoreceptors reaches the RPE at a high enough concentration to provide protection from free radical damage in the RPE cells. Because free radicals are produced constantly and their uncontrolled production may occur in any number of insults, including during aging, it is therefore possible that melatonin provides protection from these damaging effects.

References


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