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Ceramide metabolism regulates autophagy and apoptotic-cell death induced by melatonin in liver cancer cells

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Abstract

Autophagy is a process that maintains homeostasis during stress, although it also contributes to cell death under specific contexts. Ceramides have emerged as important effectors in the regulation of autophagy, mediating the crosstalk with apoptosis. Melatonin induces apoptosis of cancer cells; however, its role in autophagy and ceramide metabolism has yet to be clearly elucidated. This study was aimed to evaluate the effect of melatonin administration on autophagy and ceramide metabolism and its possible link with melatonin-induced apoptotic cell death in hepatocarcinoma (HCC) cells. Melatonin (2 mM) transiently induced autophagy in HepG2 cells through JNK phosphorylation, characterized by increased Beclin1 expression, p62 degradation and LC3II and LAMP2 colocalization, which translated in decreased cell viability. Moreover, *ATG5*-silencing sensitized HepG2 cells to melatonin induced-apoptosis, suggesting a dual role of autophagy in cell death. Melatonin enhanced ceramide levels through both *de novo* synthesis and acid sphingomyelinase (ASMase) stimulation. Serine palmitoyl transferase (SPT) inhibition with myriocin prevented melatonin induced autophagy and ASMase inhibition with imipramine impaired autophagy flux. However, ASMase inhibition partially protected HepG2 cells against melatonin while SPT inhibition significantly enhanced cell death. Findings suggest a cross-talk between SPT-mediated ceramide generation and autophagy in protecting against melatonin, while specific ASMase-induced ceramide production participates in melatonin-mediated cell death. Thus, dual blocking of SPT and autophagy emerge as a potential strategy to potentiate the apoptotic effects of melatonin in liver cancer cells.

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Keywords

melatonin; hepatocarcinoma; autophagy; ceramides; apoptosis; serine palmitoyltransferase; acid sphingomyelinase

Introduction

Hepatocellular carcinoma is the fifth most common neoplasm and the third most frequent cause of cancer death [1]. Surgery remains the most effective treatment for patients with HCC; however, it is limited to selected cases [2], and new curative modalities are required. Hepatocarcinogenesis is a complex multistep process in which many signaling cascades are altered, leading to a heterogeneous molecular profile. The main mutations include the gene for β catenin and tumor suppressor p53. Other mutations less frequent such as chromosomal amplifications and deletions, or epigenetic alterations also affect important oncogenes and tumor suppressors [3]. As a result of these alterations, several signaling cascades related to cell survival and proliferations are modified. For instance, ceramide metabolism and signaling are usually suppressed by overexpression of ceramide-metabolizing enzymes or downregulation of ceramide-generating enzymes [4, 5]. Moreover, alterations such as deregulation of the hepatocyte growth factor (HGF) and c-MET pathway, low levels of human forkhead box class O (FoxO) transcription factors or high levels of vascular endothelial growth factor (VEGF) are associated with poor prognosis in cancer patients, and seem to confer chemotherapy resistance [6, 7]. Finally, the MTOR (mammalian target of rapamycin) pathway, related to autophagy, is disrupted in 40–50% of liver cancers owing to inactivation of the tumour suppressor PTEN, or mutations of phosphoinositide-3-kinase [8, 9].

Autophagy is a dynamic process that triggers the digestion of damaged organelles and misfolded proteins. Cell components are engulfed in double-membrane vesicles called autophagosomes, which after maturation fuse with lysosomes, leading to the formation of an autophagolysosome, whose contents are degraded by lysosomal enzymes. The role of autophagy in cancer is dual. At early stages of tumour development, autophagy acts as a suppressor mechanism [10], promoting the recycling of defective organelles and unfolded proteins, prevention of oxidative stress and maintenance of genome stability [11]. However, autophagy can also favour tumour promotion via oncogene-mediated cancer development [12] and cellular adaptation to different stress, such as hypoxia or starvation [13, 14]. Although the crosstalk between autophagy and apoptosis is not well defined, a relationship has been established due to the interaction of different autophagy and apoptosis-related proteins [15, 16]. Moreover, both processes are regulated by common transcription factors and signalling pathways, such as nuclear factor kappaB (NF-κB), tumor protein p53 (p53), phosphatidylinositide 3-kinases, protein kinase B (PI3K/Akt), or c-Jun N-terminal kinase (JNK) [17].

Ceramides are critical components of membrane bilayers and play an important role in cellular responses, including inflammation, cell proliferation, differentiation, apoptosis, cell migration, senescence and autophagy [18, 19]. Although ceramide is a well-established

apoptosis inducer, more recent studies have also implicated ceramide in the induction of autophagy through the activation of JNK, upregulation of Beclin-1 or BNIP3 and downregulation of nutrient transporter proteins [20–22]. Thus, ceramides have emerged as important effectors in the regulation of the autophagic pathway, mediating the crosstalk between apoptosis and autophagy. Cells generate ceramides by *the novo* byosinthesis in the endoplasmic reticulum (ER) with the condensation of serine and palmitoyl-CoA catalysed by serine palmitoyl transferase (SPT) [23]. In addition, ceramides can be generated through sphingomyelin hydrolysis by sphingomyelinases (SMase) [24]. Acid SMase (ASMase) is activated in response to various proinflammatory and proapoptotic stimuli [22, 25], and it contributes to apoptotic death of tumour cells. Recent evidence demonstrates that ASMase regulates key mechanisms involved in autophagy [26]. On the other hand, although ceramide promotes early autophagy and apoptotic cell death, the cytotoxic effect of C2 ceramide is enhanced when autophagy is inhibited [27]. In addition, an increase of endogenous levels of ceramides is related to apoptosis, and these pro-apoptotic effects are mediated in part by an early autophagy induction [28].

Melatonin is the main product of the pineal gland and plays a protective role in several pathophysiological contexts, including cancer, where it acts as an effective oncostatic drug [29, 30]. Previous studies from our group showed the anti-proliferative, pro-apoptotic, anti-invasiveness and anti-angiogenic effect of melatonin in HepG2 cells [31–33]. However, it has been previously reported a dual role of melatonin in cancer cells. For instance, in glioblastoma-initiating cells, autophagy is involved in melatonin-induced cell death [34], while in hepatoma H22-bearing mice autophagy caused by melatonin is related to apoptotic cell death resistance [35]. Other antioxidants similar to melatonin such as honokiol, quercetin or resveratrol induce autophagy and apoptosis in cancer cell lines and the disruption of autophagy enhance apoptosis-dependent cell death [36, 37]. Moreover, HepG2 cells treated with low doses of a curcumin analog, exhibited increased apoptosis following autophagy inhibition through caspase-dependent and caspase-independent pathway [38].

The role of melatonin on autophagy and its effect on apoptotic cell death in liver cancer cells is not completely understood. Moreover, no information exists on the relationship between melatonin and sphingolipid metabolism. Thus, our aim was to investigate the effect of melatonin administration on autophagy and ceramide metabolism in HepG2 cells, and its possible link with melatonin-induced apoptotic cell death.

Materials and methods

Cell culture and reagents

The HepG2 human hepatocarcinoma cell line was obtained from the American Type Culture Collection (Manassas, VA). They were cultured under controlled conditions (37°C, 5% CO_2) and grown in Dulbecco's modified Eagle's medium/low glucose, GlutaMAX™ supplement pyruvate (GIBCO, Life Technologies, Madrid, Spain) containing 10% fetal bovine serum and 100 U/mL penicillin/streptomycin. Cells were plated in 9.6 cm^2 culture dishes at a density of 0.25×10^6 cells/well. Twenty four hours after plating, cells were treated with 2 mM melatonin (Sigma, St Louis, MO), 50 μM chloroquine diphosphate salt (Sigma) or 100 nM bafilomycin A1 (Tocris, Bristol, UK). In some cases, cells were

pretreated two hours with 2.5 μM myriocin (Sigma), 10 μM imipramine (Sigma) or 10 μM SP600125 (Tocris).

Annexin V-propidium iodide assay

Apoptosis was assessed by Alexa Fluor 488 annexin V/Dead apoptosis kit (Invitrogen, Carlsbad, CA). HepG2 cells were seeded in a 6-well plate at density of 0.25×10^6 cells/well. Next day, the cells were treated with melatonin 2 mM for 48 hours. Cell pellets were resuspended in 100 μL buffer with 5 μl annexin V and 1 μL of propidium iodide, and incubated for 15 min at 25°C in the dark. 400 μL of buffer were added for a final volume of 500 μL. Cells were immediately analyzed by FACS SCAN flow Cytometer (Becton-Dickinson, San Jose, United States). 10,000 cells per sample were acquired and percentage of cell death was analyzed using Cell Quest software.

Cell viability assay

HepG2 cells were seeded on 96-well plates at 5,000 cells/well 24 hours before being treated with melatonin and ceramide synthesis inhibitors for 48 hours. Cells were incubated for 3 hours with 0.5 mg/ml of 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma) dissolved in serum free medium. After this interval, cells were washed with PBS followed by the addition of DMSO. The optical densities were measured at 560 nm spectral wavelength using microtitre plate reader (SynergyTM HT Multi-Mode Microplate Reader, Bio-Tek Instruments, Inc., Winooski, VT, USA).

Western blot analysis

After treatments, cultured cells were washed twice with ice cold PBS and lysed by adding ice cold RIPA buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.1% Triton 100X, 10% sodium deoxycholate, 10% SDS, 1 mM NaF and protease cocktail inhibitor (Roche, Basel, Switzerland) and scraped off the plate. Extracts were transferred to a microfuge tube and centrifuged for 10 min at 15,000g. Equal amounts of the supernatant protein (20 μg) were separately subjected to SDS-PAGE and transferred to a PVDF membrane (Bio-Rad, Hercules, CA). Primary antibodies (Ab) were diluted in blocking solution and incubated overnight at 4°C with polyclonal Ab to p62, LC3, Atg5, phospho-JNK, (1:1000 dilution; from Cell Signalling, Bervely, MA) phospho-mTOR (Ser2448) (1:1000 dilution, Abcam, Cambridge, UK), PARP, BAX and Beclin1 (1:100, Santa Cruz Biotechnology, Dallas, TX). Equal loading of protein was demonstrated by probing the membranes with a rabbit polyclonal anti β-actin antibody (Sigma). After washing with PBS-T, the membranes were incubated for 1 hour at room temperature with secondary HRP-conjugated antibody (1:5,000; Dako, Glostrup, Denmark) and visualized using ECL detection kit (Amersham Pharmacia, Uppsala, Sweden). The density of the specific bands was quantified employing the software ImageJ (National institute of Mental Health, Bethesda, MD) with an imaging densitometer (Scion Image, Maryland, MA).

ASMase assay

ASMase activity was determined using a fluorescent sphingomyelin analog (NBD C6-sphingomyelin (Cayman Ann Harbor, MI). HepG2 cells were lysed in sodium acetate buffer

(250 mM sodium acetate, 0.1% Triton X-100, pH 5.0). Samples (75 μg protein) were incubated for 1 hour 30 min at 37 °C in a buffer (250 mM sodium acetate, 0.1% Tri- ton X-100, pH 5.0) containing 10 μM NBD C6-sphingomyelin. Lipids were extracted with a methanol:chloroform (1:2), dried in a speed vacuum and separated by TLC (chloroform: methanol: H2O; 65:25:4, v/v). NBD-ceramide was visualized under UV light and the images were collected with the image capturing instrument, LAS4000 (GE Healthcare, Little Chalfont, UK) and analyzed with Image J free software.

Ceramide detection

Lipids were obtained and measured using HPLC as described previously [39]. Samples were extracted with methanol:chloroform (1:2, v/v). The chloroform phase extracts were dried, resuspended in 250 μl of 1 M KOH in methanol, and incubated at 100°C 1 hour. Once samples had cooled to room temperature, the methanol:chloroform (1:2, v/v) extraction was repeated. The free long-chain bases were recovered in the chloroform phase and dried. Lipids were dissolved in 50 μl of methanol and mixed with 100ul of naphthalene-2,3-dicarboxaldehyde (NDA) reagent 50 mM boric acid (pH 9), sodium cyanide 5 mM and NDA 5 mM (2:1:1, v/v). After 10 min of incubation at 50°C in the dark, 300 μl of methanol was added. Then samples were centrifuged and analyzed by HPLC in a reverse phase C18 column (Teknokroma, Barcelona, Spain) using a Gilson fluorimetric detector with an excitation wavelength of 252 nm and an emission wavelength of 483 nm. The mobile phase composition for the gradient system was 5 mM potassium phosphate buffer (pH=6.5):methanol (85:15, v/v) for mobile phase A and acetonitrile:methanol (75:25, v/v) for mobile phase B and the flow rate was 1 ml/min. The gradient program was 0–1 min 52.94 A, 47.06 B; 1–6 min 52.94-5.88 A, 47.06-94.11 B; 6–21 min 5.88 A, 94.11 B; 21–25 min 5.88-52.94 A, 94.11-47.06 B, 25–30 min 52.94 A, 47.06 B. Quantification of the ceramide peaks were calculated according to a calibration curve commercial standards.

Real-time reverse transcriptase polymerase chain reaction (RT-PCR)

Real-time reverse transcriptase polymerase chain reaction (RT-PCR) was performed as described [40]. Briefly, confluent HepG2 cells grown in complete media were replated in 6 wells culture plate, at a density of 0.25×10^6 cells/well in a total volume of 2 mL of complete medium. After treatment, total RNA was isolated with TRIzol reagent (Invitrogen). For reverse transcription, 1 μg of the total RNA was converted to first-strand cDNAs using a High-Capacity cDNA Archive Kit (Applied Biosystems, CA). Quantitative real-time PCR analysis was performed using SYBR Green (Invitrogen). Human primers used in the present study were: *ASMase* forward 5′-CTGACTCTCGGGTTCTCTGG-3′ and reverse 5′-AGGTTGATGGCGGTGAATAG-3′ and *SPT* forward 5′-TGGAAGAGAGCACTGGGTCT-3′ and reverse 5′-GCTACCTCCTTGATGGTGGA-3′. For *ATG5* determination, quantitative real-time PCR was performed using FastStart TaqMan Probe Master (Roche Diagnostics GmbH, Mannheim, Germany). TaqMan primers and probes for *ATG5* gene are derived from the commercially available TaqMan Gene Expression Assays. To normalize expression data, actin was used as an internal control gene. Relative changes in gene expression levels were determined using the $2^{-\Delta\Delta}$CT method.

Immunofluorescence and laser confocal imaging

For immunofluorescence labeling cells were cultured on 24 wells culture plates containing glass coverslips at a seeding density of 1×10^4. Briefly, HepG2 cells were fixed for 15 minutes with 4% paraformaldehyde and washed twice with PBS 1X. Cells were blocked and permeabilized with PBS 1X + 0.2 % saponin and 1% fatty acid free BSA (FFA-BSA) for 15 minutes at room temperature. After washing twice with PBS 1X, cells were incubated with a rat anti-LAMP monoclonal antibody [GL2A7] (Abcam) and rabbit anti- LC3B polyclonal antibody (Cell Signaling) diluted both 1:300 in 1X PBS with 1 % ffa-BSA O/N at 4°C and washed twice with PBS 1X followed by incubation with a secondary anti-rat IgG antibody, conjugated to Alexa 488 (1:500) and anti-rabbit IgG antibody, conjugated to Alexa 647 (1:500) for 1 hour at 25°C. Coverslips were washed twice with PBS 1X and mounted on glass slides with fluorescent mounting medium Fluoroshield™ with DAPI (Sigma) and visualized in a Leica SPE confocal laser-scanning microscope.

Statistical analysis

Results are expressed as mean values ± SD of the indicated number of experiments. One-way ANOVA followed by Bonferroni post hoc test was used to determinate differences between the mean values of the different treated groups. $p < 0.05$ was considered significant. Values were analyzed using the statistical package GraphPad Prism 5.

Results

We have previously reported that melatonin induces apoptotic cell death in HepG2 cells [32]. Since autophagy-lysosome pathway is involved in the turnover of damaged organelles and misfolded proteins and due to the importance of autophagy in cell homeostasis and its relationship with apoptosis, we first assessed if melatonin was able to trigger an autophagic response in HepG2 cells. As shown in Fig. 1., when HepG2 cells were maintained in the presence of melatonin (2 mM), LC3II levels increased gradually over time, peaking at 8–12 hours, and decreasing at 24 hours after melatonin treatment. These findings were accompanied by p62 accumulation and gradual degradation over time, as well as an early increase of Beclin 1 levels (Fig. 1A). In order to confirm the functional relationship between autophagosomes and lysosomes we evaluated the colocalization of the specific markers, LC3 and LAMP-2 by confocal microscopy. Colocalization of both markers was detected at 4 hours exhibiting a maximum after 8 hours post-treatment, which decreased by 24 hours, indicating the onset of autophagy (Fig. 1B).

To define the functional impact of melatonin on autophagy, we determined autophagy flux status. We used HepG2 cells in the presence of melatonin with and without chloroquine (50 μM) or bafilomycin A1 (100 nM). After 8 hours of melatonin treatment, an increase in LC3II was observed, which further augmented by autophagy inhibitors chloroquine and bafilomycin A1. Although, chloroquine and bafilomimycin A1 increased LC3II content, this increase was lower that observed in the presence of melatonin (Fig. 1C). These data suggest that induction of autophagy by melatonin is an early and transient effect following administration of the indol.

To elucidate the mechanism for the induction of autophagy, we decided to evaluate mTOR pathway, which is a critical regulator of autophagy at the level of autophagosome formation. As seen, melatonin failed to reduce the phosphorylation of mTOR (Fig. 1A). Phosphorylation of JNK has also been reported to contribute to autophagy induction in different contexts such as reticulum stress response to unfolded proteins accumulation, so we checked if ER stress and JNK activation were altered by melatonin. Melatonin triggered an increase in GRP78 levels from 1 to 4 hours of treatment, concomitant with JNK phosphorylation (Fig. 1D). Thus, ER stress mediated JNK activation could account for initiation of autophagy in HepG2 cells in presence of melatonin. To address this question, we administered a specific JNK phosphorylation inhibitor SP600125 (10 μM) 1 hour prior to melatonin treatment. Western blot showed that the increase in LC3II levels induced by melatonin was prevented by p-JNK inhibitor, indicating that autophagy triggered by melatonin is likely caused by JNK activation (Fig 1E).

Autophagy is described as a pro-survival mechanism and it is up-regulated in response to therapeutic agents. On the other hand, autophagy is able to promote cell death in different cellular contexts. In order to address the role of autophagy in melatonin induced cell death, the initiation of autophagy was inhibited by siRNA-mediated knockdown of *ATG5* in HepG2 cells. *ATG5* mRNA expression and protein levels were depleted after 24 hours in *ATG5*-silenced HepG2 cells (Fig. 2A, B). The *ATG5*-silencing suppressed the increase of LC3II as well as p62 degradation induced by melatonin administration (2 mM) (Fig. 2B).

Next, we assessed the effect of *ATG5* siRNA in melatonin induced cell death. The down regulation of *ATG5* enhanced significantly the cytotoxic effect of 2 mM melatonin in *ATG5* silenced cells versus control cells after incubation with 2 mM melatonin for 48 hours (Fig. 2C). Moreover, these findings were accompanied by PARP processing, a caspase-3 target, with an increase of the cleaved 89 kDa fragment after 48 hours treatment in *ATG5* silenced cells (Fig. 2D). These results suggest that autophagy induction is a survival mechanism that reduces in part apoptotic cell death induced by melatonin.

Ceramides are bioactive lipids which have been implicated in a variety of physiological functions, including cell growth arrest, differentiation, apoptosis and autophagy. In order to evaluate the role of ceramides in apoptosis and autophagy, we assessed whether melatonin increase ceramide levels. We observed an early and transient increase in total ceramide content that declined by 24–36 hours after melatonin treatment (Fig. 3A). Interestingly, ASMase activity was stimulated in presence of melatonin as early as 1 hour post-treatment (Fig. 3B), which could contribute to the initial phase of ceramide generation. This activation of ASMase, however, was accompanied by increase expression of the mRNA level detected after 8 hours of melatonin treatment (Fig. 3C). Moreover, melatonin induced the expression of SPT, which catalyzes de rate-limiting step in ceramide neo synthesis (Fig. 3C). These results suggest that melatonin is able to induce ceramide synthesis involving different pathways in HepG2 cells.

To address the role of ceramide in melatonin-induced autophagy, we tested the effect of inhibition of ASMase and SPT during melatonin treatment. Pre-incubation with imipramine, which prevents ASMase activation, led to a significant increase of LC3II levels in the

presence of melatonin, chloroquine, and the combination of both (Fig. 4A). Additionally, we measured p62 levels to monitor the status of lysosomal degradation. Intriguingly, we observed that p62 levels were significantly enhanced after pre-incubation with imipramine for all conditions, indicating dysfunctional lysosomal degradation (Fig. 4A). Pre-incubation with myriocin abrogated the induction of LC3II levels caused by melatonin (Fig. 4B), suggesting that *de novo* ceramide shyntesis is necessary for melatonin to induce autophagy. Once established that autophagy was impaired through the inhibition of ASMase, we determined the effect of imipramine on melatonin induced apoptotic cell death. Initially, HepG2 cells were pre-incubated 2 hours with imipramine and treated with melatonin and we evaluated the effect over time. As expected, LC3II was increased over time with imipramine as well as the combination of imipramine and melatonin (Fig. 4C), which was accompanied by reduced cleaved PARP in response to melatonin administration (Fig. 4C). Moreover, in conditions of genetic autophagy antagonism by *ATG5* silencing, ASMase inhibition by imipramine decreased melatonin induced PARP cleavage and BAX induction, suggesting an ASMase-induced apoptotic pathway in response to melatonin (Fig. 4E). In contrast, LC3II levels were not modified when cells were pre-treated with myriocin, although it increased PARP-cleaved and BAX levels (Fig. 4D). To further explore these effects on HepG2 viability, cells were pre-incubated with imipramine and treated with 2 mM of melatonin during 48 hours. Surprisingly, cells pre-treated with imipramine prevented the cytotoxic effect of melatonin respect to cells treated only with melatonin (Fig. 5A, B). Together, this data support the idea that ASMase inhibition impairs the autophagy-lysosomal degradation pathway, while rescuing cell viability in response to melatonin, highlighting the important role of ceramides in melatonin induced apoptotic cell death. On the other hand, myriocin pre-treatment enhanced melatonin induced cell death (Fig. 5A, B). Interestingly, ASMase expression was enhanced in cells co-treated with myriocin plus melatonin (data not shown). These results suggest that the loss of viability in HepG2 could be related not only to autophagy suppression, but also to induction of ASMase, which enhances apoptotic cell death.

Discussion

Results from the present study indicate that exposure of HepG2 to melatonin transiently induces autophagy and an increase in ceramide levels, which are related to apoptotic cell death possibly by induction of *de novo* synthesis and ASMase activity. Moreover, the inhibition of ceramide synthesis regulates the autophagic response and has consequences on melatonin-induced apoptosis. Thus, ceramide metabolism modulated by melatonin is proposed to play an important role in autophagy regulation as well as in apoptotic cell death.

First, we observed that melatonin increases the expression of LC3II and Beclin1, and causes the colocalization of LC3II with LAMP-2. These findings are in line with other studies in different cell types and H22 bearing mice [34, 35]. Lower doses of melatonin have been tested in other types of cells, such as HTC116 colon cancer cells or corneal dystrophy type 2 (GCD2) fibroblasts, showing a non-continuous induction of autophagy [41, 42]. On the other hand, previous studies from our laboratory indicate that melatonin administration improves survival and reduces the autophagic response in a viral model of fulminant hepatic failure [43]. In addition, treatment with melatonin also reduces autophagy in the liver of

ob/ob mice, or in morphine-treated rat neurons [44, 45], and in both cases autophagy reduction is beneficial. Therefore, melatonin modulation of autophagy seems to be cell type and context-dependent, similarly to other antioxidant molecules with analog properties [38, 46].

Given the key role of mTOR in autophagy regulation [34, 35, 41, 42], we monitored the effect of melatonin in mTOR phosphorylation. Unexpectedly, no changes in p-mTOR protein levels were observed at different time points in cells treated with melatonin. Our data suggest that induction of autophagy could occur upstream of mTOR signaling. ER stress has a pivotal role in cancer and some interactions between autophagy and ER stress have been reported [47–49]. On the other hand, activation of ER stress has been related with JNK phosphorylation and autophagosome formation in SK-N-SH cells [50] or BRAFV600E melanoma cells [48]. Previous research has shown that Beclin1 interacts with anti-apoptotic proteins, such as Bcl-2 or Bcl-XL, inhibiting autophagy [20, 51, 52]. In different situations that induce autophagy, p-JNK is able to phosphorylate Bcl-2, promoting the disruption of Bcl-2/Beclin1 complex and consequently, the initiation of the autophagic process [48, 52]. Our results demonstrate that melatonin administration induces an increase in GRP78 levels paralleled by JNK phosphorylation. Moreover, Beclin1 levels are enhanced from early stages of melatonin administration. These observations are consistent with an induction of autophagy mediated by ER stress, JNK activation and high levels of Beclin1 as necessary elements for autophagosome formation. In fact, inhibition of p-JNK prevented the increment of LC3II protein levels by melatonin. Based on these findings and previous reports, we hypothesize that melatonin could induce autophagy through phosphorylation of Bcl-2 and disturbance of its interaction with Beclin1.

The role played by autophagy in cell survival and cell death in cancer is still unclear. Autophagy functions as a tumour suppressor mechanism by removing damaged organelles/proteins and limiting cell growth and genomic instability, and protects against tumorigenesis by limiting necrosis and chronic inflammation in the early stage [53]. In established tumour cells, autophagy can be used as a survival mechanism in response to chemotherapeutic or nutritional stress. Autophagy modulation as a potential therapeutic target in cancer has been reported in ovarian cell lines, colorectal cancer stem-like cells, immortalized baby mouse kidney (iBMK) cells and the triple-negative breast cancer [54–57]. In the present study, autophagic inhibition assessed by *ATG5* silencing increased melatonin-induced apoptotic cell death, suggesting that autophagy favours cell survival in HepG2 cells.

Melatonin exerts its biological actions by receptor-mediated and independent-receptor mechanisms. Two plasma membrane-bounded melatonin receptors, MT1 and MT2, and a nuclear receptor of the orphan family have been described. Moreover, melatonin can also bind to calmodulin and drive antiproliferative effects [58]. MT1 and MT2 are widely expressed in different human tissues, and its implication in melatonin-related antiproliferative action has been reported in different cancer cell lines and *in vivo* models [59]. Our group has demonstrated that inhibition of MT1 prevents cytostatic and cytotoxic effects of pharmacological concentrations of melatonin on HepG2 cells, probably associated to alterations in cAMP intracellular concentrations [60]. Activation of MT receptors requires the formation of homo or heterodimers. It has been shown that MT1 and MT2 interaction is

necessary for melatonin inhibitory action on cAMP formation when nanomolar concentrations are used [61]. However, MT2 receptor is not expressed in HepG2 cells [62], which could explain that higher concentrations of the indole are necessary for its apoptotic and autophagic effect on this HCC cell line.

Ceramides regulate many cellular processes and play an important role as a lipid mediator of apoptotic cell death. Interestingly, ceramide has been also reported to promote the formation of autophagic vacuoles by up-regulation of Beclin1, ER stress and JNK phosphorylation [63, 64]. Here, we show an early induction of ceramide synthesis in response to melatonin treatment. To our understanding, there are no previous reports that demonstrate alterations in ceramide metabolism due to melatonin, but other compounds with similar effects on cancer cells have demonstrated to induce endogenous ceramides accumulation. For instance, resveratrol induces an increase in ceramide levels via *de novo* biosynthetic pathway in breast cancer cells [65]. Stichoposide c also increases ceramide production through ASMase activation in mouse CT-26 subcutaneous tumours and HL-60 leukaemia xenograft models or colorectal cancer cells [66]. The increase in ceramide levels by melatonin is due in part to the ASMase activity observed early after melatonin treatment, although we cannot discard the contribution of the *de novo* synthesis because of the observed increase in the expression of key enzymes in the *de novo* synthesis. To address the implication of ceramide metabolism on autophagy induced by melatonin, we prevented activation of the enzymes SPT and ASMase with chemical inhibitors. When ASMase activity was inhibited with imipramine, we observed that melatonin was able to induce LC3 lipidation but not p62 degradation, indicating an accumulation of autophaghosomes and, consequently, an incomplete autophagic process. These results are in accordance to those observed in ASMase deficient mice, where induction of autophagy in liver due to high fat diet feeding is not complete [67]. In addition, KO ASMase mice exhibit a defect in the fusion of autophagosomes with lysosomes in arterial smooth muscle cells [67]. In line with these findings, ASMase was necessary for the upregulation of *ATG5* expression and autophagy induction in HepG2 cells [64]. Moreover, other inhibitors of ASMase such as desipramine, disturbe lysosome function in HTC hepatoma cells [68]. Lysosomal membrane permeabilization (LMP) has been suggested to be related to impaired autophagic flux because of the release of lysosomal enzymes to the cytoplasm, altering lysosomes function [69, 70]. In addition, the lack of ASMase activity is also associated to LMP and blocking of autophagy [67, 71]. So, our observations in HepG2 cells treated with melatonin and imipramine suggest a LMP regulation through ASMase activity.

De novo synthesis of ceramides is necessary for autophagy induction in different cell types and contexts. For instance, inhibition of SPT with myriocin in MCF-7 suppressed Beclin1 expression and reduced resveratrol induced autophagy [63]. Moreover, myriocin pre-treatment prevents C16 ceramide accumulation, ER stress and autophagy induced by resveratrol in human nasopharyngeal cancer cells [72]. In addition, overexpression of SPT in liver has been observed to induce autophagy [73]. In line with these findings, our experiments with the SPT inhibitor myriocin showed that this enzyme is necessary for autophagy induction due to melatonin. However, the mechanism by which myriocin pre-treatment prevents melatonin-induced autophagy in HepG2 cells remains unclear.

To elucidate the interplay between autophagy and apoptosis, and the implication of both ceramide synthesis pathways in these processes, we employed myriocin 2.5 μM and imipramine 10 μM. ASMase is an enzyme that mediates stress response and apoptosis. It is activated in response to a variety of proinflammatory and proapoptotic stimuli such as cytokines, ROS and oxidative stress [25, 74, 75]. Our results show that inhibition of ASMase activity recovers partially the apoptotic cell death induced by melatonin. Moreover, ASMase inhibition in autophagy-defective cells reduces apoptotic markers such as PARP and BAX versus cells without imipramine pre-treatment. These results taken together suggest that ASMase activity plays an important role in melatonin-induced apoptotic cell death.

When SPT was blocked, cell death induced by melatonin was potentiated, which could be explained by the fact that autophagy as a survival mechanism is not induced. In human breast cancer cells, vitamin E analogs stimulate apoptosis mediated by ceramides, which in turn activate the JNK/CHOP pathway; however when *de novo* synthesis of ceramides is abolished, no toxic effects are observed, showing a relationship between *de novo* pathway and cell death [76]. In bovine endothelial cerebral cells, apoptosis induced by TNF-α is due to ceramide accumulation by *de novo* synthesis stimulation [77]. Resveratrol, another molecule with similar properties to melatonin, reduces the expression of cleaved PARP when SPT is inhibited with myriocin in human nasopharyngeal carcinoma cells [72]. These data contrast with our results, which demonstrate that inhibition of SPT reduces HepG2 cells viability and increases apoptosis when melatonin is administered. On the other hand, it has been observed that inhibition of *de novo* ceramide synthesis with fumonisin B1 in mouse liver activates ASMase at the same time that sphyngomielin levels are reduced [78]. Moreover, administration of fumonisin B1 in mice increases apoptosis although ceramide levels are not altered [79], which suggests that ASMase could enhance its activity in order to maintain ceramide amounts. In addition, SPT inhibition in insulinoma cells did not affected induced apoptosis, and it was proposed that ceramides from sphyngomieline hydrolysis was the key factor for cell death [80]. SPT inhibition in HepG2 cells causes autophagy suppression, so the protective effect induced in response to melatonin is not activated, which could also explain this enhancement in cell death. In addition, myriocin pre-treatment and melatonin administration enhanced significantly ASMase RNA levels versus only melatonin.

In summary, these results suggest that the inhibition of *de novo* ceramide synthesis could be activating sphingomyelin hydrolysis through ASMase activity as a compensatory mechanism. Additionally, the results presented here indicate that ASMase activity stimulated by melatonin regulates autophagy. Moreover, when both autophagy and ASMase were inhibited, apoptosis was lower compared to melatonin alone. These data suggest that ASMase changes induced by melatonin have a more powerful effect on apoptosis and cell viability that its implication in the regulation of autophagic flux. However, autophagy suppression by targeting SPT is important to enhance the cytotoxic effect of melatonin. Our findings indicate that inhibition of autophagy could increase the beneficial effects of melatonin in cancer treatment, but the blockade of this mechanism should be made with caution considering the implication of different intracellular messengers in cell death.

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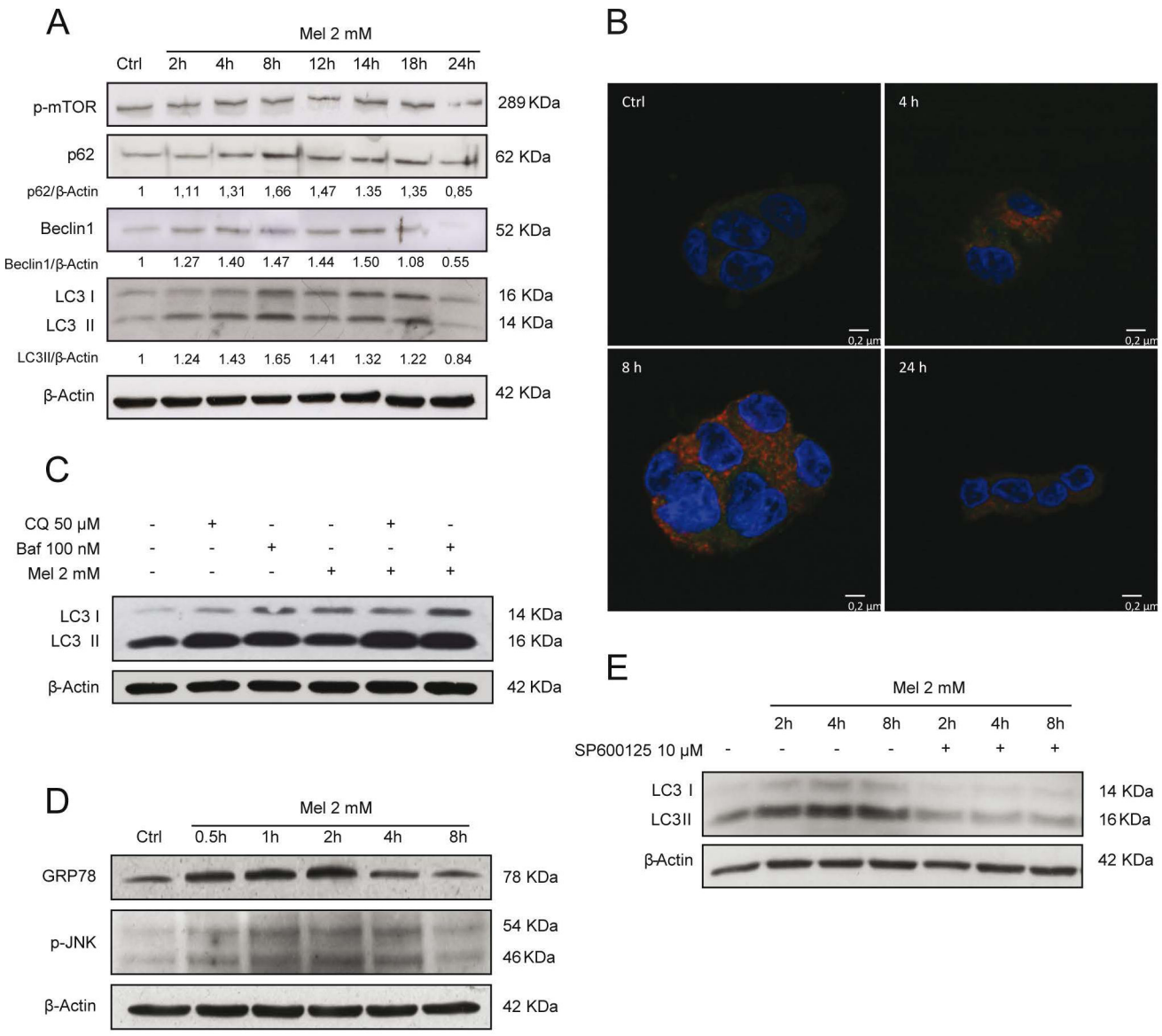

Fig. 1.

Melatonin (mel) administration (2 mM) induces an autophagic response in HCC HepG2 cells. Representative immunoblots of p-mTOR, p62, Beclin1 and LC3I/II after melatonin treatment for different time points showing a transient autophagic response characterized by Beclin1 and LC3II increased expression and p62 degradation. No changes were observed in p-mTOR levels (A). Representative confocal images of LAMP-2 (red) and LC3 (green) immunofluorescence labelling showing a maximal colocalization of LC3 and LAMP-2 at 8 hours after melatonin administration, which disappears at 24 hours (B). Effect of melatonin 2 mM on autophagic flux. Representative immunoblots of LC3I/II from HepG2 cells treated for 8 hours with melatonin in presence of the autophagic inhibitors chloroquine 50 µM (CQ) and bafilomycin 100 nM (Baf) for the last 4 hours of treatment. LC3 levels increased following treatment with melatonin as well as with CQ and Baf, with a higher effect following the combination of melatonin and the autophagic inhibitors (C). Expression levels

of ER stress proteins. Time-course with melatonin 2 mM showed enhanced levels of BIP and p-JNK from 30 minutes of treatment until 2–4 hours (D). Prevention of JNK phosphorylation with SP600125 10 μM reduced LC3II formation (E). Images of immunoblots are representative for experiments performed in triplicate.

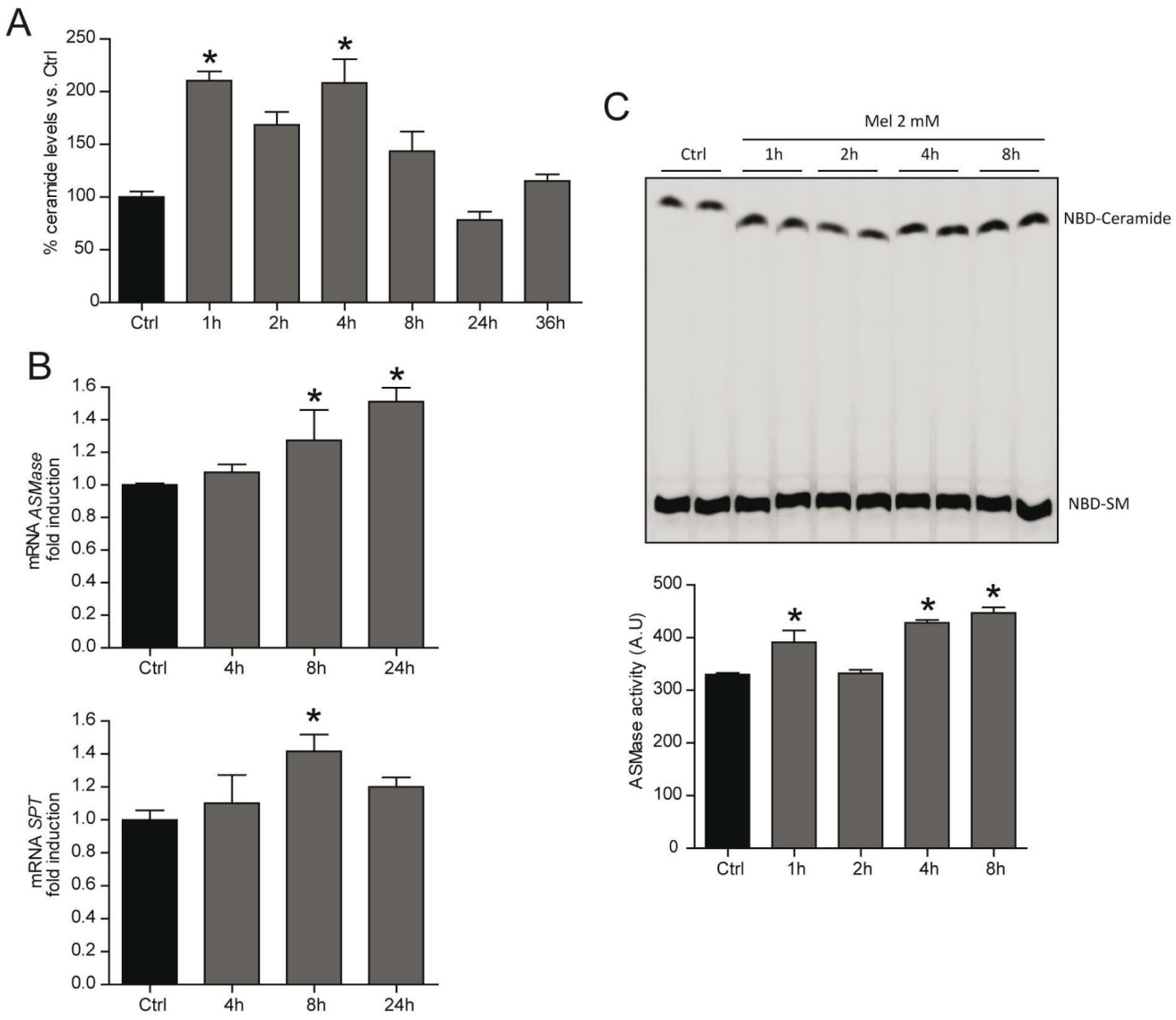

Fig. 2.

Autophagic response induced by melatonin 2 mM in HepG2 cells is essential for cell survival. Silencing of *ATG5* for 24 hours reduces *ATG5* mRNA levels and autophagy induced by melatonin 2 mM treatment for 8 hours (A and B). Representative immunoblots showed not increased levels of Atg5 and LC3, as well as p62 accumulation. After 48 hours, MTT assays revealed lower viability of *ATG5*-silenced cells in presence of melatonin in a dose-dependent manner (C). Autophagy blocks the increased apoptotic response to melatonin 2 mM treatment. Representative immunoblots of cleaved-PARP, a marker of apoptosis, showed that *ATG5*-silenced cells are more susceptible to apoptosis in presence of melatonin (D). Images of immunoblots are representative for experiments performed in triplicate. Data are expressed as a percentage of mean values ± SEM of three independent experiments. **P* < 0.05 significant differences *vs* non-silenced cells ***P* < 0.05 significant differences between treated and untreated silenced cells #*P* < 0.05 significant differences between control-treated cells and silenced-treated cells cells.

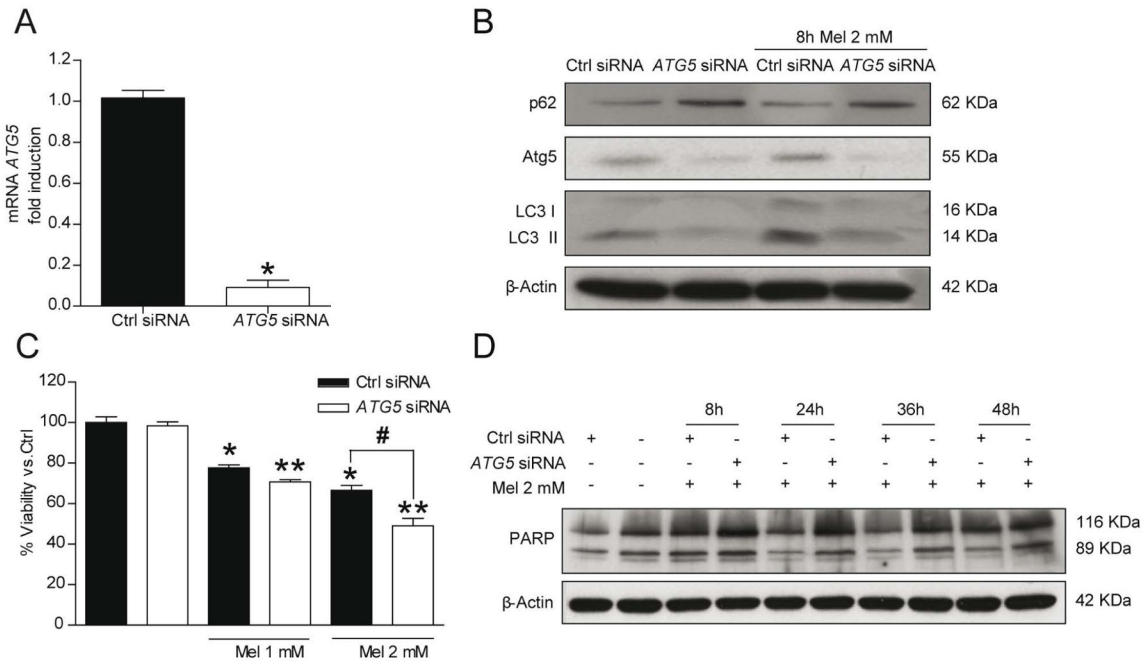

Fig. 3.

Sphingolipid metabolism is enhanced in HepG2 cells in response to melatonin 2 mM treatment. Total ceramide levels analyzed by HPLC in HepG2 cells treated with melatonin 2 mM for different time points showing enhanced ceramide synthesis from 1 hour (A). Representative TLC of ASMase activity displaying the enhanced ASMase activity from 1 hour later melatonin 2 mM treatment. Arbitrary units are densitometric values of NBD-Ceramide bands representing the specific product normalized with the values of the corresponding NBD-SM bands (B). mRNA levels of *SPT* and *ASMase* after incubation with melatonin 2 mM at the indicated time points analyzed by quantitative RT-PCR. mRNA values were normalized to β-actin RNA and reported as relative levels compared to the expression in control (Ctrl) HepG2 cells (C). Data are expressed as a percentage of mean values ± SEM of experiments performed in triplicate.$^{*}P < 0.05$ versus Ctrl HepG2 cells values by unpaired one-way analysis of variance with the Bonferroni post hoc test.

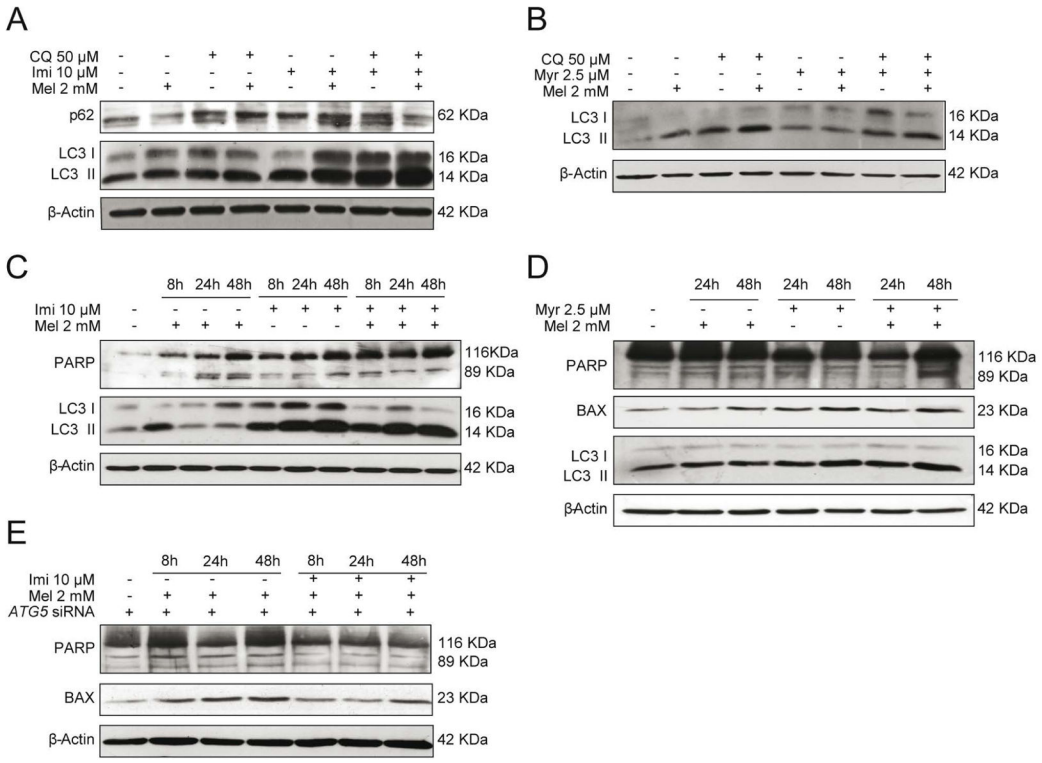

Fig. 4.

Effect of ceramide synthesis inhibition in HepG2 cells on autophagy and apoptosis induced by melatonin 2 mM. Representative immunoblots of LC3 and p62 in HepG2 cells pre-incubated with imipramine 10 µM 2 hours and treated with melatonin 2 mM in the absence or presence of CQ 50 µM for the last four hours of treatment. LC3II protein levels were enhanced with imipramine pre-treatment as well as p62, suggesting an impairment in autophagic flux (A). Representative immunoblots of LC3 in HepG2 cells pre-incubated with myriocin 2.5 µM (Myr) 2 hours and treated with melatonin 2 mM in the absence or presence of CQ 50 µM for the last four hours of treatment. Pre-treatment with myriocin prevented an increase of LC3 levels in response to melatonin administration (B). Representative immunoblots of PARP and LC3 in HepG2 cells pre-incubated with imipramine (Imi) 10 µM for 2 hours and treated with melatonin 2 mM, showing that pre-incubation with imipramine enhanced LC3 levels and prevented PARP cleavage in response to melatonin administration (C). Representative immunoblots of PARP, BAX and LC3 in HepG2 cells pre-incubated with myoricin 2.5 µM for 2 hours and treated with melatonin 2 mM. Pre-incubation with myriocin enhanced LC3, PARP cleavage and Bax levels in response to melatonin treatment (D). Representative immunoblots of PARP and BAX in *ATG5*-silenced HepG2 cells pre-incubated with imipramine 10 µM 2 hours and treated with melatonin 2 mM, showing that pre-incubation with imipramine prevented PARP cleaved and reduced BAX levels in response to melatonin administration (E). Data are expressed as a percentage of mean values ± SEM of experiments performed in triplicate.

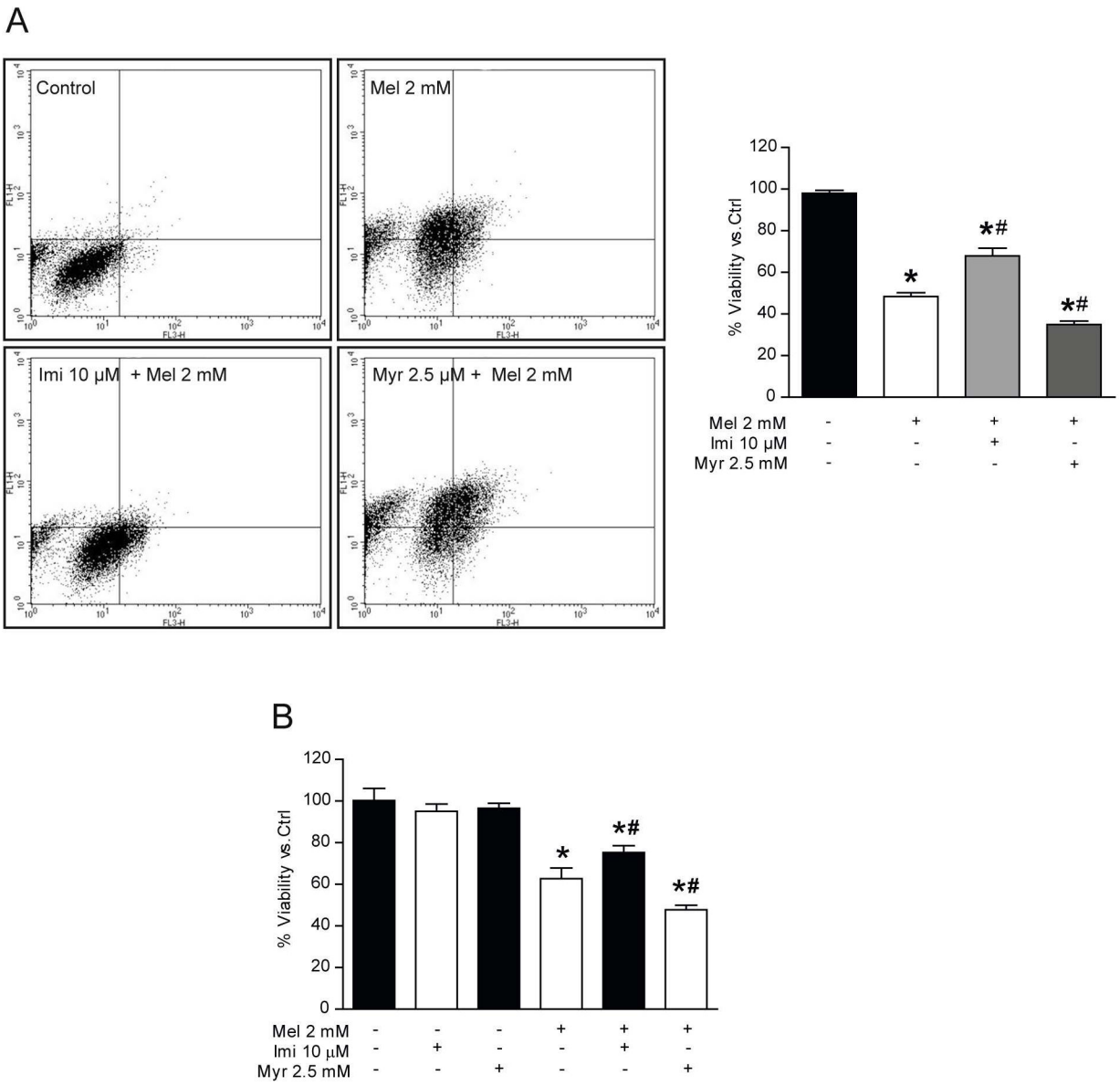

Fig. 5.

Effect of SPT and ASMase inhibition on cell viability and cell death in melatonin-HepG2 treated cells. Cells pre-treated with imipramine 10 μM or myriocin 2.5 μM were incubated with melatonin 2 mM until 48 hours. Annexin V-propidium iodide assay determinated low cell viability when cells were pre-treated with myriocin and a parcial rise when ASMase was inhibited (A). Viability was determined by MTT assay, showing a decrease in cell viability in myriocin plus melatonin group, but a recovery of viability in imipramine co-treated cells (B). Data are expressed as a percentage of mean values ± SEM of three independent experiments. **P* < 0.05 significant differences *vs* control cells #*P* < 0.05 significant differences *vs* melatonin-treated cells.