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Melatonin inhibits the sphingosine kinase 1/sphingosine-1-phosphate signaling pathway in rabbits with fulminant hepatitis of viral origin

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Running head: Sphingosine kinase 1/sphingosine-1-phosphate pathway and melatonin

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Abstract: The sphingosine kinase (SphK)1/sphingosine-1-phosphate (S1P) pathway is involved in multiple biological processes, including liver diseases. This study investigate whether modulation of the SphK1/S1P system associates to the beneficial effects of melatonin in an animal model of acute liver failure (ALF) induced by the rabbit hemorrhagic disease virus (RHDV). Rabbits were experimentally infected with $2x10^4$ hemagglutination units of a RHDV isolate and received 20 mg/kg of melatonin at 0 hr, 12 hr and 24 hr postinfection. Liver mRNA levels, protein concentration and immunohistochemical labelling for SphK1 increased in RHDV-infected rabbits. S1P production and protein expression of the S1PR1 receptor were significantly elevated following RHDV infection. These effects were significantly reduced by melatonin. Rabbits also exhibited increased expression of toll-like receptor (TLR)4, tumor necrosis factor alpha (TNF-α), interleukin (IL)-6, nuclear factor-kappa B (NF-κB) p50 and p65 subunits and phosphorylated inhibitor of kappa B (IκB)α. Melatonin administration significantly inhibited those changes and induced a decreased immunoreactivity for RHDV viral VP60 antigen in the liver. Results obtained indicate that the SphK1/S1P system activates in parallel to viral replication and the inflammatory process induced by the virus. Inhibition of the lipid signaling pathway by the indole reveals novel molecular pathways that may account for the protective effect of melatonin in this animal model of ALF, and supports the potential of melatonin as an antiviral agent.

Introduction

Sphingolipids represent a major class of lipids that form components of cellular membranes and play important roles in diverse cellular events, such as cell growth arrest, senescence, apoptosis, or inflammation [1]. Sphingosine-1-phosphate (S1P), a pleiotropic bioactive lipid mediator, is formed by the phosphorylation of sphingosine by sphingosine kinases (SphK) and regulates an array of biological activities in various cell types [2]. Two isoforms of mammalian SphK (SphK1 and SphK2) have been cloned and characterized. SphK1, the predominant isoform in many cells, plays a central role in the so-called 'sphingolipid rheostat' as it controls the balance between the levels of the sphingolipids ceramide, sphingosine, and S1P [3]. Different stimuli activate SphK1 by inducing its phosphorylation and translocation to the plasma membrane, where S1P is produced [4]. Most of the characterized actions of S1P are mediated through a family of five G protein-coupled receptors (S1PR types 1-5) [5]. S1PR1, S1PR2, and S1PR3 are expressed by a wide variety of tissues, while S1PR4 expression is confined to lymphoid and haematopoietic tissue and S1PR5 expression to the central nervous system [6]. Tumor necrosis factor (TNF)α-dependent activation of SphK1 and binding of S1P to S1PR1 receptor, has been reported to promote nuclear factor-kappa B (NF-κB) activation and signal transducer and activator of transcription 3 (STAT3)-mediated interleukin (IL)-6 formation, contributing to a feed-forward amplification loop which plays an important role in inflammation and cancer [4,7].

In the last few years a number of studies have approached the biological significance of the SphK1/S1P signalling pathway in liver diseases. A role of S1P receptors has been reported in the wound healing response to liver injury induced by carbon tetrachloride and in cholestasis-induced liver fibrosis, respectively [8,9]. It has been shown that S1P plays a significant role in regeneration

and fibrosis in diethylnitrosamine-treated mice [10], and that intracellular S1P contributes to collagen expression of hepatic miofibroblasts in human fibrosis [11]. SphK1 also mediates inflammation in nonalcoholic fatty liver disease [12] and it has been very recently reported that deletion of SphK1 ameliorates hepatic steatosis in obese mice [13]. Acute liver failure (ALF) is an unusual, severe and often fatal, condition resulting of rapid hepatocyte injury occurring over days or a few weeks, and encompassing multiple etiologies. Liver transplantation is the only therapy of proven benefit, but the rapidity of progression and the variable course of the disease limit its use [14]. Therefore, identification of new molecular targets may be useful in AFL therapy. However, the exact functional role of S1P as well as the therapeutic potential of strategies aimed at SphK1/S1P signalling in AFL is still unknown.

Viral infections due to hepatitis B virus, hepatitis A virus, and hepatitis E virus are common causes of ALF, mainly in particular geographical areas of the world [15]. Recently, several studies have addressed the role of S1P in viral infections and shown that bovine viral diarrhea virus [16] or dengue virus [17] inhibit SphK1 activity, leading to enhanced virus replication. In contrast, respiratory syncytial virus [18], human cytomegalovirus [19], and influenza virus [3,20] increase the activation of SphK1. Therefore, SphK1 activity is differentially regulated depending on the type of virus, presumably because viruses have developed specialized strategies to employ host cellular machinery for their own advantage [21,22]. Our research group has described a new animal model of ALF using experimental infection of rabbits with the rabbit hemorrhagic disease virus (RHDV) which reproduces the most representative biochemical and histologic parameters and clinical signs of the human disease [23,24]. Different studies with the RHDV have demonstrated that melatonin reduces liver damage in RHDV-infected rabbits by a combination of anti-oxidant, anti-inflammatory and anti-apoptotic effects, being also able to reduce viral replication [25-28]. However, information on the relationship between melatonin and sphingolipid metabolism is

scarce, and only one research indicating an association of melatonin with SphK1 inhibition in prostate cancer cells has been published until now [29]. In the present work we first investigated if RHDV infection induces changes in the SphK1/S1P signalling pathway. Subsequently, the potential contribution of this system to the protective effects of melatonin was analysed. Results obtained support the usefulness of melatonin as an antiviral agent and the interest of the SphK1/S1P system as an attractive target for therapeutic interventions in AFL.

Materials and methods

Virus and experimental model

Nine-week-old male New Zealand white rabbits were kept in the animal facility of the University of León with 12-hr light cycle at 21-22ºC and 50% relative humidity. They were given a standard dry rabbit food and water *ad libitum*. Effects of melatonin were studied by sacrificing control rabbits and batches of infected animals at 18, 24, 30 and 36 hr post infection (hpi). Infection was induced by i.m. injection of $2x10^4$ hemagglutination units of an RHDV isolate [23]. Melatonin was given (20 mg/kg body weight i.p.) at 0, 12 and 24 hpi; untreated animals received 4 mL of vehicle at 0, 12 and 24 hpi. Melatonin (Sigma, St Louis, MO, USA) was dissolved into absolute ethanol and further dilutions were made in saline. The final concentration of ethanol was 5%.

The study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and was specifically approved by the Ethics Committee of the University of León.

Real-time RT-PCR

Total RNA was extracted from frozen rabbit liver using a Trizol reagent (Life Technologies, Madrid, Spain) and quantified using NANO DROP 1000 spectrophotometer (Thermo Scientific,

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Wilmington, DE). Residual genomic DNA was removed by incubating RNA with RQ1 RNase-free DNase (Promega, Madison, WI). RNA integrity was confirmed by formaldehyde gel electrophoresis. Total RNA (1 μg) was reverse transcribed as described [30] and mRNA was determined by real time PCR analysis using SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany) and the appropriate primers (5′-GCTGGCCGCTTCTCTGAAC-3′ and 5′-GCTGGCCGCTTCTCTGAAC-3′ for SphK1 and 5′-TGGCATCCTGACGCTCAA-3′and 5′-TCGTCCCAGTTGGTCACGAT-3′ for β-Actin). Relative changes in gene expression levels were determined using the $2^{-\Delta\Delta ct}$ method as described [25]. The cycle number at which the transcripts were detectable (Ct) was normalized to the cycle number of β-Actin gene detection, referred to as ΔCt.

Western blot analysis

For Western blot analysis liver tissue (0,2 gr) was homogenised in 1 mL RIPA buffer containing protease and phosphatase inhibitor cocktails (Roche Diagnostics GmbH), maintaining temperature at 4ºC throughout all procedures. Then the homogenate was incubated on ice for 30 min and finally the samples were centrifuged at 13,000g for 30 min at 4ºC. The supernatant fraction was stored at -80ºC in aliquots until use. Protein concentration was measured by Bradford assay. Equal amounts of protein extracts (30 μg) were separated by 7-12% sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and transferred electrically to polyvinyllidene difluoride membranes (Millipore; Bedford, MA, USA). The membranes were then blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBST) for 30 min at 37ºC and probed overnight at 4ºC with polyclonal anti p50, p65 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho-IκBα, IκBα (Cell Signaling Technology, Danvers, MA, USA) and anti TNF-α, TLR4,

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IL-6, SphK1 and S1PR1 (Abcam; Cambridge, UK) antibodies at 1:200-1:1,000 dilution with PBST containing 2.5% non-fat dry milk. Equal loading of protein was demonstrated by probing the membranes with a rabbit anti Actin polyclonal antibody (1:2,000; Sigma). After washing with TBST, the membranes were incubated for 1 hr at room temperature with secondary HRP conjugated antibody (Dako, Glostrup, Denmark, 1:5,000), and visualized using ECL detection kit (Amersham Pharmacia; Uppsala, Sweden) [26]. The density of the specific bands was quantified with an imaging densitometer (Scion Image J Software 1.46a; Bethesda, MD, USA).

Immunohistochemistry

Tissue samples were recovered, fixed in 10% buffered formalin and embedded in paraffin. Sections (4 μm) were dewaxed and hydrated through graded ethanol, cooked in 25 mM citrate buffer, pH 6.0, in a pressure cooker for 10 min, transferred into boiling deionized water and let to cool for 20 min. Tissue sections were then treated with 3% hydrogen peroxide to inactivate endogenous peroxidase activity. The slides were incubated with rabbit anti SphK1, S1PR1 (Abcam), and mouse anti-VP60 (Ingenasa, Madrid, Spain) antibodies overnight at 4°C. Subsequently, the sections were incubated for 30 min using the EnVision+ system and developed with a solution of 3-3-diaminobenzidine (DAB) (Vector Lab; Burlingame, CA, USA). The slides were stained with hematoxylin for 10 s and mounted. The specificity of the technique was evaluated by negative controls (omitting the incubation with the primary antibody and incubating it with non-immune sera). Pathological findings were assessed by one of the authors blinded to the group allocations.

Assay for S1P levels

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S1P was tested by ELISA using a commercial kit (Echelon Biosciences, UT, USA). Quantitative S1P assays were done in duplicate on all liver homogenate samples according to manufacturer's instructions. The levels were expressed as pmol/μg of protein.

Results

In order to confirm if the SphK1/S1P axis was induced in RHDV-infected rabbits, immunohistochemistry for SphK1 was performed. Immunoreactivity was negative in liver sections from control rabbits, and increased progressively in the RHDV-infected group (Fig. 1A, B). Parallel changes were observed in the mRNA levels and protein concentration of the enzyme (Fig. 1C, D). All these effects were significantly abrogated in animals receiving melatonin (Fig. 1A-D).

To investigate whether RHDV-induced SphK1 activation increases intracellular S1P (the product of SphK1), S1P was tested by ELISA at several time points after RHDV infection. Our data indicate that RHDV infection significantly increased the level of S1P in rabbit liver (Fig. 2D). However, melatonin administration significantly dampened the elevation of S1P level.

Since binding of S1P to the S1PR1 receptor has been shown to be playing a role in the activation of inflammatory pathways, we first investigated whether this receptor is expressed in rabbit cells. Western blot analysis indicated that S1PR1 was detectable in control rabbits and its expression increased significantly in animals infected with the RHDV (Fig. 2C). This issue was further confirmed by S1PR1 immunohistochemical labelling (Fig. 2A, B). Protein concentration and immunoreactivity were significantly lower in animals treated with melatonin (Fig. 2A-C).

Given the well-known role of the SphK1/S1P system on inflammation, expression of different inflammatory markers was studied in RHDV-infected and melatonin-treated rabbits. As shown in Fig.3 expression of TNF-α and IL-6 was significantly elevated in infected rabbits and this effect

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associated to an increased expression of TLR4. Changes in the NF-κB-mediated signalling pathway were shown by a significant increase in the concentration of p50 and p65 NF-κB subunits, a reduced expression of IκBα, and an enhanced phosphorylation of the inhibitor. These effects were significantly attenuated in rabbits treated with melatonin.

Finally, to analyse the relationship between changes in the SphK1/S1P pathway and viral replication, the presence of the virus in the liver of RHDV-infected rabbits was detected by immunohistochemical staining of the viral VP60 antigen. Results obtained show a progressive increase in the extent of labelling in RHDV-infected animals. Decreased immunoreactivity in rabbits treated with melatonin confirms the inhibitory role of the indol on RHDV replication (Fig. 4A, B).

Discussion

S1P is now recognized as a powerful mediator of many vital cellular processes, and a pletora of roles have emerged in the regulation of phenomena as inflammation or viral replication. The development of the sphingosine analogue FTY720 has proven the potential beneficial effects of targeting SphK1/S1P signaling in human diseases and the interest of developing a sphingolipid-centered therapeutics [31]. Mechanisms responsible for liver injury in acute severe hepatitis leading to AFL are complex and include the virus cytopatic effect, the damage induced by an exacerbated immune response, and the citotoxicty of immune effectors [32]. The development by our group of a highly reproducible model of virus-induced ALF has offered a valuable tool to test protective therapies in this condition and to identify the responsible mechanisms [33]. Previous studies have shown that in rabbits with fulminant hepatitis induced by the RHDV a combination of effects contribute to reduce liver damage [25-27]. Data obtained in the present research indicate that

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inhibition of SphK1/S1P signaling may be a novel mechanism contributing to the benefits of melatonin treatment in this animal model of ALF.

S1P has been linked to the production of proinflammatory cytokines and inflammation, and different research suggests that pharmacological agents targeting the function of S1P and its receptors show therapeutic potential for treating a wide range of inflammatory disorders [4,7]. We demonstrate in our study that hepatic SphK1 activity is upregulated in RHDV-infected rabbits, with an increased production of S1P and enhanced protein level of S1PR1, which associate to a higher TNF-α and IL-6 expression. Melatonin significantly reduced, as previously reported [34], expression of the proinflammatory markers, and also resulted in a clear inhibition of the SphK1/S1P system. Some studies have shown that suppression of the SphK1/S1P pathway contributes to the anti-inflammatory effect of different therapeutic agents. Thus, the flavone apigenin decreases serum levels of IL-1β, IL-6 and TNF-α in a lipopolysaccharide-induced endotoxemic rat model, and this effect relates to a lowered SphK1 activity and S1P level in heart; in addition, apigenin also inhibits the SphK1/S1P system, resulting in a lowered inflammatory response, in rat embryonic heart-derived H9c2 cells [35]. In murine models of inflammatory arthritis, chemical inhibition or siRNA knockdown of SphK1 results in reduced inflammation [36]. The synthetic sphingosine analog FTY720 phosphate, by binding to and inducing functional antagonism of the S1PR1, reduces proinflammatory cytokine release by astrocytes in mice with multiple sclerosis [37].

NF-κB is a transcription factor acting as a known pleiotropic regulator of various genes involved in inflammatory responses. Many biological stimuli result in the phosphorylation and degradation of the IκBα inhibitor, causing the nuclear translocation of NF-κB heterodimers to the nucleus [38]. SphK1 and S1P are necessary for the phosphorylation of IκBα and IKKα, an upstream regulator of IκBα degradation, leading to NF-κB activation, which has therefore been

proposed to be crucial for connecting the activated SphK1/S1P signaling pathway with inflammation [39]. High fructose feeding in rats or stimulation of BRL3A cells with fructose induces an elevation of SphK1 activity and S1P production, with a subsequent increased phosphorylation of IκBα and IKKα, NF-κB activation, and increase of IL-1β, IL-6 and TNF-α protein levels; inhibition of the ShpK1/S1P pathway by morin, results in a reduction of NF-κB activation and pro-inflammatory cytokine levels in both experimental models [40]. Glabridin has been shown to inhibit TNF-α-induced ICAM-1 expression in HUVEC cells by blocking SphK1 activity and S1P generation, an effect associated to IκBα phosphorylation and NF-κB activation [41]. In addition, experimental diabetic nephropathy models, berberine inhibits SphK1 activity and S1P production, also suppressing NF-κB activation [42]. Our finding of a parallel inhibition of the SphK1/S1P and NF-κB pathways, suggest that NF-κB activation mediated by the S1P system may play role in the protective effect of the flavonoid on hepatic inflammation. Moreover, melatonin action could also be connected to the observed downregulation of TLR4, a transmembrane protein which may ignite cascades of proinflammatory mediators, thus aggravating hepatocellular damage in severe forms of acute liver disease [34,43]. Previous studies have shown that lipopolysaccharide stimulation of TLR4 in macrophages increases SphK1 mRNA and activity, resulting in generation of S1P [44], and blockade of SphK1 has been found to inhibit TLR-induced NF-κB activation and production of proinflammatory cytokines in septic mice [45].

Understanding of the role of sphingolipids at the level of viral pathogenicity is still in its infancy and may vary considerably depending on the virus and type of disease [22]. Nevertheless, a number of studies have addressed in the last years the roles of SphK1 in viral infection. Stimulation of SphK1 activity is associated with increased viral replication and cell survival of syncytial virus, human cytomegalovirus or influenza virus [20,46]. However, reduction in SphK1 activity is

required for successful viral infection by the bovine viral diarrhea virus, and in dengue virus it has been suggested that therapeutic inhibition may have dual befits in the disease by reducing production of inflammatory mediators and assisting clearance of virus-infected cells [16,46]. We have previously reported that melatonin reduces VP60 immunohistochemical labelling and VP60 mRNA expression in hepatocytes from RHDV-infected rabbits [27]. Data from the present study confirm a decreased presence of the viral protein VP60 in the liver. In cells infected with the measles virus it has been previously shown that SphK1 inhibition impairs viral protein expression and infectious viral production, being this effect mediated by modulation of the NF-κB signal pathway, because the use of NF-κB specific inhibitors blocks viral replication [47]. It is also known that influenza virus increases expression/activation of SphK1 and inhibition of the enzyme suppresses the activation of NF-κB to reduce viral RNA synthesis [20]. Thus, results here obtained support a potential pro-viral role of the SphK1/S1P axis in the replication of the RHDV virus, and its modulation by the NF-κB pathway. Regulation of the sphingolipid pathway could play a role in the inhibition of viral replication by melatonin, although other mechanisms previously reported to by modulated by the indole, such as decreased activity of antioxidant enzymes, activation of Nrf2 signaling and changes in the autophagic response may also make a significant contribution [26,27].

In conclusion, we here describe novel features of the SphK1/S1P axis within the liver, providing evidence that the hepatic SphK1/S1P system activates in rabbits experimentally infected with the RHDV and that inhibition of this signaling pathway by melatonin associates to an anti-inflammatory effect and to a lowered RHDV replication induced by the indole. Melatonin has very high safety profile and no deaths or serious toxicity associated to its usage has been reported. Moreover, different studies have found melatonin being effective in fighting viral infections in a variety of experimental animal and *in vitro* studies [48,49]. Although further studies are required to

a better knowledge of melatonin effects on virus-host interactions, data obtained reveal novel molecular pathways that may account for the protective effect of melatonin in virus-induced ALF, and support a possible therapeutical potential for melatonin in human virus-induced disorders.

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Figure legends

Fig. 1. Liver expression of SphK1 in RHDV-infected and melatonin-treated rabbits. (A): Photomicrographs of sections of liver samples taken from (a) Control; (b) Control + Mel; (c) RHDV 18 hr; (d) RHDV + Mel 18 hr; (e) RHDV 24 hr; (f) RHDV + Mel 24 hr; (g) RHDV 30 hr; (h) RHDV + Mel 30 hr; (i) RHDV 36 hr; (j) RHDV + Mel 36 hr. Paraffin-embedded sections were stained with SphK1 antibody. Original magnification: 200x. (B): Image analysis of the area of SphK1 staining. Image analysis was performed using the ImageJ software v3.91. (C): Levels of SphK1 mRNA analyzed by real-time PCR assay and normalized against β-Actin. (D): Western blot of SphK1. Proteins from liver extracts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by immunoblotting. Equal loading of proteins is illustrated by β-Actin bands. Values are expressed as means S.E.M (n=6). $^{a}p < 0.05$, compared with Control. $^{b}p < 0.05$, compared with RHDV same period.

Fig. 2. Liver expression of S1PR1 in RHDV-infected and melatonin-treated rabbits. (A): Photomicrographs of sections of liver samples taken from (a) Control; (b) Control + Mel; (c) RHDV 18 hr; (d) RHDV + Mel 18 hr; (e) RHDV 24 hr; (f) RHDV + Mel 24 hr; (g) RHDV 30 hr; (h) RHDV + Mel 30 hr; (i) RHDV 36 hr; (j) RHDV + Mel 36 hr. Paraffin-embedded sections were stained with S1PR1 antibody. Original magnification: 200x. (B): Image analysis of the area of S1PR1 staining. Image analysis was performed using the ImageJ software v3.91. (C): Western blot of S1PR1. Proteins from liver extracts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by immunoblotting. Equal loading of proteins is illustrated by β-Actin bands. (D): Level of S1P in rabbits liver homogenates analyzed by ELISA. Values are expressed as

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means S.E.M (n=6). [a] $p < 0.05$, compared with Control. [b] $p < 0.05$, compared with RHDV same period.

Fig. 3. Liver expression of TNF-α, IL-6, TLR4, p50, p65, phospho-IκBα and IκBα in RHDV-infected and melatonin-treated rabbits. Protein from liver extracts was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by immunoblotting. Equal loading of proteins is illustrated by β-Actin bands. Left upper panel shows representative Western-blot photographs. Histograms represent the result of densitometric quantification. Values are expressed as means S.E.M (n=6). [a] $p < 0.05$, compared with Control. [b] $p < 0.05$, compared with RHDV same period.

Fig. 4. Liver expression of the RHDV capsid protein VP60 in RHDV-infected and melatonin-treated rabbits. (A): Photomicrographs of sections of liver samples taken from (a) Control, (b) Control + Mel (c) RHDV 18 hr; (d) RHDV + Mel 18 hr; (e) RHDV 24 hr; (f) RHDV + Mel 24 hr; (g) RHDV 30 hr; (h) RHDV + Mel 30 hr; (i) RHDV 36 hr. (j) RHDV + Mel 36 hr. Paraffin-embedded sections were stained with VP60 antibody. Original magnification: 200x. (B): Image analysis of the area of VP60 staining. Image analysis was performed using the ImageJ software v3.91. Values are expressed as means S.E.M (n=6). [a] $p < 0.05$, compared with Control. [b] $p < 0.05$, compared with RHDV same period.

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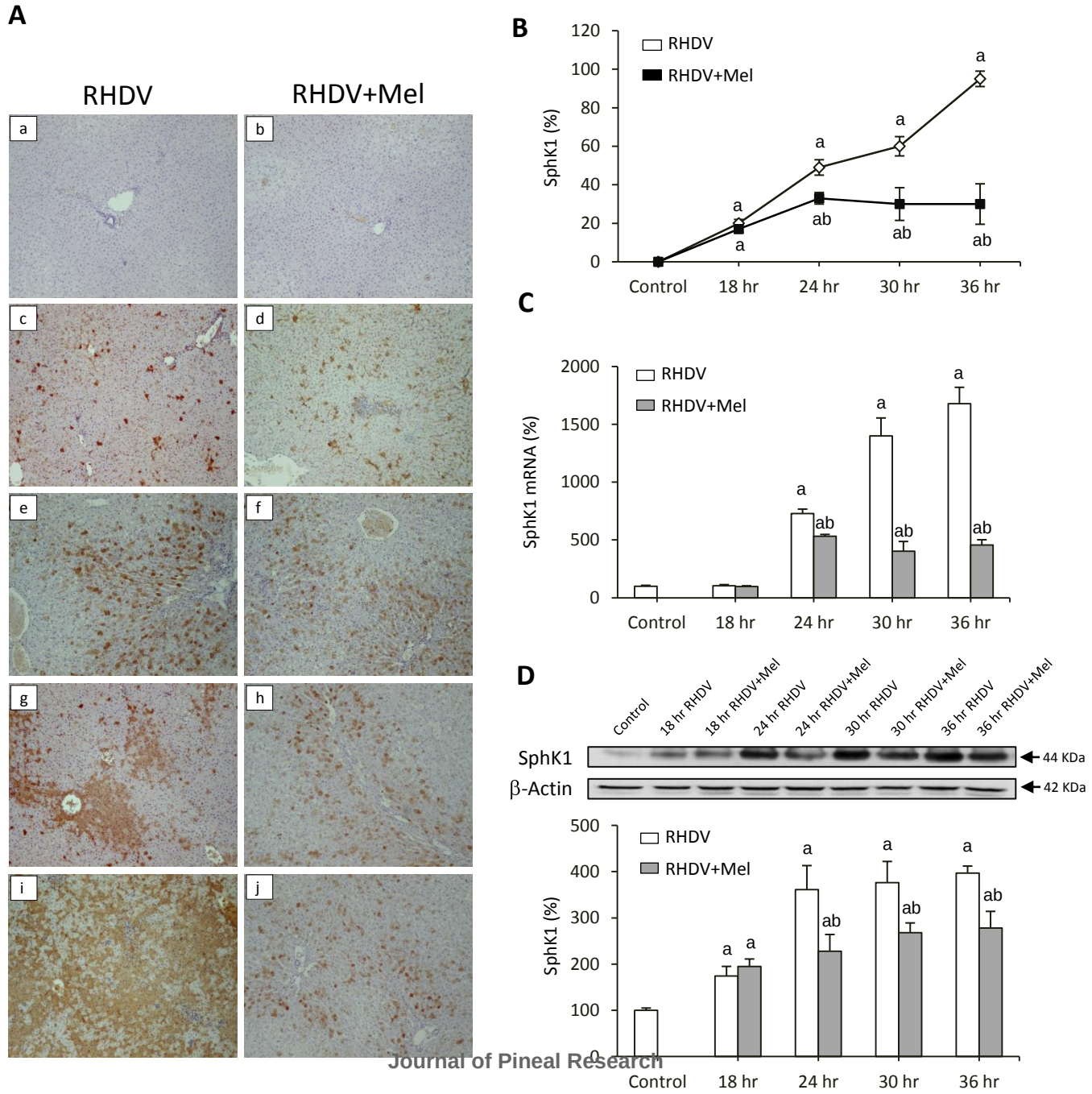

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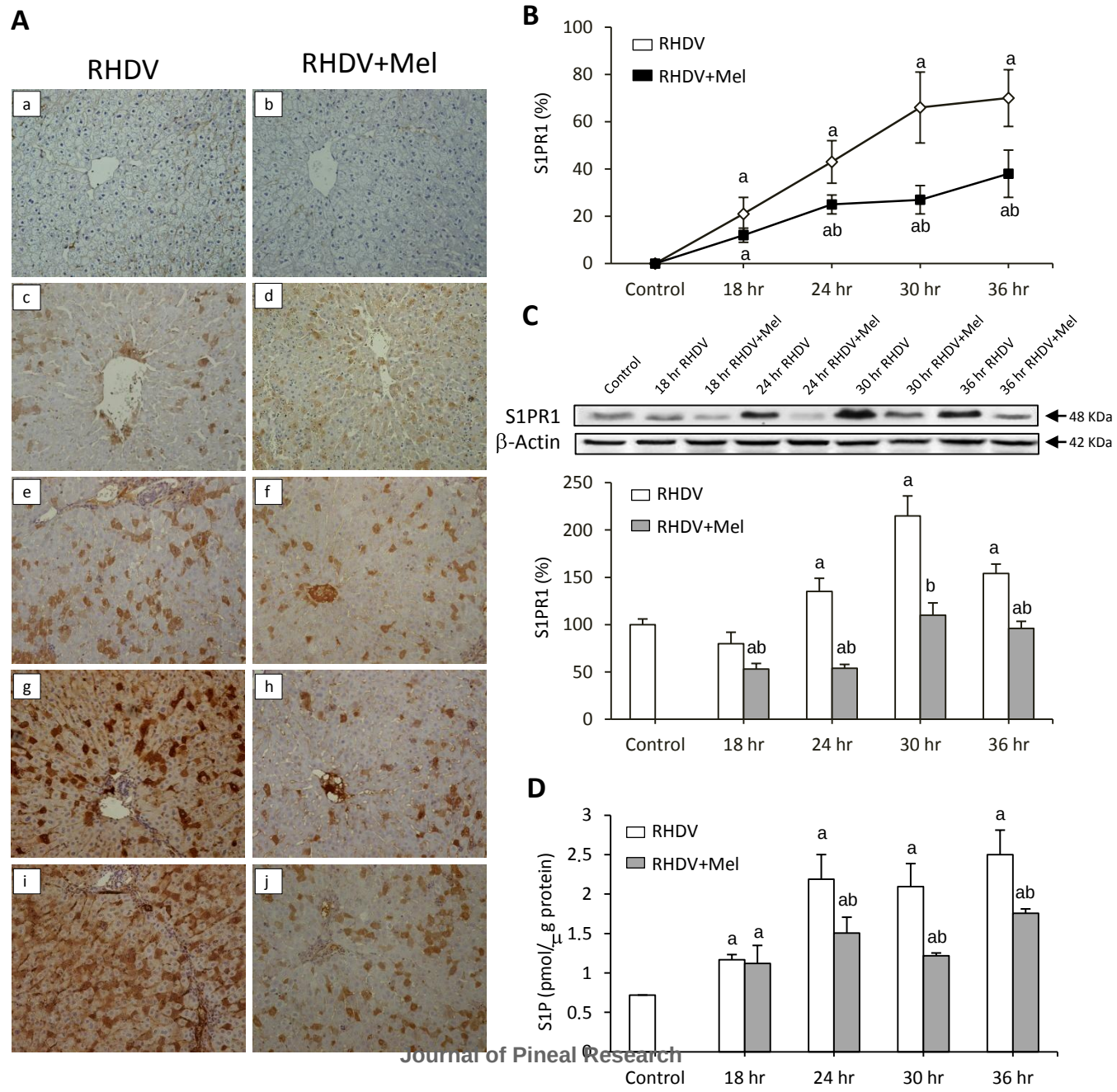

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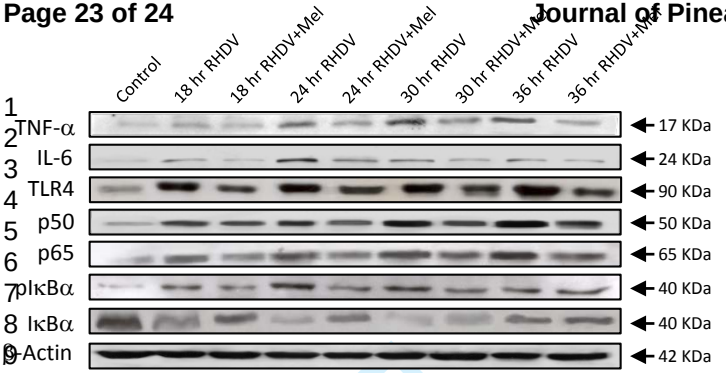

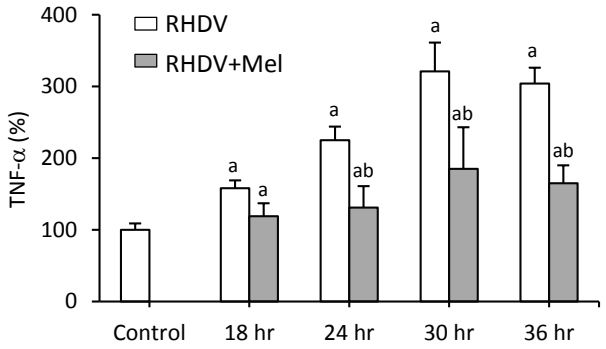

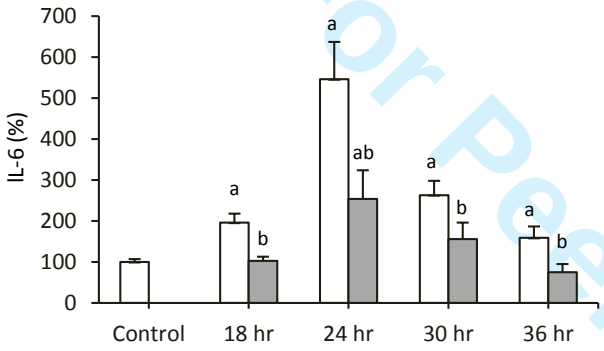

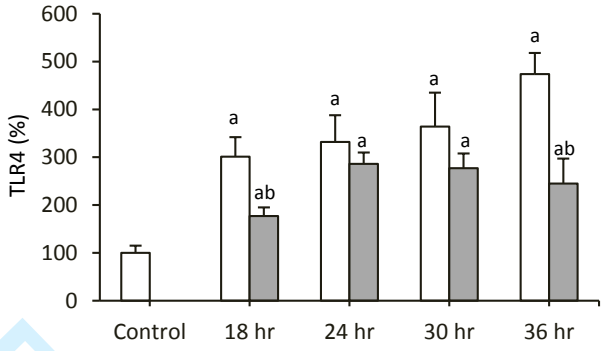

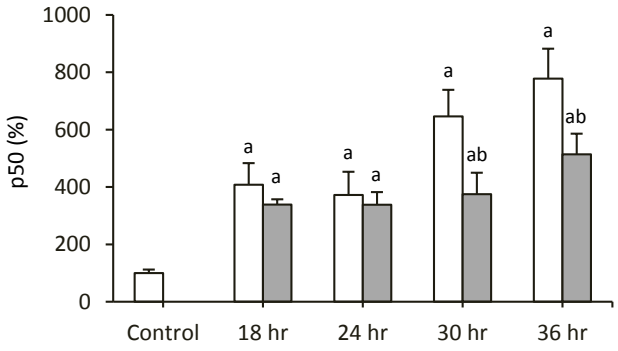

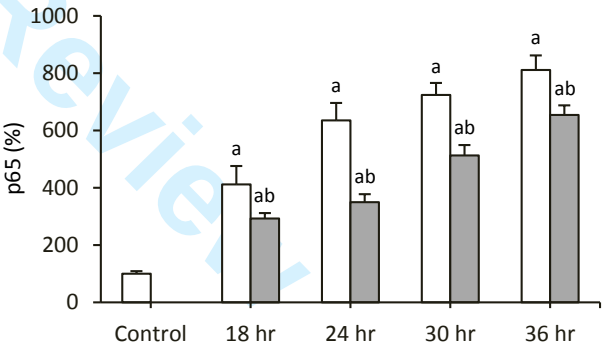

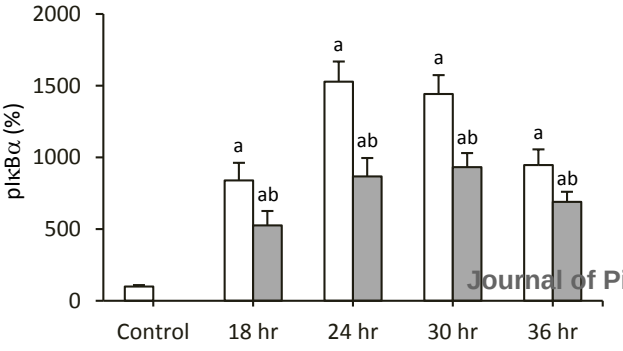

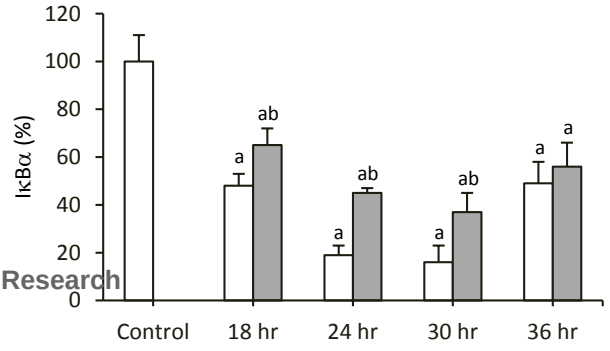

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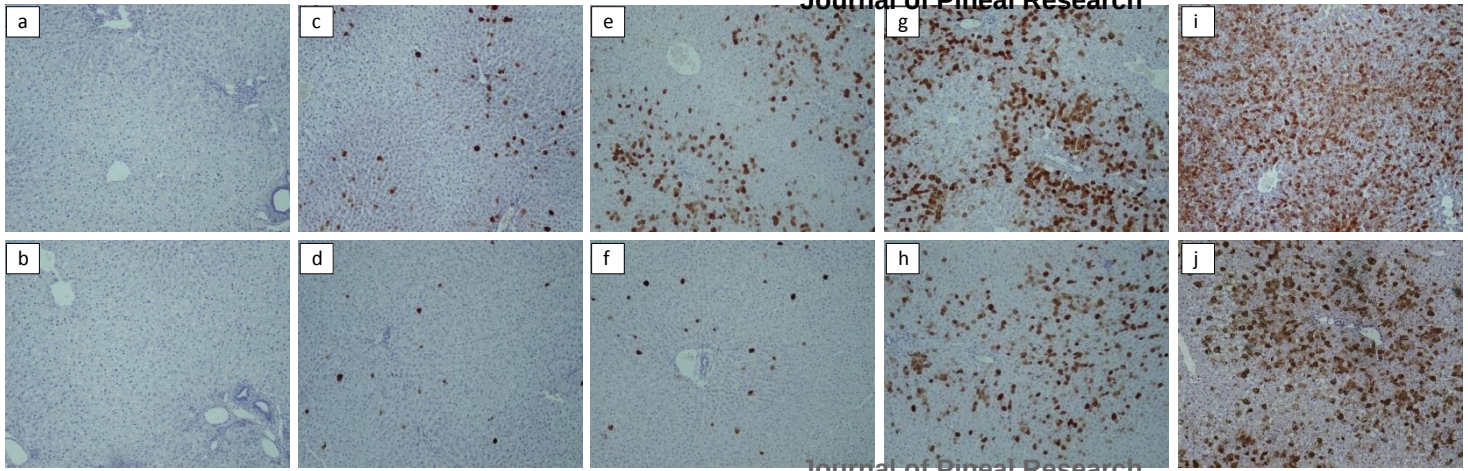

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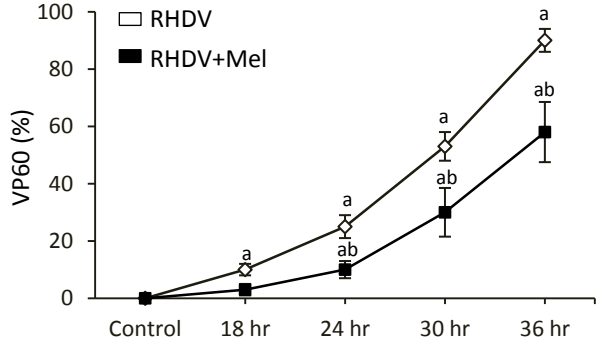

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