Melatonin attenuates vascular calcification by activating mitochondrial fusion and mitophagy via an AMPK/OPA1 signaling pathway

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Research

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Abstract

Background: Mitochondrial fusion/mitophagy play a role in cardiovascular calcification. Melatonin has been shown to protect against cardiovascular disease. This study sought to explore whether melatonin attenuates vascular calcification by regulating mitochondrial fusion/mitophagy via an AMP activated protein kinase/ Optic atrophy 1 (AMPK/OPA1) signaling pathway.

Methods: The effects of melatonin on vascular calcification were investigated in vascular smooth muscle cells (VSMCs). Calcium deposits were visualised by Alizarin red staining. Calcium content and alkaline phosphatase (ALP) activity were used to evaluate osteogenic differentiation. Western blots were used to measure expression of runt-related transcription factor 2 (Runx2), mitofusin 2 (Mfn2), mito-light chain 3 II (LC3II) and cleaved caspase3.

Results: Melatonin markedly reduced calcium deposition and ALP activity. Runx2 and cleaved caspase3 were found to be down-regulated and Mfn2 or mito-LC3II was found to be enhanced in response to melatonin, together with a decrease in mitochondrial superoxide levels. Melatonin also maintained mitochondrial function and promoted mitochondrial fusion/mitophagy via OPA1 pathway. But OPA1 deletion abolished the protective effects of melatonin on VSMC calcification. Melatonin treatment significantly increased the p-AMPK and OPA1 protein expression. Treatment with compound C ablated the benefit observed with melatonin treatment. **Conclusions:** Melatonin protects VSMC against calcification by promoting mitochondrial fusion/mitophagy via AMPK/OPA1 pathway.

Background

Vascular calcification (VC) is prevalent in coronary artery disease, and the extent of VC predicts cardiovascular risk [7]. The causes of calcification in atherosclerosis include dysregulated matrix metabolism, epitaxial mineral deposition, inflammation, oxidative stress, and apoptosis [13]. VC mainly mediated by vascular smooth muscle cells (VSMCs) [15]. It is shown that mitochondrial fusion and mitophagy play a role in the development of vascular calcification [12, 22]. Optic atrophy 1 (OPA1) is a key regulator of mitochondrial fusion, and the AMP activated protein kinase (AMPK)/OPA1 pathway is associated with mitochondrial fusion/mitophagy during cardiovascular disease [2, 20]. The phosphate-AMPK protein levels were decreased in VC, ghrelin improved VC through AMPK activation [18]. Metformin inhibited β -GP-induced impairment of mitochondrial biogenesis via AMPK activation in VC [12]. Melatonin is the main indoleamine produced by the pineal gland; it is known recently to have anti-inflammatory, anti-cancer and antioxidant activities [4]. Melatonin was found to exert a substantial influence on mitochondrial fusion/mitophagy [3, 6]. The aim of the present study was to investigate whether melatonin reduces VSMC calcification by regulating mitochondrial fusion/mitophagy through the AMPK/OPA1 signaling pathway.

Materials And Methods

VSMCs isolation, culture and calcification

VSMCs were isolated from the aortas of Sprague–Dawley rats (4 weeks) using the explant method described in the previous study [1]. For calcification, VSMCs were cultured with dulbecco's modified eagle medium containing 10% fetal bovine serum and 10 mM β -glycerophosphate (β -GP) for 14 days [5]. The medium was changed every 3 days. For drug treatment, melatonin was added before inducing calcification and used for 14 days. To evaluate whether the AMPK pathway was involved in the protective effect of melatonin, VSMCs were treated with compound C (1 μ M, Sigma, USA) [18] before inducing calcification and used for 14 days (n = 6/group).

Measurement of calcium deposition and alkaline phosphatase activity

Alizarin Red S staining was performed to measure the formation of mineralized matrix (Shanghai Gefan Biological Technology Co., Ltd, Shanghai, China). Cells were decalcified with 0.6 mol/L HCl for 24 hours at 37 °C, and the calcium content was determined using a calcium colorimetric assay kit (Nanjing Jiancheng Biological Engineering Institute, Nanjing, China). Alkaline phosphatase (ALP) activity was measured using an ALP kit ((Beyotime Institute of Biotechnology, Shanghai, China).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen) and reverse transcribed with the one-step RT-PCR kit (TransGen Biotech Co., Ltd., China) according to the manufacturer's instructions. Quantification of gene expression was performed using the ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA) with SYBR Green (TransGen Biotech Co., Ltd., China). mRNA levels were determined by qRT-PCR in triplicate for each of the independently prepared RNAs and were normalized to β -actin expression.

ELISA

The monocyte chemotactic protein (MCP-1), tumor necrosis factor α (TNF α) and interleukin-6 (IL-6) levels were analyzed by a sandwich ELISA (BioCheck, Foster City, CA). The glutathione peroxidase (GPx) assay kit, glutathione (GSH) assay kit, and superoxide dismutase (SOD) assay kit had been used to evaluate the GPx, GSH and SOD levels respectively. Caspase-3 activity was monitored using a caspase-3 assay kit (Beyotime Institute of Biotechnology, Shanghai, China). Glucose uptake assay kit is used for the measurement of glucose uptake (#KA4086, Abnova, Taiwan, China). Lactate assay kit has been used to determine the concentrations of lactate (#ab65331, Abcam, Cambridge, MA, USA).

MitoSOX red mitochondrial superoxide indicator, mitochondrial membrane potential, mitochondrial permeability transition pore (mPTP) opening, mitochondrial morphology and mitochondrial respiratory assays

Mitochondrial superoxide production was detected using MitoSOX Red mitochondrial superoxide indicator (YESEN, Shanghai, China). JC-1 Kit was used to evaluate the the mitochondrial membrane potential (Beyotime, Nanjing, China). The mPTP was measured as the rapid dissipation of tetramethylrhodamine ethyl ester fluorescence. Arbitrary mPTP opening time was assessed as the time to the loss of average tetramethylrhodamine ethyl ester fluorescence intensity by half between the initial and residual fluorescence intensity. Cellular ATP levels were determined using an ATP Assay Kit (Beyotime Institute of Biotechnology, China). Mitochondrial morphology was assessed using MitoTracker Red images in conjunction with NIH ImageJ software. Mitochondrial State 3 or State 4 respiration was achieved by adding ADP according to the general method described previously [8].

Western blots

Following experimental treatment, VSMCs were lysed with RIPA lysis buffer containing protease inhibitor (Beyotime, China) for 30 minutes and centrifuged at $14,000 \times g$ for 30 minutes. BCA protein estimation kit was used to measure the protein concentrations (Beyotime Institute of Biotechnology, Shanghai, China). Equal amounts of protein (50ug) were loaded into wells of a 10% sodium dodecyl sulfate-polyacrylamide gel. Proteins were separated by gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, MA, USA). Membranes were blocked with 5% milk in Tris-buffered saline containing 0.05% Tween 20 (TBST) at room temperature for 1 hour followed by overnight incubation at 4°C with the following primary antibodies: anti-runt-related transcription factor 2 (Runx2, 1:1000, Abcam, #ab76956), anti-OPA1 (1:1000, Abcam, #ab42364), anti-cleaved-caspase3 (1:1000, Abcam, #ab13847), anti-mitochondrial fission protein 1 (Fis1, 1:1000, Abcam, #ab71498), anti-mitofusin 2 (Mfn2, 1:1000, Abcam, #ab56889), anti-light chain 3 II/I (LC3 II/I, 1:1000, Cell Signaling Technology, #3868/#4599), anti-Beclin1 (1:1000, Abcam, #ab62557), anti-AMPK (1:1000, Abcam, #ab131512), anti-p-AMPK (1:1000, Abcam, #ab23875) and anti- β -actin (1:1000, Abcam, #ab8227). After overnight incubation, membranes were washed with TBST and further incubated with the appropriate secondary antibody at room temperature for 60 minutes. Membranes were developed with an enhanced chemiluminescence reagent.

Immunofluorescence and TUNEL method

For immunofluorescence, cells were fixed with 4% paraformaldehyde for 30 minutes, followed by permeation using 0.5% Triton X-100 for 10 minutes. Then cells were blocked with 5% BSA for 1 hour, and incubated with primary antibody against Runx2 (1:200, Cell Signaling Technology), OPA1 (1:200, Cell Signaling Technology), cleaved caspase3 (1:200, Cell Signaling Technology), LC3II (1:200, Cell Signaling

Technology), p-AMPK (1:200, Cell Signaling Technology) overnight at 4 °C. The next day, cells were incubated with secondary antibody (1:200, Cell Signaling Technology) for 1 hour at 37°C. Images were taken using a fluorescence microscope (OLYMPUS DX51; Olympus, Tokyo, Japan). Apoptosis was detected using a TUNEL assay (Roche, Germany) according to the manufacturer's instructions. The apoptosis index was calculated by counting the percentage of TUNEL-positive cells against total nucleated cells stained by DAPI.

Transfection

The scrambled siRNA against OPA1 or autophagy protein 5 (ATG5) was purchased from Santa Cruz Biotechnology. For the RNAi knockdown, cells were seeded in plates containing medium without antibiotics for 24 hours before transfection. The siRNAs were transfected into the cells using Lipofectamine 2000 (Invitrogen, Burlington, ON, Canada) in serum-free Opti-MEM (Invitrogen), according to the manufacturer's instructions. Recombinant OPA1 adenovirus (Ad-OPA1) was purchased from Cyagen Company (Sunnyvale, CA, USA).

Statistical analysis

Data are described as the mean $\pm$ standard deviation (SD), and were analyzed by one-way analysis of variance followed by Tukey's test. The limit of statistical significance between treated and control groups was $P < 0.05$.

Results

Melatonin attenuated β -GP-induced VSMC calcification through OPA1 pathway

As shown in Fig. 1A and 1B, 5 μ M of melatonin significantly reduced calcium content and decreased ALP activity in calcifying VSMCs. Therefore, most experiments were performed at the concentration of 5 μ M of melatonin. The Alizarin Red S staining indicated that β -GP promoted the calcification of VSMCs and melatonin significantly inhibited β -GP-induced calcification ($P < 0.05$) (Fig. 1C-D). ALP activity was significantly increased in response to β -GP, and melatonin significantly reduced ALP activity (Fig. 1E). However, OPA1 deletion reduced the protective effects of melatonin on VSMC calcification.

Immunofluorescence assay was used to evaluate the Runx2 and OPA1 expression in VSMCs. The Runx2 protein was increased in the β -GP group while decreased in the β -GP + melatonin group. We also found that β -GP decreased OPA1 expression, while melatonin treatment significantly upregulated OPA1 expression. But OPA1 knockout reversed this phenomenon (Fig. 1F-H). The western blot results showed

that the Runx2 and OPA1 expression were similar to those in Fig. 1F among the control, β -GP and β -GP + melatonin groups (Fig. 1I-J). Overall, these data show that melatonin protects VSMC against calcification by promoting OPA1 expression.

Melatonin reduced VSMC inflammatory response and oxidative stress through OPA1 pathway

Quantitative real-time polymerase chain reaction analysis showed that the mRNA expressions of inflammatory factors were increased in the β -GP group and were decreased in the β -GP and melatonin co-treatment group. However, deletion of OPA1 significantly increased these levels despite treatment with melatonin (Fig. 2A-C). The ELISA results further confirmed this finding. Melatonin significantly reduced the inflammatory factors levels in β -GP-induced calcified VSMCs, and the loss of OPA1 nullified the effect of melatonin on VSMC inflammation (Fig. 2D-2F).

To investigate the relationship between the melatonin-mediated vascular protection and oxidative stress, we measured the levels of mitochondrial superoxide in VSMCs. β -GP increased the levels of mitochondrial superoxide, while melatonin reduced the levels of mitochondrial superoxide through OPA1 regulation (Fig. 2G-H). Moreover, melatonin promoted the levels of antioxidant compounds (GPx, GSH, and SOD), while OPA1 ablation might inhibit these effects (Fig. 2I-K). Taken together, these results suggest that melatonin could reduce inflammation and oxidative stress via OPA1 regulation in calcifying VSMCs.

Melatonin protected VSMCs against apoptosis through OPA1 pathway. Immunofluorescence staining results showed that the expression of cleaved caspase-3 was increased in the β -GP-treated cells, whereas melatonin significantly reduced the expression of cleaved caspase-3. But the loss of OPA1 significantly promoted cleaved caspase-3 expression in the β -GP-treated VSMCs (Fig. 3A-C).

The TUNEL assay was used to evaluate the incidence of apoptosis in VSMCs. Compared with the β -GP group, melatonin treatment significantly inhibited apoptosis in VSMCs (Fig. 3D-E). In addition, cleaved caspase-3 protein expression was found to be decreased in the melatonin group (Fig. 3F). But OPA1 deficiency abolished the protective effects of melatonin on VSMC apoptosis. Thus, these data indicate that melatonin protects against VSMC apoptosis via OPA1 pathway.

Melatonin maintained mitochondrial function through OPA1 pathway

The $\Delta\Psi_m$ dissipation plays a key role in mitochondrial dysfunction. In this study, the $\Delta\Psi_m$ was dissipated by β -GP treatment, and melatonin reversed β -GP-induced $\Delta\Psi_m$ dissipation via OPA1 (Fig. 4A-B). The opening of mPTP was promoted by treatment with β -GP; however, this β -GP-induced promotion can be attenuated by simultaneous supplementation of melatonin (Fig. 4C). Melatonin also increased cellular ATP levels after β -GP treatment via OPA1 (Fig. 4D).

Mitochondrial respiratory function was also evaluated in this study. β -GP decreased the mRNA expression of mitochondrial respiratory complex (CIII-core2, CII-30 and CIV-II) and they were increased when VSMCs were treated with melatonin; however, when OPA1 knockout was present, the expression levels of mitochondrial respiratory complex were returned to the level of the β -GP group (Fig. 4E-G). Melatonin

treatment significantly promoted the glucose uptake and lactic acid production, but deletion of OPA1 attenuated this promotion in β -GP-treated VSMCs (Fig. 4H-I). In addition, the State 3/4 mitochondrial respiratory rates were found to be increased by melatonin via OPA1 regulation (Fig. 4J-K).

Melatonin maintained structural integrity through OPA1 pathway

As shown in the polymerase chain reaction results, β -GP promoted the Drp1 and Fis1 mRNA expression, and melatonin significantly inhibited β -GP-induced promotion. The levels of Mfn1 and Mfn2 mRNA expression were significantly decreased in response to β -GP, and melatonin significantly increased Mfn1 and Mfn2 mRNA expression. However, these effects were negated in the OPA1-deleted VSMCs. (Fig. 5A-D). This phenomenon was also confirmed by western blot analysis of Fis1 and Mfn2 (Fig. 5E-F).

Mitochondria fragmentation was significantly increased by β -GP, but mitochondria fragmentation was reduced by treatment with melatonin via a OPA1-dependent manner ($P < 0.05$) (Fig. 5G-H). These data confirmed that melatonin promotes mitochondrial fusion via OPA1 pathway.

Melatonin promoted protective mitophagy through OPA1 pathway

The western blot results revealed that β -GP slightly increased the mito-LC3II and beclin1 protein expression, and melatonin significantly promoted mito-LC3II and beclin1 protein expression. However, OPA1 deletion abrogated these effects (Fig. 6A-C). The effects of melatonin on Beclin1 mRNA expression, were consistent with the western blot data (Fig. 6D-E). Mitophagy (interaction between mitochondria and LC3II) was slightly upregulated by β -GP, and melatonin treatment significantly increased mitophagy via OPA1 pathway ($P < 0.05$) (Fig. 6F-G).

To investigate the effects of OPA1 or mitophagy on VSMC calcification, OPA1 overexpression (Ad-OPA1) or siRNA against ATG5 was used in this study. Melatonin or OPA1 overexpression significantly reduced ALP activity. However, the effects of melatonin were mitigated by OPA1 deletion or ATG5 knockout (Fig. 6H). These data indicate that melatonin protects VSMC against calcification via OPA1-related mitophagy.

Melatonin attenuated β -GP-induced VSMC calcification through AMPK/OPA1 pathway

Immunofluorescence staining results showed that melatonin treatment increases p-AMPK and OPA1 expression in calcifying VSMCs. We also found that compound C may inhibit the effect of melatonin on OPA1, as indicated by the decreased OPA1 and p-AMPK signal in the compound C + melatonin group compared with melatonin group (Fig. 7A-C). This result was also supported by western blot analysis (Fig. 7D-E). Melatonin significantly reduced calcium deposition, Runx2 mRNA expression, interleukin-6 level, and caspase-3 activity in β -GP-induced calcified VSMCs (Fig. 7F-J). But compound C reduced the protective effects of melatonin on VSMC calcification. Overall, these data indicate that melatonin attenuates β -GP-induced VSMC calcification via the AMPK/OPA1 signaling pathway.

Discussion

In the present study, we investigated the effects of melatonin on VSMC calcification and the molecular mechanism by which these effects were produced. Our results suggest that the decrease of VSMC calcification by melatonin is mediated, at least in part, by AMPK/OPA1 signaling.

The effect of melatonin on calcification has been investigated more recently [14, 10, 21]. Son et al. found that melatonin could promote osteoblastic differentiation and mineralization of preosteoblastic MC3T3-E1 cells under hypoxic conditions [14]. But Kumar and Naidu showed that melatonin significantly antagonized cyclosporine-induced renal impairment. Microcalcification in corticomedullary junction seen with cyclosporine was prevented by melatonin [10]. Zhang et al. demonstrated that melatonin treatment suppresses oxidative stress-induced calcification and apoptosis in endplate chondrocytes [21].

Mitochondrial fusion and mitophagy have been demonstrated to be involved in vascular calcification. A study by Kim et al. showed that the disruption of mitochondrial structural integrity was found in calcifying vascular smooth muscle cells. α -Lipoic acid inhibited the VSMC apoptosis and calcification by promoting mitochondrial fusion [9]. Ma et al. found that the mitochondrial mass was decreased after β -GP exposure, whereas metformin treatment increased the mitochondrial fusion compared to the β -GP group. Metformin attenuated β -GP-induced vascular calcification through enhancing mitophagy. Inhibition of autophagy by small interfering RNA targeting Atg5 aggravated β -GP-induced calcification [12].

OPA1 has been shown to inhibit reperfusion injury in the heart and brain by promoting mitochondrial fusion. Wei et al. showed that melatonin increased OPA1 expression, enhanced mitochondrial fusion and reduced neuron death during brain reperfusion injury. This protective effects of melatonin were negated by OPA1 knockout [17]. The findings of Ma, et al. suggest that melatonin attenuated cardiac ischemia reperfusion injury by upregulating OPA1-related mitochondrial fusion. OPA1 depletion abolished the protective effects of melatonin on cardiac reperfusion injury [11]. Zhang et al. found that melatonin attenuated cardiac reperfusion injury by activating OPA1-related mitochondrial fusion and mitophagy. ATG5 deletion abolished this protective effects [20]. Our experiments demonstrated that melatonin increased OPA1-related mitochondrial fusion/mitophagy and reduced VSMC calcification. Furthermore, OPA1 knockout reduced the effects of melatonin on mitochondrial fusion/mitophagy and inhibited the effects of melatonin on VSMC calcification. Our data suggest that melatonin inhibits VSMC calcification through OPA1-related mitochondrial fusion/mitophagy.

Mitochondrial fusion/mitophagy is related with AMPK, which is a key energy sensor and regulates cellular metabolism to maintain energy homeostasis [16, 19]. Cui et al. showed that melatonin treatment reduced the apoptosis of human umbilical vein endothelial cells via promoting mitochondrial fusion by activating the AMPK pathway [6]. Zhang et al. showed that melatonin increased OPA1 expression via the AMPK pathway, compound C reduced OPA1 expression and negated the effects of melatonin [20]. Our experiments demonstrated that melatonin activated AMPK protein expression, promoted OPA1-required mitochondrial fusion/mitophagy and reduced VSMC calcification. Furthermore, we used the AMPK inhibitor to research the effects of melatonin on VSMC calcification. We found that compound C reduced

the effects of melatonin on AMPK/OPA1 and increased VSMC calcification. Taken together, melatonin activated the expression of AMPK, which in turn enhanced OPA1 and subsequently promoted mitochondrial fusion/mitophagy. Activation of mitochondrial fusion/mitophagy reduced apoptosis, oxidative stress, inflammation and calcium deposition. These effects subsequently inhibited VSMC calcification (Fig. 8).

There are a few limitations to our study. First, the findings are only based on in vitro experiments. Second, we only observed the caspase-3 activation in the study, other apoptosis signals can be evaluated in the future study. Third, AMPK knocking down system may be better to validate the role of AMPK in melatonin-reversed VSMC calcification induced by β -GP.

Conclusions

Our study demonstrated that melatonin played an important and protective role in VSMCs by inhibiting calcification via the AMPK/OPA1 system.

Abbreviations

AMPK, AMP activated protein kinase; OPA1, Optic atrophy 1; VSMC, vascular smooth muscle cell; ALP, alkaline phosphatase; Runx2, related transcription factor 2; Mfn2, mitofusin 2; VC, vascular calcification; β -GP, β -glycerophosphate; MCP-1, monocyte chemotactic protein; TNF α , tumor necrosis factor α ; IL-6, interleukin-6; GPx, glutathione peroxidase; GSH, L-glutathione; SOD, superoxide dismutase; LC3, light chain 3; ATG5, autophagy protein 5; Drp1, dynamin-related protein1; Fis1, fission protein 1.

Declarations

Competing interests

The authors declared no potential conflicts of interest with respect to the research, authorship, or publication of this article.

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Authors' contributions

All authors have substantially contributed to the manuscript in terms of conception and design, analysis and interpretation of data, drafting the article, revising it critically for important intellectual content. All authors read and approved the final manuscript.

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Figures

Figure 1

Melatonin reduced β-GP-induced calcium deposition via OPA1 in VSMCs (n = 6/group). VSMCs were cultured with dulbecco's modified eagle medium containing 10% fetal bovine serum and 10 mM β-GP for 14 days. (A-B) Result of different concentration of melatonin on calcium content and Alkaline

phosphatase (ALP) activity. (C) Result of melatonin (5 μ M) on Alizarin red staining. (D) Result of calcium concentration. (E) Result of ALP activity. (F-H) Result of Immunofluorescence assay. (Red signal represents Runx2, green signal represents OPA1). (I-J) Results of Runx2 and OPA1 protein expression. * $P < 0.05$ vs Con, # $P < 0.05$ vs β -GP, & $P < 0.05$ vs β -GP+Mel.

Figure 2

Effects of melatonin on inflammatory response and oxidative stress in β -GP-treated VSMCs (n = 6/group). (A) Result of matrix metalloprotein 9 (MMP9) mRNA expression. (B) Result of macrophage inhibitory protein-1 α (MIP1 α) mRNA expression. (C) Result of interleukin-8 (IL-8) mRNA expression. (D) Result of monocyte chemotactic protein (MCP-1) level. (E) Result of tumor necrosis factor α (TNF α) level. (F) Result of interleukin-6 (IL-6) level. (G-H) MitoSOX for mitochondrial superoxide formation. (I-K) Results

of glutathione peroxidase (GPx), L-glutathione (GSH) and superoxide dismutase (SOD) levels. *P<0.05 vs Con, #P<0.05 vs β -GP group, &P<0.05 vs β -GP+Mel.

Figure 3

Effects of melatonin on the apoptosis in β -GP-treated VSMCs (n = 6/group). (A-C) Confocal microscopy of immunofluorescence staining of cleaved caspase-3 (red) and OPA1 (green). (D-E) The apoptosis of VSMC was determined by TUNEL staining. (F) Results of cleaved caspase-3 expression. *P<0.05 vs Con, # P<0.05 vs β -GP, &P<0.05 vs β -GP+Mel.

Figure 4

Effects of melatonin on mitochondrial membrane potential, mitochondrial permeability transition pore (mPTP) opening time, ATP production and mitochondrial respiratory function in β -GP-treated VSMCs ($n = 6/\text{group}$). (A-B) The change of membrane potential ($\Delta\Psi_m$) by JC-1 staining. (C) Result of arbitrary mPTP opening time. (D) Result of cellular ATP levels. (E-G) Results of mitochondrial respiratory complex (CIII-core2, CII-30, CIV-II) mRNA expression. (H-I) Results of glucose uptake and lactic acid production. (J-K)

Results of mitochondrial state 3/4 respiratory rate. *P<0.05 vs Con, #P<0.05 vs β -GP group, &P<0.05 vs β -GP+Mel.

Figure 5

Effects of melatonin on mitochondrial fission in β -GP-treated VSMCs (n = 6/group). (A) Result of dynamin-related protein1 (Drp1) mRNA expression. (B) Result of mitochondrial fission protein 1 (Fis1) mRNA expression. (C) Result of mitofusin 2 (Mfn2) mRNA expression. (D) Result of mitofusin 1 (Mfn1) mRNA expression. (E-F) Results of Fis1 and Mfn2 protein expression. (G-H) Mitochondrial morphology was observed with the Mitotracker-red. The yellow arrows indicate the fragmented mitochondria. *P<0.05 vs Con, #P<0.05 vs β -GP group, &P<0.05 vs β -GP+Mel.

Figure 6

Effects of melatonin on mitophagy in β -GP-treated VSMCs ($n = 6/\text{group}$). (A-C) Results of LC3II/I, mito-LC3II and Beclin1 protein expression. (D) Result of Beclin1 mRNA expression. (E) Result of P62 mRNA expression. (F-G) The co-location of LC3II and mitochondria in VSMCs. (H) Result of OPA1 overexpression (Ad-OPA1) or autophagy protein 5 knockout (si-ATG5) on ALP activity. * $P < 0.05$ vs Con, # $P < 0.05$ vs β -GP group, & $P < 0.05$ vs β -GP+Mel.

Figure 7

Effects of melatonin and AMPK pathway inhibitor (Compound C, 1 μ M) on β -GP-induced calcification in VSMCs ($n = 6/\text{group}$). (A-C) Results of Immunofluorescence assay. (Red signal represents p-AMPK, green signal represents OPA1). (D-E) Results of p-AMPK and OPA1 protein expression. (F) Result of calcium concentration. (G) Result of Runx2 mRNA expression. (H) Result of interleukin-6 (IL-6) level. (I) Results of

SOD level. (J) Results of caspase3 activity. *P<0.05 vs Con, #P<0.05 vs β-GP group, &P<0.05 vs β-GP+Mel.

Figure 8

Schematic representation showing that melatonin regulates VSMC calcification through an AMPK/OPA1 signaling pathway. Melatonin activated the expression of AMPK, which in turn enhanced OPA1 and subsequently promoted mitochondrial fusion/mitophagy. Activation of mitochondrial fusion/mitophagy

reduced apoptosis, oxidative stress, inflammation and calcium deposition. These effects subsequently inhibited VSMC calcification.