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REVIEW

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Oxidative/nitrosative stress, autophagy and apoptosis as therapeutic targets of melatonin in idiopathic pulmonary fibrosis

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ABSTRACT

Introduction: Idiopathic pulmonary fibrosis (IPF) is a fatal interstitial lung disease associated with disruption of alveolar epithelial cell layer and expansion of fibroblasts/myofibroblasts. Excessive levels of oxidative/nitrosative stress, induction of apoptosis, and insufficient autophagy may be involved in IPF pathogenesis; hence, the targeting of these pathways may ameliorate IPF.

Areas covered: We describe the ameliorative effect of melatonin on IPF. We summarize the research on IPF pathogenesis with a focus on oxidative/nitrosative stress, autophagy and apoptosis pathways and discuss the potential effects of melatonin on these pathways.

Expert opinion: Oxidative/nitrosative stress, apoptosis and autophagy could be interesting targets for therapeutic intervention in IPF. Melatonin, as a potent antioxidant, induces the expression of antioxidant enzymes, scavenges free radicals and modulates apoptosis and autophagy pathways. The effect of melatonin in the induction of autophagy could be an important mechanism against fibrotic process in IPF lungs. Further clinical studies are necessary to determine if melatonin could be a candidate for treating IPF.

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Pulmonary fibrosis; melatonin; oxidative stress; autophagy; apoptosis

1. Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic lung disease characterized by continuous alveolar epithelial cell injury and expansion of fibroblasts/myofibroblasts, resulting in excessive production of fibrin and abnormal wound repair [1]. Repetitive micro-injuries and subsequent dysregulated wound repair of alveolar epithelial cells are considered as a key player in the pathobiology of IPF [2]. Fibrotic process has been shown to be associated with apoptosis of alveolar epithelial cells; apoptosis is implicated as an important mediator of lung fibrosis [3]. The alveolar epithelium is organized by two morphologically distinct cell types including alveolar epithelial type I cells (AECs I) and type II cells (AECs II). AECs I cover approximately 90–95% of the alveolar surface area and facilitate the fast exchange of gases, whereas AECs II are responsible for producing and secreting surfactant. AECs I are more susceptible to injury when compared with AECs II. In the initial stages of injury, AECs I apoptosis are associated with AECs II hyperplastic proliferation and migration to cover the exposed alveolar basement membranes. In normal process of tissue repair, these events are accomplished through regulated apoptosis of hyperplastic AECs II and trans (differentiation) of remaining cells into AECs I [2]. However, recurrent injury to the epithelial alveolar cells leads to increased susceptibility of AECs II to apoptosis. The loss of AECs II can lead to the accumulation of resident intrapulmonary fibroblasts in damaged areas, proceeding to secrete extracellular matrix (ECM) components [4]. Excessive production of oxidants has been proposed to be a mechanism for epithelial cell apoptosis. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) cause direct damage to the intracellular components. Furthermore, free radicals function as important intracellular signaling molecules and activate a variety of pro-fibrogenic factors such as transforming growth factor (TGF)-β and molecules involved in the apoptotic signaling pathways [5]. Macro-autophagy (hereinafter referred to as autophagy) has been described as a cellular defense against cellular stressors including oxidative stress, starvation, and endoplasmic reticulum (ER) stress; autophagy has been found to reduce in human IPF lungs [6–10]. Herein, we survey the signaling pathways of oxidative stress, apoptosis, and autophagy in the pathophysiology of IPF and review the role of melatonin in ameliorating IPF through the regulation of these signaling pathways.

2. Oxidative/nitrosative stress in IPF and the influence of melatonin

2.1. The role of oxidative/nitrosative stress in the progression of IPF

Oxidative/nitrosative stress results from an imbalance between the cellular production of ROS/RNS and antioxidant

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Article highlights

- Idiopathic pulmonary fibrosis (IPF) is associated with apoptosis of alveolar epithelial cells and expansion of fibroblasts/myofibroblasts in lung tissue.
- Excessive level of oxidative/nitrosative stress and insufficient autophagy are proposed to be a mechanism for epithelial cell apoptosis.
- Melatonin is a potent antioxidant stimulating the expression of GSH and antioxidant enzymes and scavenging free radicals.
- Melatonin has protective effects on oxidant/antioxidant status of the lungs in various pathologic conditions.
- The regulatory effect of melatonin on apoptosis and autophagy processes has been reported in various *in vivo* and *in vitro* experiments.
- The antioxidant properties of melatonin and its modulatory effect on apoptosis and autophagy pathways suggest that melatonin could be a promising agent for IPF treatment.

This box summarizes key points contained in the article.

defenses. Although ROS/RNS may regulate intracellular signaling pathways and post-translational processing of proteins, increased levels of ROS/RNS cause severe damage to cellular and intracellular structures, including cell membranes, proteins, DNA, and mitochondria [11–14]. Accumulating evidence from several studies suggest that increased oxidative/nitrosative stress may play a major role in the progression of IPF [15]. In lungs, ROS/RNS are endogenously produced by alveolar inflammatory cells including lymphocytes, macrophages, and neutrophils. In addition to inflammatory cells, fibroblasts and myofibroblasts from IPF patients produce high levels of ROS/RNS in response to cytokines and growth factors [16,17]. Exogenous oxidizing agents such as toxins, air pollutant, hyperoxia, radiation, cigarette smoke, and drugs also induce inflammatory cells to produce free radicals. Exogenous or endogenous generated of ROS/RNS may cause direct damages to the alveolar epithelium, predisposing individuals to lung fibrosis [14]. The main sources of ROS/RNS production may include nicotinamide adenine dinucleotide phosphate oxidases (NADPH oxidases, NOXs), myeloperoxidase, xanthine oxidase, nitric oxide synthase (NOS), and the mitochondrial electron transport chain [11,14].

ROS generated by the NOX family of enzymes, all of the NOX isoforms except NOX3, have now been found to play a central role in the pathogenesis of pulmonary fibrosis [13,18]. The isoform NOX4 is strongly expressed in fibroblastic foci and hyperplastic type II cells from lungs of humans with IPF. NADPH oxidase 4-derived ROS has been shown to be involved in TGF-β1-induced myofibroblast differentiation, AEC death and ECM production during the development of pulmonary fibrosis; TGF-β1 is the most studied pro-fibrotic cytokine related with IPF development [13,18–20]. Recent findings suggest that NOX4-generated ROS triggers Nalp3 inflammasome activation in inflammatory cells. This inflammasome promotes the maturation of inflammatory cytokines such as IL-1β in silica and asbestos-induced pulmonary injury [21]. In addition to the induction of NOXs expression by TGF-β, NOX-dependent redox pathways could activate TGF-β/Sma- and Mad-related (Smad) signaling though a feed-forward mechanism. Recent findings indicate that antioxidant agents and NOX4 gene silencing suppress TGF-β-induced Smad2/3 phosphorylation [22].

An excess of various RNS, such as peroxynitrite ($ONOO^-$) can be formed through superoxide reaction with nitric oxide (NO·, the main nitrogen species produced by NOS). Nitric oxide is synthesized by three isoforms of NOS, including constitutive isoforms, endothelial (eNOS) and neuronal (nNOS), and an inducible isoform (iNOS). Inducible NOS produces the highest levels of NO· in comparison with constitutive isoforms [23]. The elevated level of iNOS and nitrotyrosine modify proteins in the lung of IPF patient indicating that iNOS plays a critical role in IPF pathogenesis by induction of nitrosative stress [24]. Although nitric oxide has beneficial effects such as relaxation of smooth muscle cells in the cardiovascular and pulmonary systems, large amounts of NO· and NO-derived species covalently interact with biological molecules and modify their function [25]. Furthermore, findings from animal studies have been shown that NO· signaling pathway activates TGF-β/Smad signaling cascade, thereby increasing the expression of collagen type I and heat-shock protein (HSP) 47 in pulmonary fibroblasts. On the basis of these observations, NO· signaling pathway has a positive effect on the progression of pulmonary fibrosis via inducing pro-fibrotic cytokine expression [26,27].

The lung however has a wide variety of antioxidant resources to protect against cellular damage induced by ROS and RNS. The endogenous antioxidant defense system includes small-molecular-weight antioxidants (vitamin E, melatonin, glutathione (GSH), uric acid, etc.), classic antioxidant enzymes (superoxide dismutases (SODs), catalase, and glutathione peroxidase (GPx)), phase II detoxifying enzymes (glutathione-*S*-transferase (GST) isozymes, NADP(H), quinone oxidoreductase (NQO1), etc.), stress-response proteins (heme oxygenase (HO)-1, ferritin, etc.), mucins, and metal binding proteins (lactoferrin, transferrin, metallothionein, etc.) [28]. Furthermore, nuclear factor-erythroid 2 p45 subunit-related factor 2 (Nrf2) is essential for activating antioxidant responses in the lung through inducing the expression most of antioxidant and detoxifying enzymes. These enzymes are expressed and localized in bronchial and alveolar epithelial cells and macrophages of human lungs in a cell specific manner [14].

Catalase is mainly found in AECs and inflammatory cells of the lung and exerts antioxidant activity via reducing hydrogen peroxide (H_2O_2), thus inhibiting H_2O_2-mediated fibroblast activation in IPF lungs [17]. Intratracheal administration of catalase in asbestos-treated wild-type mice protects them against the development of pulmonary fibrosis by inhibiting NADPH oxidase-induced generation of H_2O_2. Moreover, extended intravenous infusion of polyethylene glycol (PEG)-conjugated catalase reduces collagen deposition and fibrosis in the lung, in a rat model of asbestosis [29,30].

All three isoforms of superoxide dismutase, including extracellular-SOD (EC-SOD), manganese-SOD (Mn-SOD) and copper/zinc-SOD (Cu/Zn-SOD) are expressed in interstitium, vascular endothelium, macrophages and bronchial and alveolar epithelium of the lung; these protect the lung from oxidative stress by decomposing superoxide anion to H_2O_2

[11,31,32]. EC-SOD exists in high levels in the lung and has a critical role in bleomycin-, asbestos-, and radiation-induced pulmonary fibrosis by preventing oxidative stress [33–35]. The EC-SOD$^{-/-}$ mice exhibit more pulmonary fibrosis following exposure to bleomycin and asbestos [36]. Furthermore, the level of EC-SOD decreased in an animal model of asbestos-induced pulmonary fibrosis and accumulated in proteolysed form, suggesting depletion of EC-SOD from the fibrotic lung. Thus, EC-SOD appears to be important in inhibiting fibrotic processes in the lung [37]. There is evidence indicating that EC-SOD exerts anti-fibrotic effects in lungs via preventing the oxidative degradation of ECM, leading to the inhibition of the destructive effects of ECM degradation products on lung epithelial and mesenchymal cells [38]. Although the expression level of EC-SOD around alveolar macrophages and airway epithelial cells from fibrotic lung is very similar to normal lung, EC-SOD is not detectable by immunohistochemistry in fibrotic areas and fibroblastic foci of lungs from IPF patients. Given that TGF-β inhibits the expression of EC-SOD in AECs, smooth muscle cells and fibroblasts, could be a reason for the loss of EC-SOD in fibrotic areas of human IPF lung tissues [39]. In contrast to EC-SOD, Mn-SOD and Cu/Zn-SOD are lower in the lung compared with other vital organs. The roles of these latter two SOD isoforms are less well understood in the pathogenesis of pulmonary fibrosis. Cu/Zn-SOD and Mn-SOD are mainly localized in AEC II, alveolar macrophages and bronchial epithelial cells in lungs [11]. Administration of human recombinant Mn-SOD inhibits the bleomycin-induced lung fibrosis in rats, while Cu/Zn-SOD administration in these condition did not show a significant effect [40]; this effect is related to more anti-inflammatory effect of Mn-SOD compared with Cu/Zn-SOD. Furthermore, in asbestosis patients, the expression and activity of Cu/Zn-SOD in alveolar macrophages is higher than in normal subjects, resulting in the increased generation of H_2O_2. In contrast, alveolar macrophages of asbestosis patients have similar Mn-SOD expression and activity as compared with normal subjects [41]. Moreover, knockout mice lacking Cu/Zn-SOD have less oxidative stress following exposure to asbestos [42]. These findings suggest that Cu/Zn-SOD accelerates development of pulmonary fibrosis via increasing H_2O_2 production in asbestosis patients. Asbestos has been reported to induce translocation of Cu/Zn-SOD into the mitochondrial intermembrane space in alveolar monocytic inflammatory cells [43]. The function of Cu/Zn-SOD in the mitochondrial intermembrane space is similar to its function in the cytosol. However, in the oxidative stress condition, the scavenging systems in the mitochondrial intermembrane space may be insufficient to eliminate produced H_2O_2 contributing to mitochondrial damage [44].

Reduced glutathione (GSH) is a major low-molecular-weight antioxidant, synthesized by bronchial epithelial cells and alveolar macrophages. This antioxidant is found in high levels in epithelial lining fluid of lungs [45]. Glutathione reductase (GSH-Rd), γ-glutamyl cysteine synthase and glutathione synthase are responsible for maintaining GSH at optimal cellular levels. The level of GSH substantially decreases in epithelial lining fluid in IPF lungs, whereas oxidized glutathione (GSSG) rises. Furthermore, the immunoreactivity of γ-glutamyl cysteine synthase, a rate limiting enzyme participating in GSH synthesis, is increased in areas of regenerating bronchiolar epithelium of human lung fibrosis, but is reduced in fibrotic areas and fibroblastic foci [46,47]. Administration of *N*-acetyl-cysteine (NAC, a GSH precursor) augments lung GSH levels and subsequently reduces oxidative stress, primary inflammatory events and development of lung fibrosis induced by bleomycin [48].

The transcription factor, Nrf2, is the principal ARE-binding protein, inducing the expression of antioxidant enzymes. Under normal conditions, Nrf2 is kept in the cytoplasm through binding to Kelch like-ECH-associated protein 1 (Keap1). In response to stress signals, Nrf2 is released from Keap1 and translocated into the nucleus, where it triggers the induction of hundreds of genes, including HO-1, NQO1, antioxidant enzymes involved in the glutathione biosynthesis, SOD, GPx, and catalase. In addition, Nrf2 regulates the expression of genes involved in inflammatory and fibrotic responses [49]. Recent studies have demonstrated that Nrf2 may be one causative factor that regulates pulmonary fibrosis. Nrf2 protects lung against bleomycin-induced fibrosis and Nrf2 knockout mice show enhanced susceptibility to bleomycin-induced lung fibrosis [50,51]. There is compelling evidence indicating that fibroblasts cultured from IPF patients have a reduced level of Nrf2 expression and elevated level of α-SMA and type 1 collagen expression. Sulfaphane and another Nrf2 activator inhibit TGF-β1-induced pro-fibrotic effects in IPF fibroblasts and attenuate bleomycin- and paraquat-induced pulmonary fibrosis in animal models [52].

2.2. The effect of melatonin on oxidative/nitrosative stress

Melatonin is a potent antioxidant with lipophilic and hydrophilic characteristics, allowing this indoleamine to pass through all biobarriers with ease. Melatonin antioxidant actions are both receptor-dependent and independent [53–55]. Under chronic stress conditions, the expression of melatonin receptors, MT1 and MT2, are up-regulated in lung tissues to limit the harmful effects of stress [56]. Various studies have shown that melatonin stimulates the expression of GSH and antioxidant enzymes such as catalase, SOD, GPx, GSH-Rd in different organs and tissues; this action likely mediates through binding to membrane (MT1 and MT2) or nuclear (RZR and ROR) receptors [57–59]. Furthermore, melatonin elevates Nrf2 nuclear translocation in various models of oxidative stress [60]. Melatonin attenuates the excessive activity of NOX in various tissues and different pathologic conditions such as diabetic nephropathy, β-amyloid-activated microglia, bile duct ligation-induced cholestasis and oxidative stress, angiotensin II-induced vascular endothelial damage, hypoxia-induced endothelial dysfunction and myocardial injuries [61–66].

Receptor-independent properties of melatonin are described by scavenging free radicals and interacting with cytosolic proteins. Melatonin neutralizes the hydroxyl radical (·OH), H_2O_2, $ONOO^-$, ·NO, and superoxide anion ($O_2{\cdot}^-$) [67]. Melatonin interaction with free radicals results in the generation of active metabolites such as N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK) and N^1-acetyl-5-methoxykynuramine

(AMK), known to be potent free radical scavengers. Moreover, melatonin and its metabolites, AFMK and AMK, down-regulate the expression of pro-oxidative enzymes such as iNOS and COX-2 [68]. In cytosol, melatonin interacts with calmodulin (CaM), a ubiquitous calcium-binding protein, resulting in the inhibition of calcium binding to CaM. This action of melatonin contributes to the prevention of signal transduction pathways such as NOS, which require CaM for activation. Although, the affinity of CaM to melatonin is insufficient to interact at physiological concentrations, this considerably increases upon binding of CaM to CaM kinase II [69]. Melatonin has been reported to reduce lung fibrosis and injury induced by various toxic agents through suppressing oxidative stress and inflammation. Different doses of melatonin have been evaluated in these studies. In Ccl4, hyperbaric oxygen, nicotine and pancreatic fluid-induced pulmonary injury, treatment of rats with melatonin (10 mg/kg/day) reduces the level of lung fibrosis, infiltration of inflammatory cells, interstitial hemorrhage and tissue edema; these effects were associated with reduced level of lipid peroxidation and increased antioxidant capacity in the lung tissue [70–73]. The effect of single dose of melatonin has been examined in occlusion–reperfusion and X-ray radiation (12 Gy)-induced lung injury [74,75]. Treatment with melatonin (200 mg/kg) before exposure to X-ray radiation increases SOD and catalase activity and GSH content as well as reducing cytokine production and lipid peroxidation. Furthermore, melatonin (20 mg/kg) reduces MDA level and MPO activity in aortic ischemia–reperfusion-induced lung damage.

Melatonin (1 mg/kg) has protective effects on oxidant/antioxidant status of the lungs in smoke exposed rabbits [76]; melatonin (10 μM) inhibits inflammatory responses induced by acrolein, as a constituent of cigarette smoke, via suppressing ERK1/2 and PI3K/Akt signaling pathways in human pulmonary fibroblasts [77]. Melatonin alleviates lung damage induced by nitrogen mustard, at 20 mg/kg or 40 mg/kg, and respiratory syncytial virus, at 5 mg/kg; this protective effect results from the inhibition of ROS, RNS and pro-inflammatory cytokine production, and elevation of antioxidant enzymes activities [78,79].

Charão et al. evaluated the effect of melatonin-loaded lipid-core nanocapsules on paraquat-induced toxicity in the alveolar epithelial cells. Melatonin incorporated in lipid-core nanocapsules (10 μg/mL) enhanced cell viability and reduced DNA damage induced by paraquat. It was suggested that the antioxidant properties of melatonin may increase when incorporated into lipid-core nanocapsules [80]. Therefore, melatonin with potent antioxidant effects can be useful in pulmonary fibrosis in different formulation. However, further detailed studies will be needed to determine the potential therapeutic mechanisms of melatonin in IPF lungs.

3. The role of autophagy and apoptosis in IPF and the influence of melatonin

3.1. Autophagy pathway and apoptosis processes: a short overview

Autophagy is a complicated process, which is divided into three different types including chaperone-mediated autophagy, micro-autophagy and macro-autophagy. Macro-autophagy (hereinafter referred to as autophagy), is classified into non-selective and cargo-specific autophagy. Non-selective autophagy occurs in response to nutritional deprivation, while cargo-specific autophagy occurs in nutrient-rich cells targeting aggregated proteins or useless/damaged organelle [10,81]. The selective degradation of mitochondria by autophagy is termed mitophagy, which prevents cellular damage mediated by mitochondrial dysfunction. Autophagy consists of five sequential steps, including: (a) initiation complex formation and double-membrane phagophore (nucleation) maturation, (b) membrane elongation and autophagosome formation sequestering cargo, (c) fusion with lysosome, (d) inner membrane disruption leading to degradation of cargo by hydrolases, and (e) macromolecular component utilization [82]. These steps of autophagy pathway are regulated by more than 35 autophagy-related genes (ATGs) and proteins which most of them function in complexes (Figure 1).

3.2. Regulation of autophagy and its interactions with apoptosis

The initiation phase of autophagy is negatively regulated by mammalian target of rapamycin (mTOR) which resides in a functional complex of mTOR proteins, mTOR complex 1 (mTORC1). The protein complex of mTORC1 includes GβL, regulatory-associated protein of mTOR (raptor), PRAS40 and mTOR, which is activated by nutrient and growth factor-associated signals [83]. This complex inhibits the initiation phase of autophagy, which is regulated by Unc-51-like kinase 1 (mammalian homologs of Atg1, ULK1)-Atg13-Atg101-FIP200 (mammalian homologs of Atg17) protein complex. Unc-51-like kinase 1 phosphorylates and activates Beclin-1 (mammalian homolog of Atg6). Beclin-1 is a part of multiprotein-complex, class III PI3-kinase Vps34-p150 (mammalian homolog of Vps15)-Atg14-like protein (Atg14L)-Beclin-1, promoting nucleation [82,84,85]. mTOR complex 1 phosphorylates and inhibits ULKs and ATG13 contributing to the inhibition of autophagy initiation [86].

Growth factors activate PI3K/Akt/PKB (protein kinase B) pathway that inhibits TSC (tuberous sclerosis complex) 1/TSC2 complex preventive effects on Rheb, an activator of mTORC1 [83,87]. Activated-Akt phosphorylates and subsequently inactivates transcriptional factor protein Forkhead box O3 (FoxO3), which is involved in the induction of autophagy gene expression [88,89]. Energy deprivation, which can be a result of hypoxia, is one of the factors promoting autophagy via the activation of adenosine monophosphate (AMP)-activated protein kinase (AMPK). AMPK may sense the decreased ratio of ATP/AMP leading to deactivation of mTORC1 [83,90,91]. Furthermore, anti-apoptotic proteins including Bcl2 and Bcl-XL bind to Beclin 1 during normal physiological conditions and prevent the formation of Beclin 1 complex and subsequently inhibit autophagy. Under energy deprivation conditions, Beclin-1 phosphorylation by DAP kinase (DAPK), or Bcl-2 phosphorylation by c-Jun NH2-terminal kinases (JNK) results in Bcl-2/Bcl-XL-Beclin 1 dissociation and induction of autophagy [82,92]. However, long term cellular

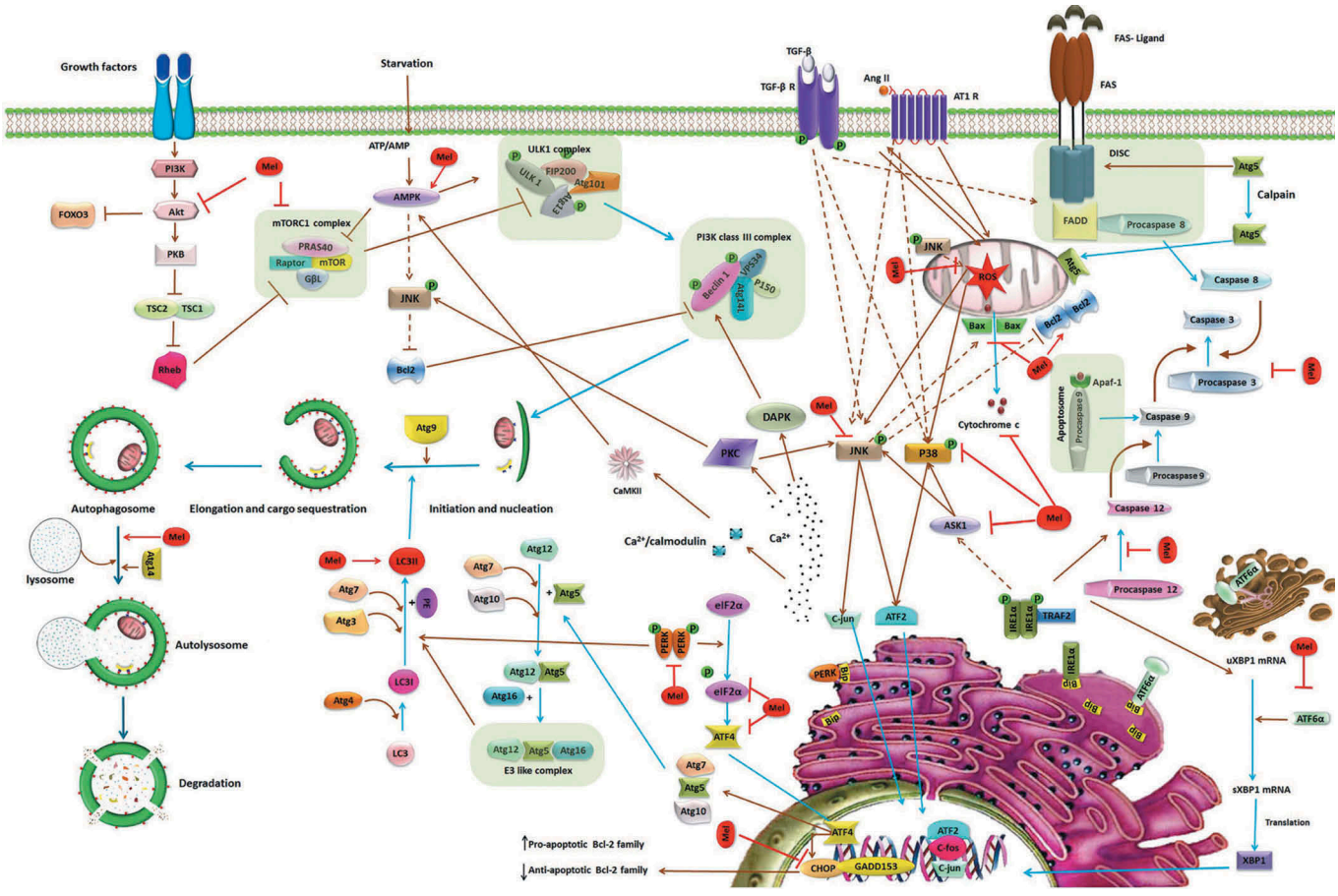

Figure 1. Insufficient autophagy is reported in human lungs with idiopathic pulmonary fibrosis (IPF); this is assessed by elevated level of mammalian target of rapamycin (mTOR) and reduced levels of LC3-phosphatidylethanolamine conjugate (LC3-II), Beclin-1, Forkhead box O3 (FoxO3) and PTEN-induced kinase 1 (PINK1) [6–9,118]. Increased unfolded protein response (UPR)-activity in IPF lungs indicates endoplasmic reticulum (ER) stress in alveolar epithelial type II cells (AECs II) [6]. This pathway induces compensatory responses to stressors. Under ER stress, the increased level of cytosolic Ca^{2+} and activation of UPR signaling pathways contribute to autophagy through inducing the expression of autophagic proteins [102,104], dissociation of Bcl2-Beclin1 [105,106,108], induction of cytosolic form of LC3 (LC3-I) conversion to LC3-II [103], inhibition of mTOR [107] and activation of protein kinase C (PKC) pathway [109,110]. However, in prolonged ER stress, UPR signaling fails to restore ER function and promotes apoptotic pathways through activating mitogen-activated protein kinases (MAPKs) and increasing the expression of pro-apoptotic proteins [111–114]. Increased AECs apoptosis is a consistent finding in IPF lungs [121]. Transforming growth factor (TGF)-β and angiotensin II (Ang II) act as potential mediators of apoptosis in lung epithelial cells in IPF through increasing pro-apoptotic protein expression and Fas-receptor clustering, resulting in the caspases cascade activation [122,131,132]. Roles of melatonin in these pathways are marked in red.

starvation is associated with the prolonged JNK1 activation and higher levels of Bcl-2 multi-site phosphorylation; these contribute to the dissociation of Bcl-2 from Bax and induction of apoptosis [93].

The elongation phase is regulated by two ubiquitin-like conjugation systems, Atg12 and LC3 (mammalian homolog of Atg8). In the first conjugation system, Atg12 is activated by Atg7 (E1-like enzyme), transferred to Atg10 (E2-like enzyme) and conjugated to Atg5. The Atg12-Atg5 conjugates further couples with Atg16 (Atg16L in mammals) to form the E3-like complex. In the LC3 conjugation system, LC3 is cleaved by a cysteine protease, Atg4, forming cytosolic form of LC3 (LC3-I). Thereafter, LC3-I is activated by Atg7 (E1-like enzyme), transferred to Atg3 (E1-like enzyme) and conjugated to phosphatidylethanolamine (PE) to form LC3-phosphatidylethanolamine conjugate (LC3-II); this process is facilitated by the E3-like complex. This lipidated form of LC3, LC3-II, is recruited to the autophagosome membrane. Finally, the Atg9 dependent pathway promotes autophagosome membrane expansion [82,84,85]. In addition to autophagy, Atg5 enhances susceptibility of cells to apoptosis. The Atg5 protein interacts with the adaptor protein FADD and activates disc, which initiates caspase signaling cascade [94]. Furthermore, cleavage of Atg5 by calpain allows its translocation to mitochondria, where Atg5 induces mitochondrial outer membrane permeabilization by interacting with Bcl-XL, resulting in cytochrome c release and caspase activation [95].

Fusion of autophagosome with lysosome is the next step of autophagy. The inner vesicle is degraded by lysosomal hydrolases, including cathepsin B, D (a homolog of proteinase A), and L. The degradation products are released to the cytosol and used in different anabolic pathways [96].

In mitophagy, dysfunctional mitochondria are recognized by PTEN-induced kinase 1 (PINK1, a mitochondrial serine/threonine-protein kinase) and E3 ubiquitin ligase Parkin (a protein encoded by the *PARK2* gene) for mitophagy clearance [97]. Under normal conditions, PINK1 is imported to the inner mitochondrial membrane and proteolytically cleaved by several proteases. Mitochondrial membrane potential is necessary

for the import of proteins such as PINK1 into the inner mitochondrial membrane. Loss of mitochondrial membrane potential prevents PINK1 translocation and degradation, which causes stabilization of PINK1 on the outer mitochondrial membrane. Therefore, PINK1 induces Parkin translocation from the cytosol to dysfunctional mitochondria, promoting removal of mitochondria via mitophagy [98,99]. However, when the number of dysfunctional mitochondria exceeds the removal ability of the mitophagy process, apoptosis could be the dominant pathway. Dysfunctional mitochondria produce excessive ROS resulting in the reduction of $\Delta\psi m$, release of cytochrome c, and activation of caspases.

3.3. The role of endoplasmic reticulum stress in autophagy and apoptosis

ER is an entry site for protein delivery. ER performs numerous functions including folding and post-translational modification of proteins, and regulation of intracellular calcium levels. The ER function disorder, referred to as ER stress, occurs when the client proteins load in the lumen of ER outweighs the folding capacity of this organelle, resulting in the aggregation of unfolded/misfolded proteins in the ER lumen. To overcome the deleterious effects of ER stress, the unfolded protein response (UPR) occurs. This signaling pathway senses ER stress and induces compensatory responses to stressors by removing the accumulated protein load and recovering normal ER function [100]. In mammals, this complex cellular response is regulated by several signaling proteins including inositol-requiring protein-1α (IRE1α), activating transcription factor 6α (ATF6α) and protein kinase RNA (PKR)-like ER kinase (PERK). Under physiological conditions, these ER stress sensors are maintained in an inactive state through binding to the ER chaperone proteins, Bip (78-kDa glucose regulated protein, GRP78). Following aggregation of unfolded proteins in the lumen of ER, UPR signaling molecules are dissociated from Bip, which causes activation of IRE1α and PERK by oligomerization and autophosphorylation, and ATF6 by proteolytic cleavage [69]. ER stress can induce autophagy by activation of UPR pathways and Ca^{2+} release from ER into the cytosol [101]. Activated PERK enhances the activation and expression of activating transcriptional factor 4 (ATF4), inducing the expression of ATG genes such as ATG5, ATG7, and ATG10 [102]. Furthermore, the PERK pathway induces LC3-I conversion to LC3-II [103]. On activation, IRE1α cleaves X-box binding protein 1 (XBP1) mRNA which is induced through ATF6 pathway. Thereafter, translated spliced XBP1 translocates to the nucleus and enhances the transcription of several genes such as Beclin1 [104]. Activated IRE1α induces the phosphorylation of Bcl-2 by JNK, leading to Bcl-2-Beclin 1 dissociation [105,106]. Under ER stress, an increase in cytosolic Ca^{2+} contributes to autophagy through several mechanisms, including (a) inhibition of mTOR via Ca^{2+}/calmodulin dependent kinase kinase-β-mediated activation of AMPK [107], (b) activation of DAPK which directly phosphorylates Beclin 1, leading to Bcl2-Beclin1 dissociation [108], and (c) activation of protein kinase c (PKC) pathway that has been shown to mediate an mTOR-independent pathway of autophagy [109,110]. However, when ER stress is prolonged, UPR signaling fails to restore ER homeostasis and represents a switch from the adaptive to pro-apoptotic pathway [111].

In addition to the maintenance of cell homeostasis, UPR signaling promotes the apoptotic pathway (Figure 1). The complex of IRE1α-TNF receptor-associated factor 2 (TRAF2) activates JNK and p38 mitogen-activated protein kinases (MAPKs) via recruiting apoptosis signal-regulating kinase 1 (ASK1). Furthermore, IRE1α-TRAF2 complex activates caspase-12 triggering the activation of caspase-9, independent from the apoptosome pathway [112,113]. The protein ATF4 induces the expression of C/EBP homologous protein (CHOP), known as growth arrest and DNA damage-inducible 153 (GADD153), while CHOP/GADD153 inhibits the expression of anti-apoptotic proteins of Bcl-2 family and increases the expression of pro-apoptotic Bcl-2 family members [114].

3.4. Decreased autophagy and increased apoptosis in IPF lungs

Autophagy has been shown to decrease in human IPF lung tissues compared with normal lungs. Defective autophagic processes have been assessed by the elevation of the mTOR activity as well as the reduction of LC3-II, Beclin-1 and FoxO3 expression [6–9]. Treatment of human lung fibroblasts with TGF-β1 has been shown to inhibit autophagic processes; this can be a mechanism for promoting fibrogenesis in IPF [8]. Moreover, silencing of LC3 and Beclin-1 genes or pharmacological inhibition of autophagy augments TGF-β1-induced expression of α-SMA and fibronectin in human lung fibroblasts [8]. These findings indicate that TGF-β1 is also involved in the pathogenesis of IPF via inhibiting autophagy processes in addition to the regulation of fibrotic processes. Interestingly, autophagy inhibition by the deletion of LC3 and ATG5 genes induces fibroblast differentiation to myofibroblasts, without the addition of TGF-β [8,115]. In bleomycin-induced experimental IPF, rapamycin-induced autophagy decreases the production of hydroxyproline and protects animals from fibrosis [8]. Inhibition of autophagy in mice with alveolar epithelial cell specific knockdown of Tsc1, exacerbates lung injury induced by bleomycin [116]. In lung samples from IPF patients, PINK1 deficiency has been observed in AECIIs, but not in fibroblasts. Knockdown of PINK1 expression in lung epithelial cells potentiates TGF-β-induced mitochondrial depolarization, ROS production, pro-fibrotic factor expression and cell death, while overexpression of PINK1 ameliorates accumulation and depolarization of mitochondria [117,118]. These observations suggest that mitophagy induction may alter the course of IPF via improving mitochondrial function of pulmonary epithelial cells in the lungs of patients. Insufficient autophagy, especially observed in aging individuals, may be responsible for epithelial cell senescence and myofibroblast differentiation, which is resulted from chronic cellular stress characterized by DNA damage, increased UPR and heat-shock proteins accumulation [6]. Although, ER stress induces autophagy, increased levels of ER stress promotes the differentiation of fibroblasts into myofibroblasts. Furthermore, ER stress and aging have been demonstrated to mediate changes in mitochondrial function in AECIIs through reducing PINK1 expression and mitophagy

flux. These events contribute to the accumulation of damaged mitochondria, apoptotic mitochondrial responses and activation of pro-fibrotic pathways [117].

A growing body of evidence suggests that apoptosis of specific lung cell types may play important roles in pathogenesis of lung diseases. Depending on the cell type, apoptosis could be proposed as being detrimental or beneficial in the fibrotic lung. Considering that the myofibroblasts and fibroblasts are the main source of excess ECM, apoptosis of these cells is thought to be beneficial in fibrotic lungs. In contrast, excess bronchiolar and alveolar epithelial cell apoptosis could be detrimental and sufficient to initiate a fibrotic response [119,120]. Indeed, increased numbers of injured AECs are a consistent finding in IPF lung tissues; these injured cells would be susceptible to apoptosis [121]. Excess AECs apoptosis induce alveolar basement membrane destruction, leading to the recruitment of fibroblasts to the site of damage to produce ECM components to provide structural scaffolding to reassemble AECs. Continuous AECs apoptosis results in the inefficient re-epithelialization, which promotes excess ECM deposition and pulmonary architecture destruction [4]. The analysis of human IPF lung biopsies and bleomycin-induced animal lung samples has shown an up-regulation in the level of fragmented DNA and pro-apoptotic factors p53, p21, Bax and caspase-3 in the AECs. Furthermore, findings obtained from patients with IPF have demonstrated that 'death receptor' Fas is expressed in AECs in the IPF lungs and circulating levels of soluble Fas ligand correlates with disease activity [122–125]. In animal models, up-regulation of Fas has been shown in AECs II in mice fibrotic lungs induced by bleomycin [126]. In addition to regulatory effects on fibrotic processes, TGFβ acts as a potential mediator of apoptosis in lung epithelial cells in IPF (Figure 1). TGF-β induces p53 expression, leading to Fas-receptor clustering and subsequent activation of caspases cascade [122].

The other signal transduction pathways involved in pulmonary epithelial cell apoptosis seems to be MAPKs activation. A wide variety of stimuli such as reactive oxygen radicals, growth factors, pro-inflammatory cytokines and lipopolysaccharide stimulate MAPKs activation [127]. In mammals, there are three distinct groups of MAPKs including the extracellular signal-regulated kinases (ERK), JNK, and p38. The TGF-β protein can activate all three members of MAPKs family [128]. Activated JNK and P38 MAPKs play important roles in the induction of cytokine expression and apoptosis, while activated ERK promotes cell proliferation and cell protection from apoptosis. Phosphorylated P38 MAPK has been found to increase in epithelial cells and fibroblasts at the intermediate stage of fibrosis [127,129]. Phosphorylated ERK and JNK have been shown to, respectively, decrease and increase in epithelial cells, but not in fibroblasts. These changes are accompanied by fibrosis progression [127,129].

Activated JNK regulates apoptotic signaling via distinct mechanisms. Activated JNK phosphorylates anti-apoptotic (Bcl-2, Bcl-xL, Mcl-1, and A1) and pro-apoptotic Bcl-2 family proteins (p53, Bad, Bik, Bim, and PUMA, known as BH3-only proteins) and induces Bid cleavage, leading to inactivation of anti-apoptotic proteins and activation of pro-apoptotic proteins. Once activated, BH3-only proteins induce conformational changes in Bax and Bak, resulting in Bax and/or Bak movement to the mitochondrial outer membrane, where they decrease mitochondrial membrane potential and allow cytochrome c escape from the inner membrane space of mitochondria [69,130]. Furthermore, phosphorylated JNK has been shown to translocate to mitochondria and induce ROS production by inhibiting respiratory complex I. The elevated level of ROS in mitochondria can cause cardiolipin oxidation, contributing to the loss of mitochondrial membrane potential and the release of cytochrome c. Activated JNK up-regulates the phosphorylation of c-Jun, which interacts with c-Fos and ATF2 to form 'activator protein 1' (AP-1) complex (Figure 1). This complex enhances the transcription level of pro-apoptotic proteins such as PUMA, P53, and Fas-L [69,130]. Activated p38 induces the expression of pro-apoptotic proteins and phosphorylates p53 at several sites and activates p53-dependent apoptosis. Moreover, phosphorylated p38 induces Bcl-2 anti-apoptotic protein phosphorylation and translocation from the mitochondrial membrane, thereby promoting the release of cytochrome c from the mitochondria [69].

Findings from *in vivo* and *in vitro* studies demonstrate that Angiotensin II (Ang II) induces AECs apoptosis; this effect is mediated by the angiotensin AT1 receptor. Angiotensin receptor AT1 antagonists and angiotensin-converting enzyme (ACE) inhibitors have been found to ameliorate AECs apoptosis and pulmonary fibrosis induced by bleomycin and amiodarone [131,132]. The results from *in vitro* studies have shown that ACE inhibitors (captopril), and AT1 antagonists (losartan) prevent amiodarone-induced AECs apoptosis [133]. Therefore, the angiotensin system plays an important role in AECs apoptosis and pulmonary fibrosis.

3.5. The regulatory effect of melatonin on autophagy and apoptosis

Melatonin exhibits protective effects in various conditions through the induction of autophagy (Figure 1). Melatonin induces autophagy and mitophagy by inhibiting mTOR phosphorylation and up-regulating of autophagy and mitophagy markers in the animal model of traumatic and subarachnoid hemorrhage-induced brain injury [134–136]. Treatment with melatonin (150 mg/kg) contributes to the reduction of mitochondria damage, ROS generation and inflammatory responses in rats with subarachnoid hemorrhage [135,136]. Melatonin (15 and 30 μM) promotes autophagy pathway and improves mitochondrial function in oxaliplatin-induced neurotoxicity, which results in the inhibition of apoptosis and enhancement of cell survival in peripheral nerves and dorsal root ganglion [137]. Melatonin (200 and 300 μM) enhances the clearance of mutant TGF-β-induced protein (TGFBIp) in corneal fibroblasts from patients with granular corneal dystrophy type 2. This therapeutic effect of melatonin is mediated by activating autophagy due to the inhibition of mTOR-dependent pathways and promotion of autophagosome-lysosome fusion [138]. Moreover, the effect of melatonin on autophagy has been tested in colorectal cancer cells and hepatocarcinoma cells. Melatonin (10 μM) increases autophagic flux in colorectal cancer cells through down-regulating Akt expression and

increasing Beclin1 and LC3-II levels [139]. In hepatocarcinoma cells, melatonin (2 mM) reduces cell survival by inducing autophagic flux characterized by elevated levels of Beclin1, LC3-II and phosphorylated JNK [140]. However, autophagy has dual roles in cancer; induction of autophagy may be a last attempt by cells to survive or be a cause of cell death. Autophagy plays tumor-suppressor role in the early stages of tumor development through recycling of defective organelles and unfolded proteins, prevention of oxidative stress, and maintenance of genome stability. However, autophagy could induce tumor promotion because of the cellular adaptation to different stress, such as hypoxia or starvation [140,141].

The impairment of autophagy is considered an important effector of cold storage-induced hepatic steatosis, which is associated with the inhibition of AMPK activity and increased ER stress. During cold storage, melatonin (100 μM) protects steatotic livers through increasing the phosphorylation of AMPK, stabilizing hypoxia-inducible factor 1α (HIF-1α) expression and inhibiting ER stress, which are followed by increased autophagy [142]. Administration of melatonin (20 and 40 mg/kg), in alcohol-induced fatty liver rats, also reduces the serum alanine aminotransferase and aspartate aminotransferase activities and serum and hepatic triglyceride levels; this protective effect of melatonin is associated with elevation of antioxidant capacity and AMPK phosphorylation [143].

The anti-apoptotic effects of melatonin and its metabolites have also been demonstrated in various *in vivo* and *in vitro* experiments (Figure 1). Melatonin inhibits JNK and p-38 MAPKs phosphorylation and subsequently prevents apoptosis. This is a result from its protective effects on mitochondria and its antioxidant activity [144–150]. The inhibitory effect of melatonin on JNK and p-38 MAPKs phosphorylation can be mediated through receptor-dependent pathways or receptor-independent radical scavenging activity. Thus, melatonin prevents the activation of P53 and subsequently reduces Bax/Bcl-2 ratio, cytochrome c escape and caspase activation [151]. Melatonin interferes with the intrinsic pathway of apoptosis by preserving mitochondrial outer membrane permeabilization and enhancing the expression of mitochondrial complex proteins [152].

In addition to the mitochondrial apoptotic pathway, melatonin inhibits ER stress-mediated apoptosis. Melatonin inhibits cadmium, atrazine, and arsenite-induced apoptosis through attenuating ER stress markers such as ATF6, ATF4, CHOP, and caspase-12 as well as decreasing caspase-3 activity, cytochrome c escape, Bax/Bcl2 ratio and JNK phosphorylation [153–155]. Furthermore, administration of melatonin (5 mg/kg) prevents lipopolysaccharide (LPS) induced-embryo lethality via alleviating LPS-evoked induction of iNOS expression, oxidative stress and hypoxic stress. Melatonin downregulates LPS-induced CHOP expression and JNK phosphorylation, thereby decreasing ER stress in the placenta [156]. During bleomycin-induced pulmonary fibrosis in mice, melatonin (5 mg/kg) alleviates bleomycin-induced epithelial-mesenchymal transition (EMT) and downregulates the expression of GRP78, phosphorylated eIF2α, PERK, phosphorylated IRE1α, cleaved ATF6, activated XBP-1 and phosphorylated JNK. Hence, melatonin reduces ER stress and consequent EMT during the development of pulmonary fibrosis induced by bleomycin [157]. Due to the Ang II effect on in the induction of the apoptosis in the IPF lungs, the inhibition of renin angiotensin system (RAS) by melatonin may be useful in IPF lungs. Findings from various studies indicate that melatonin ameliorates Ang II-induced cell and tissue injury by increasing antioxidant capacity and inhibiting apoptotic signaling pathways [64,158–160]. Treatment with melatonin (10 mg/100 mL drinking water) ameliorates activation of intrarenal RAS in a 5/6 nephrectomized rats; this effect is associated with the reduction of the expression of Ang II, AT1 receptor and interstitial fibrosis markers including type I collagen and α-SMA. Melatonin is suggested to exert this effect through inhibiting oxidative stress; ROS is considered as a major inducer of RAS system [161]. Melatonin (0.1, 0.5, and 1 mM) inhibits Ang II-induced oxidative stress and Jak/STAT signaling pathway in podocytes. This effect of melatonin results in the reduction of activated caspase-3 and Bax expression [159]. During the induction of lung damage by ischemia reperfusion, treatment of animals with melatonin (at 30 min [20 mg/kg], 6 h [50 mg/kg], and 24 h [50 mg/kg] after the procedure) increases the protective effect of adipose-derived mesenchymal stem by inhibiting the expression of apoptotic proteins including phosphorylated H2AX, cleaved caspase 3, and poly (ADP-ribose) polymerase (PARP). This effect of melatonin is associated with the elevation of antioxidant enzyme expression as well as reduction of the expression of Ang II receptor and pro-inflammatory cytokines [162]. Although the inhibitory effect of melatonin on RAS has been not evaluated in IPF lungs, these studies indicate the ability of melatonin to inhibit Ang II-induced AECs apoptosis and subsequent induction of fibrotic process in lungs.

4. Conclusions

The present study summarizes the molecular mechanisms of oxidative/nitrosative stress, autophagy and apoptosis involved in the pathophysiology of IPF. Oxidative/nitrosative stress, autophagy and apoptosis play important roles in the pathogenesis of pulmonary fibrosis. Because of the important etiological role of oxidative/nitrosative stress, autophagy and apoptosis, on the other hand, the antioxidant properties of melatonin as well as its regulatory effects on mitochondrial and ER function, this molecule would predictably have beneficial effects on IPF pathophysiology. However, studies investigating the protective effects of melatonin in IPF are rare and additional research is necessary to identify the function of melatonin in regulating autophagy and apoptosis pathways in IPF lungs. This review will hopefully stimulate further research related to the mechanisms of action of melatonin on oxidative/nitrosative stress, autophagy, and apoptosis in pulmonary fibrosis.

5. Expert opinion

Excessive levels of oxidative/nitrosative stress play an important role in the progression of IPF. In addition to the direct damage, ROS and RNS activate TGF-β/Smad signaling cascade which contributes to the overexpression of collagen type I, a responsible

factor for matrix stiffening in IPF [12,26,27]. Inhibition of autophagic processes and induction of apoptotic pathways are other mechanisms involved in the pathogenesis of IPF [6]. The influence of autophagy inhibition on the enhancement of collagen degradation is dependent or independent of TGF-β1 activity [8,115]. The impairment of autophagy induces apoptosis of AECs and fibroblast differentiation to myofibroblasts in lung tissue [6,115]. Therefore, inhibiting subcellular signaling pathways regulating AECs apoptosis may offer potential therapeutic targets for reducing progression of IPF. Considering the increased level of oxidative/nitrosative stress and apoptosis, and decreased level of autophagy in IPF lung, the inhibition of oxidative/nitrosative stress and apoptosis, and induction of autophagy are suggested as potential therapeutic targets in pulmonary fibrosis. It is, therefore, imperative to recognize potent therapeutic agents to inhibit lung fibrotic process through reducing oxidative/nitrosative stress and modulating apoptosis and autophagy pathways in lung tissue.

The antioxidant effect of melatonin and its modulatory effect on apoptosis and autophagy pathways demonstrate that melatonin can be useful in pulmonary fibrosis via inhibiting fibrosis process induced by these pathways. Melatonin increases the expression of antioxidant enzymes and scavenges free radicals, thereby protecting AECs from free radical-induced damage and eventually death [55]. Melatonin also exhibits anti-apoptotic effects through safeguarding ER and mitochondrial homeostasis [163,164]. Melatonin inhibits ER stress-mediated apoptosis characterized by reducing ER stress markers such as ATF6, ATF4, and CHOP [153–155]. Melatonin improves mitochondrial function through inhibiting the mitochondrial permeability transition pore, replenishing mitochondrial GSH, and enhancing the activity of complexes I and IV, ATP production, and mitochondrial biogenesis [69]. Melatonin also induces autophagy pathways [135,136], which is an important mechanism against fibrotic process in IPF lungs with insufficient autophagy. The antioxidant properties of melatonin and its modulatory effect on apoptosis and autophagy pathways suggest further clinical trials to determine whether melatonin could be a candidate for treating IPF.

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Declaration of interest

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