Ginsenoside Rg1 inhibits angiotensin II-induced podocyte autophagy via AMPK/mTOR/PI3K pathway

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Abstract

Recent researches have reported the extensive pharmacological activities of Ginsenoside Rg1 including antioxidant, anti-inflammatory, and anticancer properties. Furthermore Rg1 was also shown to protect various kinds of cells from self-digestion by its anti-autophagy activity. In previous studies, angiotensin II (Ang II), a key mediator of renin–angiotensin system, has been demonstrated to contribute to the progression of renal injury including abnormal autophagy. However, whether Rg1 can relieve Ang II-induced autophagy in podocyte as well as the underlying molecular mechanism remains to be elucidated. Here, we employed Ang II-treated podocyte as a model to investigate the effect of Rg1 on autophagy and the involved signal pathways. In the present study, we found that Ang II strongly promoted autophagy in immortalized mouse podocyte cells by observing the formation of autophagosomes and detecting the expression of autophagic marker, for example, LC3-II. Notably, compared to the Ang II-treated cells, treatment with Rg1 significantly inhibited the formation of autophagosomes and expression of autophagy-related proteins in Ang II pre-treated podocyte. Meanwhile, Rg1 downregulated the activity of AMPK and GSK-3β and upregulated the activity of P70S6K in Ang II-treated podocyte. In conclusion, these findings demonstrate that Ang II promotes autophagy in podocyte, and Rg1 effectively attenuates this process through AMPK/mTOR/PI3K pathway, suggesting that Rg1 may be beneficial to alleviate podocyte injury.

Keywords: Ang II; autophagy; ginsenoside Rg1; podocyte

Introduction

End-stage renal disease (ESRD) is the last phase of chronic kidney disease (CKD). In the last decade, the prevalence of ESRD is precipitously rising in the world, bringing heavy burden on the healthcare system to countries (El Nahas, 2005; Nugent et al., 2011). In this case, podocyte has gained much attention, because of its critical role in glomerular injury which is the initiation of CKD (Leeuwis et al., 2010). Podocyte, the highly specialized cell, is a pivotal component of the glomerular filtration barrier (GBF) that also plays a key role in maintenance of the glomerular basement membrane (GBM) (Dressler, 2006). Large numbers of researches indicated that different types of podocyte injury result in renal diseases, including focal segmental glomerulosclerosis (FSGS), minimal change disease (MCD), and collapsing glomerulopathy (CG) (Barisoni et al., 2009), accompanied with proteinuria, a clinical hallmark of podocytopathies (D’Agati, 2008), characterized by foot process effacement, dedifferentiation as well as apoptosis (Shankland, 2006; Wiggins, 2007). However, when the podocyte injuries were generated, cells triggered the formation of autophagosomes to degrade the oxidized proteins and damaged organelles (Susztak et al., 2006; Yadav et al., 2010). Autophagy, also

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Abbreviations: Ang II, angiotensin II; CG, collapsing glomerulopathy; CKD, chronic kidney disease; CL, chloroquine; ESRD, end-stage renal disease; FSGS, focal segmental glomerulosclerosis; GBM, glomerular basement membrane; GBF, glomerular filtration barrier; MCD, minimal change disease; mTOR, mammalian target of rapamycin; RAS, renin–angiotensin system
called cellular self-digestion, is a highly conserved process that is involved in protein and organelle degradation (Gozuacik and Kimchi, 2007; Mizushima, 2007). Normally, autophagy plays a cytoprotective role under most of cell stress, such as starvation and pathology; however, uncontrolled upregulation of autophagy fails to protect cells but leads to cell death (Mizushima et al., 2008), which is related to many kinds of human diseases, for example, cancer, neurodegeneration, aging, as well as CKD (Komatsu et al., 2006; Takahashi et al., 2007; Huber et al., 2012). Recent animal experiments showed that autophagy was a critical intracellular process to renal cell survival in CKD animal model (Huber et al., 2012), but excessive autophagy can lead to renal cell injury, especially to podocyte (De Rechter et al., 2016). Thus, maintaining the homeostasis of autophagy may be a way to reduce podocyte injury.

Renin–angiotensin system (RAS) plays a key role in renal pathophysiology and studies have demonstrated that inhibiting RAS relieves proteinuria and delays progression of CKD (Remuzzi et al., 2016; Yacoub and Campbell, 2015). During the last decade, the major renal cells including podocyte have been proved to generate ANG-II (Durvasula and Shankland, 2008). However, high levels of ANG-II in podocyte induce cell damage, especially promoting autophagy (Yadav et al., 2008). However, high levels of ANG-II in podocyte induce cell damage, especially promoting autophagy (Yadav et al., 2008). Therefore, inhibiting autophagy may reduce the ANG-II-induced podocyte injury.

Recently, ginsenoside-Rg1, the major active ingredient of ginseng, has attracted great attention not only in China, but also in Japan, Korea, and other western countries, due to its extensive pharmacological activities, including neuroprotective and nootropic effects, as well as anticancer, antioxidant, and anti-inflammatory properties (Zhang et al., 1990; Mochizuki et al., 1995; Li et al., 2015). Interestingly, previous studies have shown that Rg1 can reduce autophagy in cardiomyocytes treated with hypoxia/reoxygenation (Zhang et al., 2012), and in renal tubular cells exposed to aldosterone (Wang et al., 2015), suggesting the anti-autophagy property may be another pharmacological activity of Rg1, which may also inhibit ANG-II-induced autophagy in podocyte.

We previously reported that Rg1 inhibits aldosterone-induced autophagy in podocyte (Mao et al., 2014); however, the involved molecular mechanism is unclear. In this present study, podocytes were used to generate the cell model of autophagy by exposing to ANG-II, by which, we investigated the effects of Rg1 on relieving autophagy and the involved signaling pathways.

Materials and methods

Podocyte culture and treatment

The immortalized mouse podocyte cell lines (MPC5) was a gift from Peter Mundel (Mount Sinai School of Medicine, New York). Cells were cultured in RPMI-1640 (HyClone, USA) medium containing 10% FBS (HyClone), 100 mg/mL streptomycin, 100 U/mL penicillin, and 10 U/mL mouse recombinant γ-interferon (PeproTech, USA), and grown on type I collagen (BD Bioscience, MA, USA) at 33°C with 5% CO2. All experiments were performed under differentiating condition, which was induced by RPMI-1640 medium without γ-interferon at 37°C for 10 days.

Rg1 was purchased from DiDa Kexiang Biological Co., Ltd. (Guizhou, China). Ang II, rapamycin, chloroquine, and 3-Methyladenine were purchased from Sigma–Aldrich (St. Louis, MO, USA). Podocytes were treated with control, Ang II (10 nmol/L), rapamycin (1 ng/mL), rapamycin+Ang II, chloroquine (10 μmol/L), chloroquine+Ang II, 3-MA (4 mmol/L), 3-MA+Ang II, Rg1 (80 ng/mL), and Rg1+Ang II for 24 h, respectively.

Western blotting

Cells were lysed by radioimmunoprecipitation assay lysis buffer (20 μL; Sangon Biotech) with 1 mM phenylmethylsulfonyl fluoride (Beyotime Biotech, Beijing, China) on ice. BCA protein assay kit (Nanjing KeyGen Biotech Co. Ltd., Nanjing, China) was used to determine the protein concentration. Total protein (25 μg) were separated by 10% SDS–PAGE gel (Solarbio, Beijing, China) and transferred to PVDF membrane (EMD Millipore, Billerica MA, USA). The membranes were incubated with primary antibodies and corresponding secondary antibody. The relative protein levels were normalized to β-actin.

Transmission electron microscopy

MPC5 cells were washed by PBS, fixed with 3% glutaraldehyde (Sangon Biotech, Shanghai, China), post-fixed with 1% OsO4 (Sangon Biotech), dehydrated in acetone, and embedded in Epon 812 (Nissin EM, Tokyo, Japan). Ultrathin sections were stained with 2.0% uranyl acetate/lead citrate, and observed under transmission electron microscopy (Hitachi, Ltd., Tokyo, Japan).

Immunofluorescence

MPC5 cells were grown on cover slides for immunofluorescence. Subsequently, cells were fixed with 4% paraformaldehyde and incubated with primary rabbit polyclonal anti-LC3B antibody (1:400; CST, USA). The secondary antibody used was Alexa Fluor® 488-conjugated donkey anti-rabbit IgG (H+L) secondary antibody (1:400; Invitrogen Life Technologies, USA). Immunofluorescence images were observed under Nikon Eclipse 50i microscope.
Acridine orange staining was performed to detect the autophagic vacuoles. Cells were grown in coverslip-bottom dishes and stained with acridine orange (0.05 g/mL; Sigma, USA) in fresh serum-free medium with Hoechst 33342 (1 μg/mL; Invitrogen, USA) for 15 min at 37°C. Images were captured by Nikon Eclipse 50i microscope.

Statistical analysis
All results were analyzed by SPSS19.0 statistics software. Data were presented as means ± SD. P-values less than 0.05 was considered to be statistically significant.

Results
Ang II promotes autophagy in mice podocytes
To investigate the effect of Ang II on the formation of autophagosomes in podocytes, the transmission electron microscopy was used to observe the formation of double-or multiple-membrane encapsulated portions of the cytoplasm and/or organelles, which represents autophagosome. The TEM images showed that cells exposed to Ang II presented morphological changes forming more autophagosomes (Figure 1A). Compared with control, increased numbers of autophagosomes were found in Ang II-treated cells (Figure 1B). Furthermore, Western blotting was performed to detect the protein levels of LC3-I, LC3-II, and Beclin-1 in cells treated with control, Ang II, Rapamycin, Rapamycin+Ang II, Chloroquine, Chloroquine+Ang II, 3-Methyladenine, and 3-Methyladenine+Ang II, respectively (Figures 1C and 1D). Rapamycin is an inhibitor of mTOR and chloroquine is a lysosomal inhibitor, and these two drugs markedly increased the protein expression of LC3-II and Beclin-1 in MPC5 cells in the present study. Compared with rapamycin and chloroquine, Ang II also upregulated the protein levels of LC3-II and Beclin-1. Inversely, 3-Methyladenine downregulated the protein levels of LC3-II and Beclin-1 in Ang II-treated cells. All the above

Figure 1 Ang II promotes autophagy in mice podocytes. (A) Under the transmission electron microscope (original magnification ×10,000), more autophagosomes were observed in Ang II-treated cells. The black arrows point to the autophagosomes. (B) The bar chart of the number of autophagosomes in control and Ang II-treated cells, 30 cells were counted in each group (n = 3). *P < 0.05, compared with control. (C) Western blotting analysis to detect the protein levels of LC3-I, LC3-II, and Beclin-1. Cells were treated with control, Ang II, Rapamycin(Rapa), Rapamycin+Ang II, Chloroquine(CL), Chloroquine+Ang II, 3-Methyladenine(3M), and 3Methyladenine+Ang II, respectively. (D) Quantification of protein levels normalized to β-actin. *P < 0.05, compared with control.
data demonstrated that Ang II can increase autophagy remarkably.

Effects of Rg1 on Ang II-induced autophagy in Podocytes

In order to confirm the effects of Rg1 on Ang II-induced autophagy, acridine orange staining and immunofluorescence were performed (Figures 2A and 2B). Cells cultured with indicated drugs, followed by staining with acridine orange. Under microscopy, we found that the number of green-fluorescent puncta was strongly increased in cells exposed to Ang II, CL, and CL+Ang II. Compared with Ang II-treated group, cells treated with Rg1+Ang II exhibited much less green-fluorescent puncta, which means the number of

Figure 2 Effects of Rg1 on Ang II induced autophagy in podocytes. (A) MPC5 cells were exposed to the indicated drugs for 24 h, followed by staining with acridine orange (0.05 μg/mL) and Hoechst-33342 (1 μg/mL) for 15 min. The images were obtained by Nikon Eclipse 50i microscope (magnification ×800). (B) The expression of LC3-II in MPC5 cells exposed to drugs for 24 h. Immunofluorescence images were captured under fluorescence microscopy (magnification ×630). Green fluorescent puncta represent autophagic vesicles. (C) Quantitative analysis of autophagic vacuoles for Acridine orange staining (30 cells/group; n = 3). *P < 0.05, compared with control and Rg1+Ang II-treated group. (D) Quantitative analysis of autophagic vacuoles for Immunofluorescence (30 cells/group; n = 3). *P < 0.05, compared with control and Rg1+Ang II-treated group.
autophagosomes in Rg1 + Ang II-treated cells was less than in Ang II-treated cells (Figure 2C). Furthermore, the results of immunofluorescence were consistent with acridine orange staining (Figure 2D). These results suggested that Rg1 can significantly reduce autophagosomes in Ang II-treated cells.

Effects of Rg1 on expression of autophagy-related protein

Increased autophagosomes were observed in Ang II-induced MPC5 cells by acridine orange staining and immunofluorescence. Interestingly, Rg1 can efficiently decrease the autophagosomes, which were stimulated by Ang II. Therefore, we performed the Western blotting to investigate the expression level of autophagy-related protein in MPC5 cells treated with control, Ang II, Rg1, and Rg1 + Ang II, respectively (Figures 3A and 3B). Compared with control, Ang II strongly upregulated the expression of Beclin-1 and LC3-II, which are involved in the formation of autophagosome. However, when cells pretreated with Rg1 after exposing to Ang II, the expression of autophagy-related protein, Beclin-1, and LC3-II was significantly downregulated in MPC5 cells, suggesting that Rg1 may inhibit Ang II-induced autophagy.

Rg1 inhibits Ang II-induced autophagy by regulating the AMPK/mTOR/PI3K pathway

To investigate the signaling pathway involved in the inhibition of Ang II-induced autophagy by Rg1, Western blotting were performed to analysis the expression of phosphorylated (p)-AMPK and AMPK (Figure 4A), phosphorylated (p)-GSK-3β and GSK-3β (Figure 4B), as well as phosphorylated (p)-P70S6K and P70S6K (Figure 4C). AMPK, an inactivator of mTOR complex-1, can be activated by the phosphorylation of the catalytic subunit AMPKα, which promotes autophagy. In this present study, the ratio of p-AMPK/AMPK was calculated to measure the activation of AMPK (Figure 4D). Compared with control, Ang II increased the ratio of p-AMPK/AMPK, which means the expression of p-AMPK was upregulated, and AMPK was activated. However, the ratio of p-AMPK/AMPK was reversed to normal by Rg1 in Ang II-treated cells that was consistent with Compound C, an inhibitor of AMPK. In addition, glycogen synthase kinase 3β (GSK-3β) can also increase autophagy by activating mTOR signaling. Therefore, we detected the expression of p-GSK-3β and GSK-3β to analyze the activity of GSK-3β (Figure 4E). In this present study, the data showed that the ratio of p-GSK-3β/GSK-3β was significantly upregulated in Ang II-treated cells, and the GSK-3β inhibitor SB216763 downregulated the ratio of p-GSK-3β/GSK-3β in cells pretreated with Ang II. Meanwhile, Rg1 also decreased the ratio of p-GSK-3β/GSK-3β in Ang II-treated cells. P70S6K is downstream of mTOR in the insulin signaling pathway, and base on the experiments with rat hepatocytes (Blommaart et al., 1995), P70S6K was reported to inhibit autophagy, which may through a negative feedback to inhibit activation of phosphatidylinositol-3-OH kinase (PI3K) class I (Um et al., 2004). In the present study, the results showed that the expression of p-P70S6K and the ratio of p-P70S6K/P70S6K were decreased in Ang II-treated cells, and Rg1 recovered the phosphorylation of P70S6K, which reduced by Ang II (Figure 4F). A simplified pathway model of Rg1 inhibiting autophagy is illustrated in Figure 5. Taken together, all these data above suggested that Rg1 inhibited Ang II-induced autophagy by regulating the activity of AMPK, GSK-3β, and P70S6K.

Discussion

In the present study, we found that Ang II was a mediator capable of promoting podocyte autophagy as evidenced by the formation of autophagosome and expression of autophagy-related protein. Furthermore, we also found that treatment of Rg1 efficiently inhibited the Ang
II-induced autophagy. In addition, the inhibitory effect of Rg1 on podocyte autophagy was associated with the inhibition of AMPK and GSK-3β and upregulation of P70S6K activation.

Autophagy is a critical cellular process in which cytoplasmic components are transported to lysosomes for digestion, which maintains cellular homeostasis and energy production (Ravikumar et al., 2010). Growing evidence suggested that dysfunctional autophagy is involved in renal injury. On the other hand, accumulating evidences indicate that Ang II plays a key role in podocyte injury, and high concentration of Ang II has been reported to promote autophagy in podocyte in previous research. In this study, we established a cell model of autophagy by exposing podocyte to Ang II (10 nmol/L) to develop the potentially therapeutic agents. Therefore, chloroquine, the lysosomal protease inhibitor, which has been proved to promote autophagy, was used to be the positive control of autophagy. Inversely, 3-MA, a pharmacological inhibitor of autophagy, was employed to be the negative control of autophagy. Notably,

**Figure 4 Rg1 inhibits Ang II-induced autophagy in MPC5 cells via AMPK/mTOR/PI3 K pathway.** (A) Western blotting analysis to detect the protein levels of p-AMPK and AMPK. Proteins were harvested from cells treated with control, Ang II, Ang II+Compound C, and Ang II+Rg1, respectively. (B) Western blotting analysis to detect the protein levels of p-GSK-3β and GSK-3β. Proteins were isolated from cells treated with control, Ang II, Ang II+SB216763, and Ang II+Rg1, respectively. (C) Western blotting analysis to detect the protein levels of p-P70S6K and P70S6K. Proteins were isolated from cells treated with control, Ang II, Ang II+Rapamycin, and Ang II+Rg1, respectively. (D) Value of p-AMPK/AMPK. *P < 0.05, compared with Ang II-treated group. (E) Value of p-GSK-3β/GSK-3β. *P < 0.05, compared with Ang II-treated group. (F) Value of p-P70S6K/P70S6K. *P < 0.05, compared with Ang II-treated group.
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**Figure 5** A simplified pathway model of Rg1 inhibiting autophagy.

Some autophagy-related gene and proteins are critical for the initiation of autophagy. Several researches have indicated that Vps34-Vps15-Beclin 1 complex is essential for the formation of omegasome (Simonsen and Stenmark, 2008), and Unc-51-like kinase 1 and 2 (ULK1/2) complex plays a key role in the initiation of autophagy (Mizushima, 2010). Among these complex, over expression of beclin-1 reflects the occurrence of autophagy (Yue et al., 2003). Furthermore, increased conversion of LC3-I into phosphatidylethanolamine-conjugated LC3-II also suggests the upregulated autophagy (Kabeya et al., 2000). In this present study, expression of beclin-1 and conversion of LC3-I into LC3-II are strongly increased by Ang II in podocyte, leading to enhanced autophagy.

Ginsenoside Rg1 is a major active ingredient purified from Panax ginseng, which is the best-known herbal medicine in China. Increasing studies have demonstrated its beneficial actions against apoptosis and fibrosis in various animal or cellular models, including acute lung injury mice (Bao et al., 2015), cardiomyocytes hypoxia/reoxygenation (H/R) model (Zhang et al., 2012), and aldosterone-induced renal tubular cell injury model (Wang et al., 2015). Furthermore, it was reported that Rg1 has significant pharmacological effect on inhibiting autophagy in cardiomyocytes and renal tubular cells. However, the pharmacological effect of Rg1 on Ang II-induced autophagy is unclear. In this present study, Rg1 strongly reduced the formation of autophagosomes and expression of autophagy-related protein including LC3-II and Beclin-1, and inhibited Ang II-induced autophagy in podocyte.

Mammalian target of rapamycin (mTOR), a critical pathway in mammalian autophagy, has been studied in depth in the last decade. In addition to regulating autophagy, mTOR is a key signaling molecule to regulate transcription, mRNA translation, proliferation, cell growth, and so on (Sarbassov et al., 2005). mTOR pathway contains two functional complexes, one of them is a rapamycin-sensitive complex, mTOR complex 1 (mTORC1), which regulates autophagy, and the other one is mTOR complex 2 (mTORC2), which regulates autophagy indirectly. Previous studies demonstrated that inhibition of mTORC1 and induction of autophagy by rapamycin, a mTOR inhibitor, are related to decreased phosphorylation of its two downstream effectors, one of them is ribosomal protein S6 kinase-1 (S6K1), also known as P70S6K (Sarbassov et al., 2005). A previous study reported that, when TOR is inactivated in starvation model of Drosophila, P70S6K is activated to promote maximal autophagy, probably through an insulin receptor substrates (IRS)-related negative-feedback loop to inhibit PI3K pathway (Scott et al., 2004). Furthermore, the activation of P70S6K is necessary but not sufficient to induce autophagy (Scott et al., 2004). In this present study, rapamycin significantly reduced the expression of p-P70S6K, and Ang II also downregulated the expression of p-P70S6K, suggesting that Ang II promotes autophagy by inhibiting phosphorylation of P70S6K, which is consistent with rapamycin. Surprisingly, Rg1 efficiently increased the expression of p-P70S6K after pretreating with Ang II in podocyte. Thus, according to the results, we presume that P70S6K may partially inhibit autophagy in mTOR-active state by inhibiting PI3K pathway in mammalian, and Rg1 promotes the activity of P70S6K to relieve autophagy. To further investigate the involved pathway in inhibiting autophagy of Rg1, we detected the activity of AMPK and GSK-3β in Rg1-treated autophagic model of podocyte. AMP-activated kinase (AMPK), a major regulator of energy homeostasis, phosphorylates TSC2 by glycogen synthase kinase 3 (GSK-3) and reduces the activity of TSC1/2 heterodimer to inhibit mTOR (Inoki et al., 2006). Recent researches showed that inhibiting the activity of GSK-3β suppresses autophagy by activation of mTOR (Sarkar et al., 2008), and inhibition of AMPK by its inhibitor compound C also suppresses autophagy (Meijer and Codogno, 2007). In the present study, Ang II upregulated the expression of p-AMPK and p-GSK-3β, meanwhile, the AMPK inhibitor compound C decreased the activity of AMPK and the GSK-3β inhibitor SB216763 reduced the activity of GSK-3β, respectively. Consistent with the AMPK inhibitor compound C, Rg1 efficiently downregulated the expression of p-AMPK in Ang II-pretreated podocyte. Similarly, Rg1 also strongly downregulated the expression of p-GSK-3β in Ang II-pretreated podocyte, which is consistent with the GSK-3β inhibitor SB216763. All the above data suggested that Rg1 inhibits the activity of AMPK and GSK-3β to suppress autophagy by activating mTOR signaling and increases the activity of P70S6K to inhibit autophagy by a negative-feedback loop of inhibiting PI3K.
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AMPK and GSK-3β and activation of the P70S6K, the precise mechanism remains unclear and the effect of Rg1 on podocyte injury in vivo remains unknown, which warrant for further studies. In summary, our results demonstrate that Ang II promotes autophagy and Rg1 has cytoprotective effects on Ang II-induced autophagy in podocyte. In addition, Rg1 efficiently inhibits the activation of AMPK and GSK-3β and promotes the activation of P70S6K to sustain the activity of mTOR, which suppresses autophagy. These findings reveal the possible signaling pathway in inhibition of Ang II-induced autophagy of Rg1, also supplement the potentially pharmacological properties on anti-autophagy of Rg1 in another renal cells, podocyte, which may help develop new agents for therapy of kidney diseases.

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Conflict of interest

The authors declare that they have no competing interests.

References


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