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Original Paper

TRPC6-Mediated Ca^{2+} Signaling is Required for Hypoxia-Induced Autophagy in Human Podocytes

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Key Words
Trpc6 • Podocytes • Autophagy • Ca^{2+} signaling • Adenosine monophosphate-activated protein kinase

Abstract
Background/Aims: Intracellular Ca^{2+} signaling plays an important role in the regulation of autophagy. However, very little is known about the role of Ca^{2+} influx, which is induced by plasma membrane Ca^{2+} channels. Our previous study showed that transient receptor potential canonical channel-6 (TRPC6), a major Ca^{2+} influx pathway in podocytes, was activated by hypoxia. Here, we investigated whether TRPC6 is involved in hypoxia-induced autophagy in cultured human podocytes. ***Methods:*** In the present study, an immortalized human podocyte cell line was used. Fluo-3 fluorescence was utilized to determine intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), and western blotting was used to measure autophagy and protein expression. ***Results:*** We found that blockade TRPC6 by using either TRPC6 siRNA or a TRPC6 blocker attenuated hypoxia-induced autophagy, while enhancement of TRPC6 activity with a TRPC6 activator enhanced hypoxia-induced autophagy. Furthermore, TRPC6-dependent Ca^{2+} signaling is responsible for hypoxia-induced autophagy since both an intracellular and extracellular Ca^{2+} chelator abolished hypoxia-induced autophagy. Moreover, we found that blockade of TRPC6 by using either TRPC6 siRNA or a TRPC6 blocker decreased the expression of adenosine monophosphate-activated protein kinase (AMPK), an important signaling molecule in Ca^{2+}-dependent autophagy activation, which is activated under hypoxic conditions. These data suggest that the involvement of TRPC6 in hypoxia-induced autophagy is associated with AMPK signaling. ***Conclusion:*** TRPC6 is essential for hypoxia-induced autophagy in podocytes.

Introduction

Macroautophagy (referred to as autophagy) is an evolutionarily conserved process that is characterized by the formation of double-membrane structures, termed "autophagosomes,"

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that encircle cellular material destined for degradation via fusion with lysosomes, thereby maintaining cellular homeostasis [1-3]. Moreover, autophagy is essential for survival under stress conditions, such as hypoxia. Alterations in autophagy have been implicated in a number of human pathological conditions. Indeed, autophagy has been identified as a mechanism that is critical for the maintenance of glomerular podocyte homeostasis in response to injury [4, 5]. Therefore, understanding the underlying mechanisms of autophagy will lead to improvements in therapeutic approaches for many human diseases.

Intracellular Ca^{2+} is a secondary messenger that is involved in the regulation of several physiological cell functions, including autophagy [2, 3]. However, there are apparently conflicting views concerning the role of intracellular Ca^{2+} signaling in autophagy. Many reports have suggested that an elevated intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) promotes autophagy [6-10], whereas other studies have indicated an inhibitory role for Ca^{2+} in autophagy [11-13]. The different regulatory effects of Ca^{2+} signaling appear to depend on its spatiotemporal characteristics [2, 3]. Both Ca^{2+} influx and Ca^{2+} release are tightly controlled by numerous regulatory systems that determine the specific temporal characteristics of Ca^{2+} signaling. However, most studies that have identified calcium channels as autophagy regulators have focused on intracellular Ca^{2+} release channels [2, 3], such as inositol 1, 4,5-trisphosphate receptor (IP_3R), transient receptor potential mucolipin 1 (TRPML1) [14] and TRPML3 [15]. The data concerning the role of Ca^{2+} influx channels are very limited [2]. An early study suggested that exogenously introduced calcium in the form of calcium phosphate precipitates induces autophagy [10]. Most recently, transient receptor potential canonical channel-1 (TRPC1) has been demonstrated to be essential for the regulation of hypoxia and nutrient depletion-induced autophagy [16]. TRPC4, another TRPC channel, has also been implicated in autophagy induced by trans-3, 5,4-trimethoxystilbene (TMS), a potent small molecular inducer of autophagy [17]. In vascular endothelial cells, TRPC4-dependent Ca^{2+} influx activates adenosine monophosphate-activated protein kinase (AMPK) and is therefore involved in TMS-induced autophagy [17]. Indeed, AMPK is a downstream target of Ca^{2+} signaling and plays an important role in the initiation of autophagy in response to different stressors, such as hypoxia [18].

TRPC6 is a major Ca^{2+} influx pathway in podocytes, which lack t L-type Ca^{2+} channels [19]. However, the link between TRPC6 and autophagy has not been established. We have demonstrated that hypoxia induces Ca^{2+} influx and enhances TRPC6 expression in podocytes [20]. Moreover, TRPC6 is responsible for hypoxia-induced Ca^{2+} influx in podocytes because both pharmacological blockage of TRPC6 and knock down of TRPC6 by siRNA interference attenuates hypoxia-induced Ca^{2+} influx [20]. Here, we demonstrate, for the first time, that TRPC6-mediated Ca^{2+} signaling is essential for hypoxia-induced autophagy in human podocytes.

Materials and Methods

Cell culture and cell treatments

Conditionally immortalized human podocytes were cultured as previously described [21]. Briefly, cells were grown in RPMI-1640 medium (Corning, USA) supplemented with ITS (insulin, transferrin, and sodium selenate; Sigma, USA) and 10% fetal bovine serum (Sciencell, USA) at 33°C. To induce differentiation, podocytes were cultured at 37°C in the same medium for 11–14 days.

Fluorescence measurement of $[Ca^{2+}]_i$

Measurement of $[Ca^{2+}]_i$ was performed as previously described [21]. Briefly, podocytes were grown on coverslips and then added to a 1% physiological saline solution containing Fluo-3/AM (3 μM, Molecular Probes, USA) and Pluronic F-127 (0.03%, Sigma, USA) at 37°C for 45 min. The fluorescence intensity of Fluo-3 in podocytes was recorded using a laser scanning confocal microscope (FV300, Olympus, Japan). The $[Ca^{2+}]_i$ is expressed as the pseudo-ratio value of the actual fluorescence intensity divided by the average baseline fluorescence intensity. Calibrations were performed at the end of each experiment. Data from 20 to 40 cells were compiled from a single run, and at least three independent experiments were conducted.

Western blotting

Western blot analysis was performed using a standard protocol. LC3B rabbit monoclonal antibodies (1:1, 000, Sigma, USA), p62/SQSTM1 rabbit polyclonal antibodies (1:1, 000, Sigma, USA), Beclin-1 rabbit polyclonal antibodies (1:1, 000, Santa Cruz, USA), TRPC6 rabbit polyclonal antibodies (1:200, Alomone Labs, Israel), p-AMPKα (Thr172) rabbit polyclonal antibodies (1:500, Cell Signaling Technology, USA), AMPKα rabbit polyclonal antibodies (1:500, Cell Signaling Technology, USA), or actin antibodies (1:1, 000, ZSGB-Bio, China) were used as the primary antibodies. The membranes were extensively washed and incubated with fluorescence-conjugated goat anti-rabbit or goat anti-mouse IgG secondary antibodies (1:10, 000, LI-COR Biosciences, USA). Western blot bands were quantified using the Odyssey infrared imaging system (LI-COR Bioscience, USA). Then, the podocytes were stimulated with a variety of agonists or inhibitors as described in the results, including flufenamic acid (FFA, Sigma-Aldrich, USA), 5-aminoimidazole-4-carboxamide-1-β-D-riboside (AICAR, Merck, Germany),1-[2-(4-methoxyphenyl)]-2-[3-(4-meth-oxyphenyl)propoxy] ethyl-1H-imidazole hydrochloride (SKF-96365, Sigma, USA) and adenine 9-β-D-arabinofuranoside (Ara-A, Sigma, USA). Chloroquine (CQ, 1-oleoyl-2-acetyl-*sn*-glycerol (OAG), bis-(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid, tetra (acetoxymethyl) ester (BAPTA-AM) and ethyleneglycol-bis (2-aminoethylether)-N,N,N′, N-tetraacetic acid (EGTA) were purchased from Sigma (USA).

Transfection

Small interfering RNA (siRNA) was transfected into podocytes using the X-treme Gene siRNA transfection reagent (Roche, Switzerland) according to the manufacturer's instructions. The transfected podocytes were prepared for subsequent experiments 72 h post transfection. TRPC6 siRNA was purchased from Santa Cruz (USA).

Indirect immunofluorescence

Podocytes were fixed with ice-cold methanol for 5 min and permeabilized with 0.04% Triton X-100 for 1 h at room temperature. Subsequent to blocking with goat serum, cells were incubated with LC3B rabbit monoclonal antibodies (1:100, Cell Signaling Technology, USA) at 4°C for 24 h. Following incubation with DyLight 488 secondary antibodies (1:500, Molecular Probes, USA) and DAPI (1:50, BiYunTian, China), podocytes were subjected to confocal laser scanning microscopy to observe autophagosome changes. Fifteen cells from each group were analyzed.

Statistical analysis

Data were expressed as the mean ± standard deviation. An independent samples *t*-test was performed with SPSS 19.0. statistical software; $P<0.05$ was considered to represent significant difference. All of the results in this paper were obtained from at least three independent experiments.

Results

Hypoxia induces autophagy in cultured human podocytes

We first examined whether hypoxia induces autophagy in cultured human podocytes. Podocytes were incubated in 1% O_2 for different lengths of time (24, 48, or 72 h). Autophagy was assessed by western blot analysis, which detected the essential autophagy protein LC3. The lipidated form of LC3 (LC3-II) appeared as a band that had a lower molecular weight (16 kDa) than the native non-lipidated form (LC3-I, 18 kDa). LC3-I has been reported to be less immunoreactive and stable than LC3-II [22]; therefore, the level of LC3-II was used as an indication of the extent of autophagy. In all experiments, autophagy was quantified as the ratio of LC3-II over actin, the loading control. Exposure of podocytes to 1% O_2 significantly enhanced the LC3-II levels in a time-dependent manner (Fig. 1A). As shown in Fig. 1A, increased LC3-II levels were detectable following 24 h of hypoxia and reached a maximum at 48 h of hypoxia. Similarly, the expression level of Beclin 1, another autophagy marker, was also increased under hypoxic conditions (Fig. 1B). However, hypoxia-induced autophagy could be due to impaired autophagic flux or increased induction of autophagy. Therefore, we evaluated the expression of p62, which is important for the autophagic clearance of certain proteins

and seems to be degraded by autophagy pathways [22]. As expected, degradation of p62 was significantly enhanced by hypoxia (Fig. 1C). To further confirm these findings, the lysosomotropic drug chloroquine (100 μM), which inhibits the fusion of autophagosomes and lysosomes, was added to the medium under hypoxic conditions. As shown in Fig. 1D, treatment with chloroquine led to a significant increase in LC3-II levels compared to those in podocytes incubated without chloroquine, suggesting that hypoxia, rather than blockade of autophagic flux, induces autophagosome formation. Taken together, these results indicate that hypoxia induces autophagy in human podocytes.

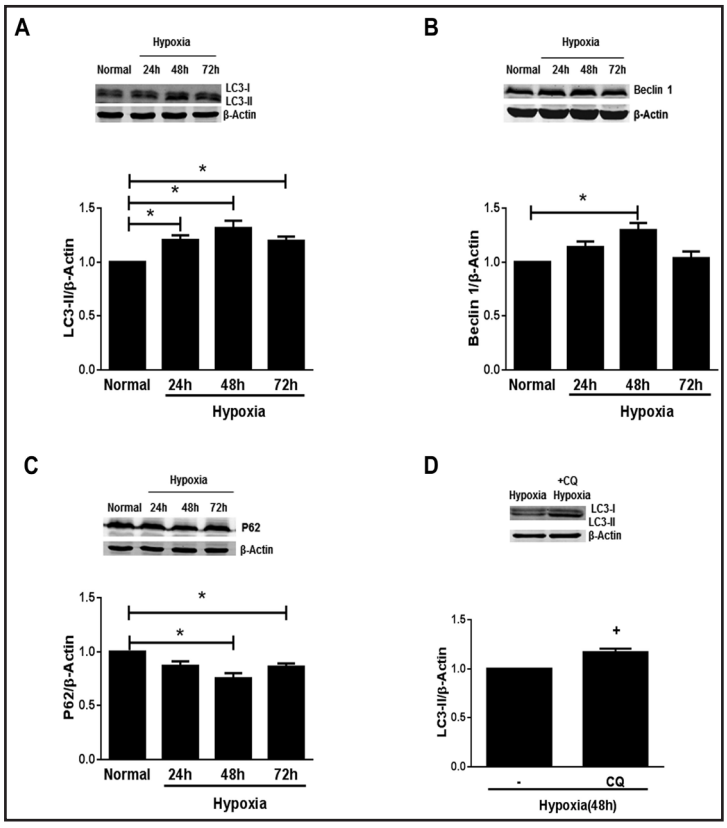

Fig. 1. Hypoxia-induced autophagy in human podocytes. The protein expression levels of LC3-II (A), Beclin 1 (B) and P62 (C) in podocytes incubated with hypoxia treatment for 24 h, 48 h or 72 h. The expression levels were normalized to β-actin. *P<0.05, compared with the normoxia. The values are the mean±SD, n=5; (D) The protein expression levels of LC3-II in podocytes were incubated with 100 μM chloroquine (CQ) before the hypoxia treatment for 48 h. $^{+}$P<0.05, compared with the podocytes treated with hypoxia treatment for 48 h. The values are the mean±SD, n=3.

Blockade of TRPC6 attenuates hypoxia-induced autophagy

To explore the role of TRPC6 in hypoxia-induced autophagy, we treated cells in the presence or absence of SKF-96365 (10 μM), a non-selective TRPC6 blocker, under 1% O_2 for 48 h. As shown in Fig. 2A, SKF-96365 abolished the effect of hypoxia on the increased LC3-II levels. Hypoxia-induced Beclin 1 expression was also reduced by SKF-96365 (Fig. 2B). Moreover, degradation of p62 induced by hypoxia was attenuated by treatment with SKF-96365 (Fig. 2C), suggesting that SKF-96365 affects autophagosome formation rather than autophagic flux. In addition, we utilized siRNA technology to downregulate TRPC6 expression in human podocytes. The efficiency of TRPC6-siRNA was confirmed by western blot analysis (Fig. 2D). The procedure did not affect the expression of other TRPC channels (data not shown). As shown in Fig. 2E, transfection with TRPC6 siRNA significantly reduced the expression of LC3-II induced by hypoxia compared to expression in cells transfected with scrambled siRNA (Fig. 2E). To further corroborate our findings, we next employed a quantitative imaging assay to assess the formation of endogenous LC3 puncta. As shown in Fig. 2F, transfection with TRPC6 siRNA significantly decreased the number of LC3 puncta per cell under hypoxia compared to transfection with scrambled siRNA. Taken together, these results suggest that TRPC6 is required for hypoxia-induced autophagy.

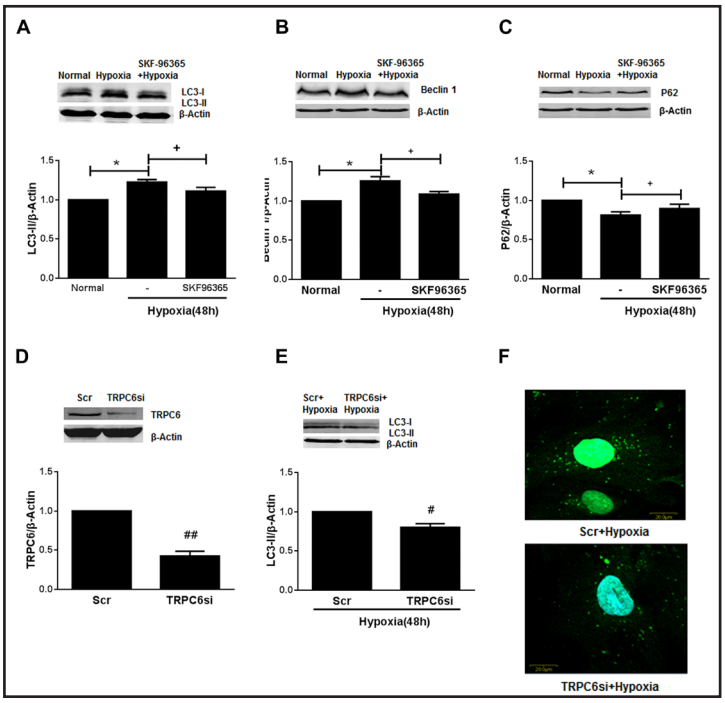

Fig. 2. Inhibition of hypoxia-induced autophagy by blockade of TRPC6. Typical bands are shown. The protein expression levels of LC3-II (A), Beclin 1 (B) and P62 (C) in podocytes incubated with 10 μM SKF-96365 before the hypoxia treatment for 48 h. The expression levels were normalized to β-actin. *P<0.05, compared with the normoxia cells; $^{+}$P<0.05, compared with the cells treated with hypoxia treatment for 48 h. The values are the mean±SD, n=4. (D) Western blot experiments showed that transfection with TRPC6 siRNA reduced TRPC6 protein expression. $^{\#\#}$p<0.01, compared with the podocytes transfected with Scramble siRNA (Scr). n = 3. (E) TRPC6 siRNA transfection inhibited protein expression levels of LC3-II after 48 hour of hypoxia. ($^{\#}$p<0.05 vs. Scr, n = 3). (F) Podocytes transfected with Scr or TRPC6si were incubated in hypoxia for 48 h by immunofluorescence using the LC3 antibody (green). n=3.

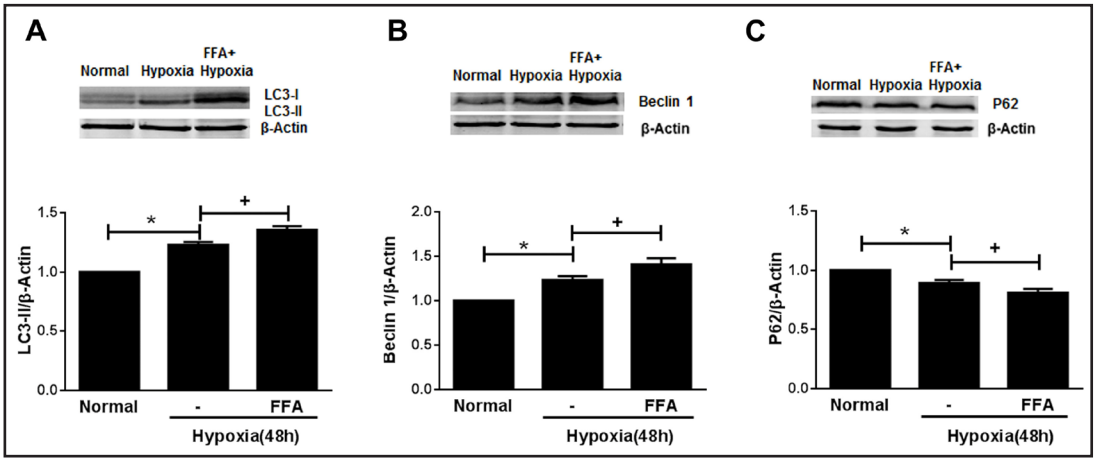

Fig. 3. TRPC6 activation up-regulates autophagy. Western blot analysis of LC3-II (A), Beclin 1 (B) and P62 (C) in cells incubated with 100 μM flufenamic acid (FFA), a known agonist of TRPC6, before the hypoxia treatment for 48 h. *P<0.05, compared with the normoxia; $^{+}$P<0.05, compared with the cells treated with hypoxia treatment for 48 h.

Activation of TRPC6 enhances hypoxia-induced autophagy

Since inhibition of TRPC6 attenuated hypoxia-induced autophagy, we investigated whether activation of TRPC6 had the opposite effect on hypoxia-induced autophagy. Flufenamic acid (FFA), a known agonist of TRPC6, activates TRPC6 in podocytes and other cell types and is widely used as a tool for studying TRPC6 [23-25]. As shown in Fig. 3A, FFA (100 μM) significantly increased the LC3-II levels induced by hypoxia compared with the controls. Similarly, the expression levels of Beclin 1 were further increased by FFA under

hypoxic conditions (Fig. 3B). As expected, degradation of p62 induced by hypoxia was also enhanced by treatment with FFA (Fig. 3C). These data confirm that TRPC6 modulates hypoxia-induced autophagy.

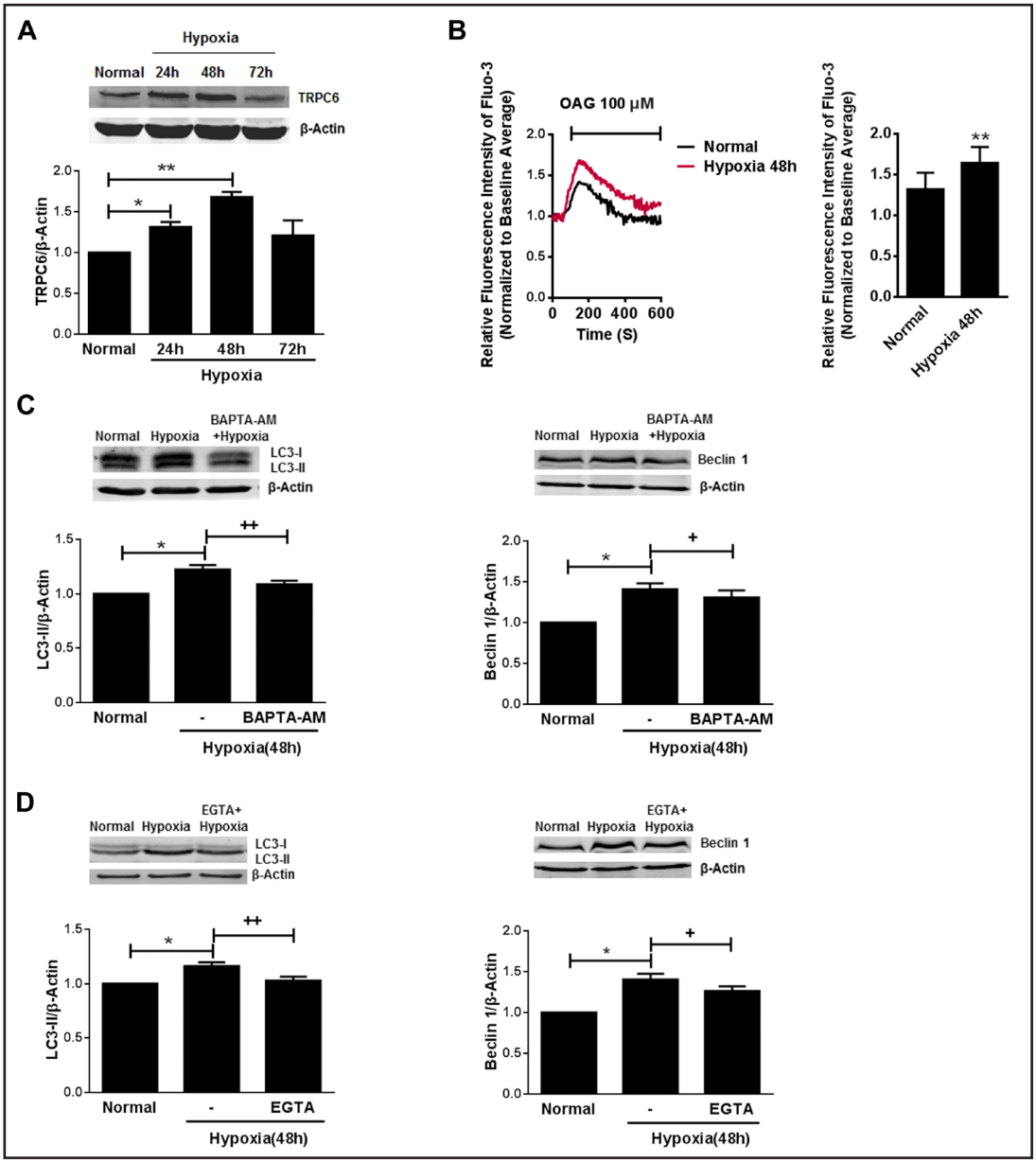

Fig. 4. TRPC6-dependent Ca^{2+} signaling was required for hypoxia-induced autophagy. (A) The protein expression levels of TRPC6 in podocytes incubated with hypoxia treatment for 24 h, 48 h or 72 h. The expression levels were normalized to β-actin. *P<0.05 and **P<0.01, compared with the normoxia. n=5 (B) Confocal microscopy using Fluo-3/AM fluorescent dye was used to observe changes in the Ca^{2+} concentration. Top panel: The concentration of extracellular Ca^{2+} was 1.8 mM. The subsequent addition of OAG (100 μM) triggered an increase in $[Ca^{2+}]_i$. OAG-induced Ca^{2+} influx was enhanced in podocytes incubated in hypoxia treatment for 48 h compared with normoxia. Bottom panel: Summary data of the same experiments. n=4. (C-D) Western blot analysis of LC3-II, Beclin 1 and p62 in cells incubated with 10 μM BAPTA-AM (an intracellular Ca^{2+} chelator) or 5 mM EGTA (an extracellular Ca^{2+} chelator) before the hypoxia treatment for 48 h. *P<0.05, compared with the normoxia podocytes; $^{+}$P<0.05 and $^{++}$P<0.01, compared with the cells treated with hypoxia. n=3.

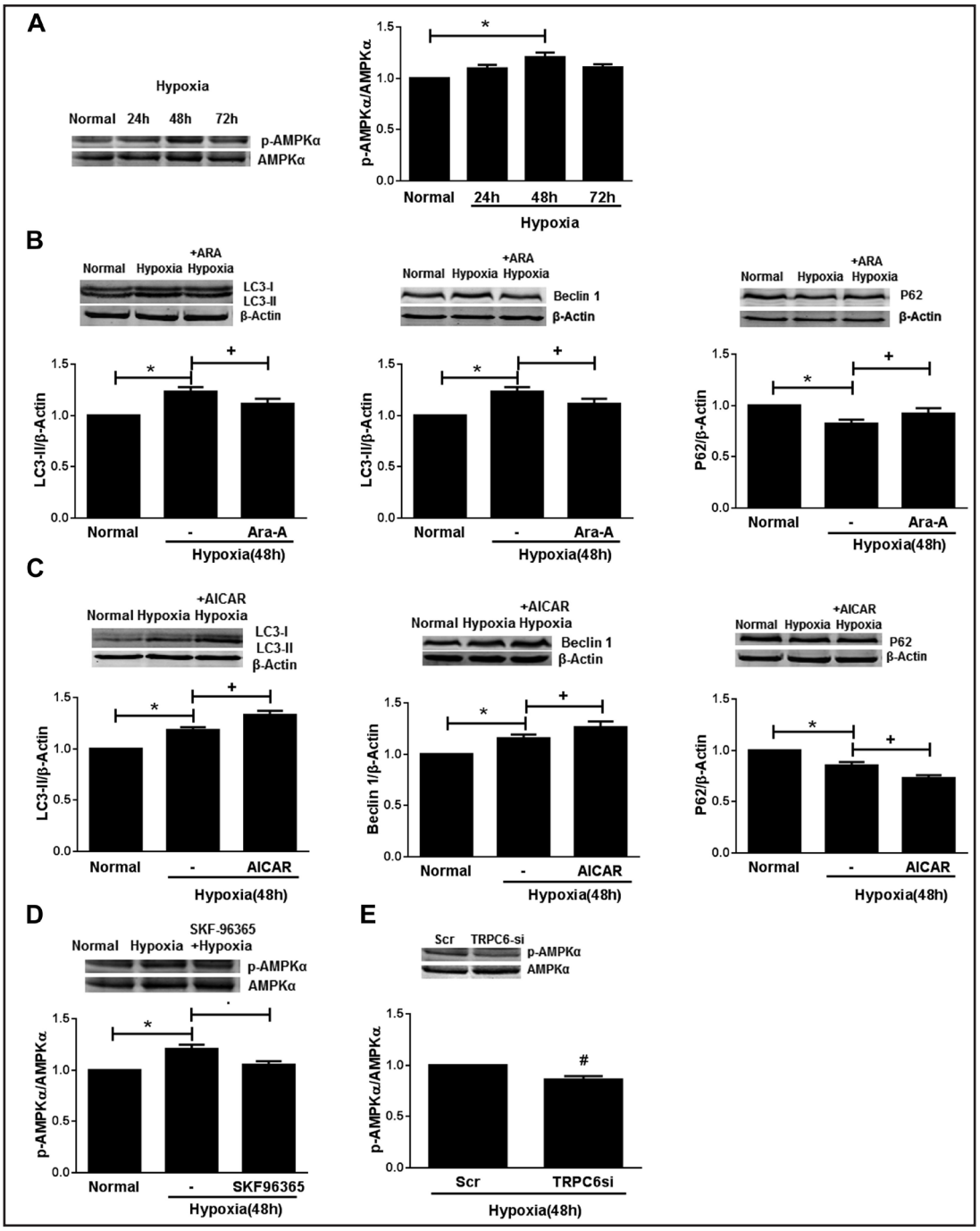

Fig. 5. AMPK involved with TRPC6 activation enhanced autophagy under hypoxia. (A) The protein expression levels of $AMPK_{\alpha}$ phosphorylation in podocytes incubated with hypoxia treatment for 24 h, 48 h or 72 h. The expression levels were normalized to β-actin. *P<0.05, compared with normoxia; n=4 (B-C) Western blot analysis of LC3-II, Beclin 1 and p62 in podocytes incubated with 1 mM adenine 9-β-d-arabinofuranoside (Ara-A, a known inhibitor of AMPK) or 1 mM 5-aminoimidazole-4-carboxamide-1-β-d-ribonucleoside (AICAR, a known agonist of AMPK) before the hypoxia treatment for 48 h. *P<0.05, compared with the normoxia cells; $^{+}$P<0.05, compared with the cells treated with hypoxia. n=3 (D) Western blot analysis of $AMPK_{\alpha}$ phosphorylation in podocytes incubated with 10 μM SKF-96365 before the hypoxia treatment for 48 h. *P<0.05, compared with the normoxia cells; $^{+}$P<0.05, compared with the cells treated with hypoxia. n=3 (E) TRPC6 siRNA transfection inhibited protein expression levels and $AMPK_{\alpha}$ phosphorylation after 48 hour of hypoxia. ($^{\#}$p<0.05 vs. Scr, n = 3).

Ca^{2+} signaling is involved in hypoxia-induced autophagy

Previously, we demonstrated that hypoxia can induce TRPC6-denpendent Ca^{2+} entry and TRPC6 expression in human podocytes [20]. To confirm this idea, we treated podocytes with 1% O_2 and measured the changes in TRPC6 expression and Ca^{2+} influx. Consistent with our previous findings [20], treatment of podocytes with 1% O_2 for 48 h significantly increased TRPC6 expression (Fig. 4A). As previously described [20], TRPC6-dependent Ca^{2+} entry was evaluated by measurement of Ca^{2+} influx induced by OAG, a membrane-permeable DAG analogue. As shown in Fig. 4B, hypoxia increased OAG-sensitive Ca^{2+} influx, in agreement with our previous study [20]. These data, along with our previous findings, demonstrated that hypoxia enhances TRPC6 expression and function. Next, we examined whether Ca^{2+} signaling plays an important role in hypoxia-induced autophagy. As shown in Fig. 4C, the increases in LC3-II and Beclin 1 levels were abolished by treating cells with BAPTA-AM (10 μM), an intracellular Ca^{2+} chelator. Alternatively, addition of EGTA (5 mM), an extracellular Ca^{2+} chelator, also inhibited the increase in LC3-II and Beclin 1 levels induced by hypoxia (Fig. 4D). Taken together, these data suggest that TRPC6-dependent Ca^{2+} influx is required for hypoxia-induced autophagy.

The involvement of TRPC6 in hypoxia-induced autophagy is associated with AMPK activation

To evaluate the role of AMPK in hypoxia-induced autophagy, phosphorylation of AMPKα was assessed by western blotting. As shown in Fig. 5A, treatment of podocytes with 1% O_2 significantly increased the relative levels of AMPKα phosphorylation (p-AMPKα) in a time-dependent manner compared with normoxia. Under hypoxic conditions, treatment of cells with 1 mM Ara-A, a selective AMPK inhibitor, led to a reduction in the expression of LC3-II and Beclin 1 along with an increase in p62 expression (Fig. 5B). By contrast, treatment with 1 mM AICAR, a selective AMPK activator, produced increases in LC3-II and Beclin 1 expression along with a reduction in p62 expression (Fig. 5C). These data suggest that AMPK contributes to autophagy that is induced by hypoxia. To reveal the possible mechanism by which TRPC6 is involved in hypoxia-induced autophagy, we observed whether TRPC6 affects AMPK activation under hypoxia. As shown in Fig. 5D, treatment of cells with SKF-96365 (10 μM), a TRPC6 blocker, under hypoxia for 48 h attenuated the increase in the p-AMPKα expression levels. Similarly, transfection with TRPC6 siRNA significantly reduced the expression levels of p-AMPKα compared with the expression levels in cells transfected with scramble siRNA (Fig. 5E). Transfection with scramble siRNA did not affect p-AMPKα expression (data not shown). These results suggest that TRPC6 is involved in hypoxia-induced autophagy via the AMPK signal pathway.

Discussion

To the best of our knowledge, the present study is the first to establish a link between TRPC6 and autophagy. In this study, reduction of TRPC6 activity, using either TRPC6 siRNA or a TRPC6 blocker, led to inhibition of hypoxia-induced autophagy, while enhancement of TRPC6 activity with a TRPC6 activator (FFA) resulted in increased hypoxia-induced autophagy. In addition, we showed that hypoxia induces increases in TRPC6 expression and Ca^{2+} influx, consistent with our previous findings [20]. Moreover, Ca^{2+} signaling is essential for hypoxia-induced autophagy since autophagy was abolished by either intracellular or extracellular Ca^{2+} chelators. Furthermore, we found that hypoxia induced an elevation in AMPK levels, which was attenuated by either pharmacological blockage of TRPC6 or knock down of TRPC6 by siRNA interference. These results suggest that the mechanisms by which TRPC6 regulates hypoxia-induced autophagy may be associated with the AMPK signaling pathway.

Intracellular Ca^{2+} signaling plays an important role in the regulation of autophagy [2, 3]. Previous studies have mostly focused on Ca^{2+} release channels, such as IP_3R [2, 3]. Changes in

intracellular Ca^{2+} signaling can result from Ca^{2+} influx through plasma membranes as well as Ca^{2+} release. The Ca^{2+} ionophore, ionomycin, which is used to promote Ca^{2+} release in previous autophagy studies [6-9, 12], can also mobilize extracellular Ca^{2+} influx. Exogenously induced Ca^{2+} influx in the form of calcium phosphate induces autophagy [10]. These studies indicated that Ca^{2+} influx, as well as Ca^{2+} release, are involved in the regulation of autophagy. Indeed, several studies have demonstrated the involvement of plasma membrane Ca^{2+} channels in autophagy, including voltage-dependent Ca^{2+} channels [11, 12], TRPC1 channels [16] and TRPC4 channels [17]. The present study supports this hypothesis and identifies a novel Ca^{2+} influx pathway involving TRPC6, which is implicated in hypoxia-induced autophagy. Our results are further supported by the recent preliminary observation that silencing TRPC6 by siRNA stabilized angiotensin II-induced autophagy [26]. The role of Ca^{2+} influx in the regulation of autophagy remains elusive. It is well known that autophagy starts with phagophore formation. Several compartments, including the endoplasmic reticulum (ER), mitochondria, Golgi complex and plasma membrane, appear to contribute proteins and lipids to phagophores, though the ER seems be a preferential location [3]. Thus, Ca^{2+} influx from the plasma membrane may be involved in the formation of phagophores originating in the plasma membrane.

Ca^{2+} signaling has been reported to have both inhibitory and stimulatory effects on autophagy [2, 3]. The dual effects of Ca^{2+} signaling seem to depend on the context of autophagy (basal *versus* stress-induced autophagy) [2, 3]. The inhibitory effect of Ca^{2+} released through IP_3R on autophagy is only relevant under basal conditions, but not under starvation [27]. IP_3R knockout results in an increase in basal autophagy [13]. Under basal conditions, Ca^{2+}, which is constitutively released through IP_3R in response to the basal levels of IP_3, is taken up by mitochondria to stimulate the production of ATP. Thus, blocking this signaling pathway reduces ATP production, resulting in activation of autophagy. When cells encounter stress conditions, autophagic stimulation induces a $[Ca^{2+}]_i$ increase, which in turn activates a variety of autophagy-stimulating proteins, thereby enhancing autophagy. Indeed, it has been reported that various types of physiological or pharmacological stresses, such as starvation [6, 16], hypoxia [16, 28], rapamycin [7] and resveratrol [17, 29], induce an increase in $[Ca^{2+}]_i$, which is stimulatory or even essential for autophagosome formation. Our study supports this hypothesis since hypoxia induces TRPC6-dependent Ca^{2+} influx, thereby promoting the induction of autophagy.

However, TRPC6 does not seem to be associated with basal autophagy given that under basal conditions, TRPC6 shows very low levels of constitutive activity in podocytes [19].

The molecular mechanisms by which Ca^{2+} signaling affects autophagy remain to be elucidated. AMPK has been considered to be an important signaling molecule in activating Ca^{2+}-dependent autophagy [1-3, 18]. Once AMPK is activated, it phosphorylates an initiator of autophagy, Unc 51-like kinase (ULK1). Additionally, ULK1 is also phosphorylated by the mammalian target of rapamycin complex 1 (mTORC1), which has an inhibitory effect on ULK1 phosphorylation, resulting in suppression of autophagy. AMPK can initiate autophagy by removing the inhibitory effect of mTORC1 on ULK1 since AMPK has been reported to suppress mTORC1 activity [1, 3, 18]. Therefore, AMPK initiates autophagy in a mTOR-dependent or -independent manner. However, there is evidence that Ca^{2+}-dependent induction of autophagy can also bypass AMPK. One study showed that nutrient starvation and pharmacological compound-modulated autophagy mobilized cellular Ca^{2+} to regulate the interaction of the WD-repeat protein with phosphoinositides (WIPI) via calmodulin-dependent kinase I (CaMKI) independent of AMPK [30]. Our study suggests that hypoxia induces autophagy via AMPK signaling in podocytes; however, whether mTORC1 is a downstream effector of AMPK requires further investigation. AMPK is activated allosterically by AMP and phosphorylation of Thr172 in the α-subunit [18]. The best-described AMPK kinase is the tumor suppressor liver kinase B1 (LKB1), which phosphorylates the α-subunit of AMPK in an AMP-dependent manner [18]. The second is calmodulin-mediated kinase kinase β (CaMKKβ). In contrast to LKB1, activation of AMPK by CaMKKβ does not require an elevation in AMP levels, but rather occurs in response to an increase in $[Ca^{2+}]_i$ [18, 31].

In that pathway, Ca^{2+} is bound by calmodulin, which activates CaMKKβ, leading to AMPK stimulation. The present study suggests that TRPC6-dependent Ca^{2+} influx mediates AMPK activation in response to hypoxia; however, the mechanisms of this mediation remain elusive. Several studies have shown that hypoxia triggers ROS production, which induces store-operated Ca^{2+} entry, leading to activation of AMPK through the CaMKKβ pathway [32, 33]. Our previous study showed that, in podocytes, hypoxia increased ROS production, which induced an increase in TRPC6 expression and TRPC6-dependent Ca^{2+} influx [20]. Therefore, similar mechanisms may be present in which TRPC6 mediates AMPK activation via the Ca^{2+}/calmodulin/ CaMKKβ pathway in a ROS-dependent manner in response to hypoxia; however, further research is needed.

Acknowledgements

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Disclosure Statement

The authors declare no conflict of interest.

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