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D2 Dopamine Receptor Antagonist Raclopride Induces Non-Canonical Autophagy in Cardiac Myocytes

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ABSTRACT

Cell death by autophagy is an important means of maintaining cellular homeostasis in adult cardiac myocytes. Autophagy was previously shown to exert a cardioprotective effect, suggesting that modulation of autophagy pathways is a potential therapeutic strategy in the treatment of heart disease. Although dopamine is known to induce autophagy in neuroblastoma cells, the underlying mechanism and the types of dopamine receptors involved in this process remain unclear. In this study, we used various dopamine receptor antagonists and agonists to identify the specific dopamine receptor that mediates induction of autophagy. We evaluated autophagy induction by the expression of autophagy markers in neonatal rat ventricular cardiac myocytes. We evaluated intracellular calcium levels using Fluo-3/AM and demonstrated autophagy-induced morphological changes in cardiac myocytes using electron microscopy. We also examined the pathway for dopamine-induced autophagy using RNAi-mediated gene knockdown. Raclopride, the well-documented D2 receptor antagonist, significantly upregulated autophagy in cardiac myocytes via an mTOR-independent pathway. There was no difference in intracellular calcium levels between raclopride-treated cells and untreated cells. siRNA-mediated knockdown of Rab9 resulted in decreased expression of autophagy markers in raclopride-treated cells. Interestingly, siRNA-mediated knockdown of Atg7 resulted in a significant increase in Rab9 levels in raclopride-treated cells, suggesting that blocking the classical autophagy pathway results in activation of an alternative pathway. Our study suggests that (1) the D2 dopamine receptor plays a role in autophagy and (2) raclopride mediated a non-canonical autophagy pathway in cardiac myocytes via Rab9. J. Cell. Biochem. 114: 103–110, 2013.

KEY WORDS: AUTOPHAGY; DOPAMINE; Rab9; CARDIAC MYOCYTES; RACLOPRIDE

Heart disease continues to be the leading cause of death in the industrialized world [Lloyd-Jones et al., 2010]. Adult cardiac myocytes that mediate the contraction of heart are terminally differentiated and cannot be fully replaced by cell proliferation [Chiong et al., 2011]. Catabolic and cell death pathways such as necrosis, apoptosis, and autophagy therefore play an important role in maintaining the cellular homeostasis of cardiac myocytes [Whelan et al., 2010].

Autophagy, primarily used for nutrient recycling and quality control, is a tightly regulated cellular process that requires the functions of multiple cellular proteins. During the process of autophagy, cellular organelles, long-lived proteins, and protein aggregates are enclosed in a double-membrane organelle, the autophagosome, and delivered to the lysosomes for degradation [Dong et al., 2010]. Proteins involved in autophagy (Atgs) are highly conserved from yeast to mammals, and interact with target of rapamycin (TOR) complex I (TORCI) and class III phosphatidylinositol-3 kinase to induce autophagy [Dong et al., 2010]. Atg5, Atg6/BECN1, Atg7, and Atg1/ULK1/2 play key roles in the formation of the autophagosome [Pattison et al., 2011], during which cytosolic LC3-I is converted to membrane-bound LC3-II [Hamacher-Brady et al., 2006b].

A significant component of autophagy, p62 is identified as a LC3-interacting protein [Bjorkoy et al., 2005; Komatsu et al., 2007; Pankiv et al., 2007]. It is a highly conserved protein and mediates a number of signaling pathways [Ichimura et al., 2008]. It is a major component of inclusion bodies and acts as a specific substrate, which is degraded via the autophagy-lysosomal pathway [Bjorkoy

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et al., 2005; Pankiv et al., 2007; Ichimura et al., 2008]. p62 interacts with ubiquitinated proteins via its C-terminal ubiquitin associated domain and self-oligomerizes via its N-terminus Phox and Bem-1 domain [Komatsu et al., 2007; Nezis et al., 2008]. p62 is found in ubiquitinated protein aggregates in a number of neurodegenerative diseases, suggesting its role in the protein degradation machinery [Pankiv et al., 2007].

The upregulation of autophagy has been shown to be cytoprotective in cell culture models of simulated ischemia-reperfusion [Yan et al., 2005; Hamacher-Brady et al., 2006ac; Gurusamy et al., 2010]. Indeed, induction of autophagy using agents such as glucosamine and mTOR inhibitors such as rapamycin has been suggested as a potential therapeutic strategy in a number of diseases [Sarkar et al., 2009; Shintani et al., 2010].

Growing evidence supports the existence of non-canonical autophagy pathways that are independent of Atg1/ULK1/2 [Cheong et al., 2011], LC3 [Nishida et al., 2009], Atg6/BECN1 [Zhu et al., 2007], or mTOR [Williams et al., 2008]. A number of Rab GTPases including Rab1, Rab9, Rab5, Rab24, and Rab7 have been shown to participate in non-canonical autophagy pathways [Nishida et al., 2009; Chua et al., 2011]. Calcium-mobilizing agents were previously shown to be potent inducers of autophagy, via inhibition of mTOR [Hoyer-Hansen et al., 2007], while calcium channel blockers mediated the induction of autophagy pathways in Huntington's disease and in the heart [Bahro and Pfeifer, 1987; Williams et al., 2008]. Interestingly, although calpain is thought to play a role in the conversion of LC3-I to LC3-II [Demarchi et al., 2006], inhibition of calpain 1 was also shown to induce autophagy in mammalian cells [Xia et al., 2010; Kuro et al., 2011], presumably via non-canonical authophagy pathways.

Dopamine (DA) is a potent cardiovascular neurotransmitter. The D1-like (D1 and D5 subtypes) and D2-like (D2, D3, D4) dopamine receptors play an important role in regulating cardiovascular hemodynamics [Cavallotti et al., 2002]. Dopamine-induced autophagy in neuroblastoma cells [Gimenez-Xavier et al., 2009], and dopamine receptor antagonists have been shown to play a role in mTOR-independent autophagy [Zhang et al., 2007]. However, the exact mechanisms underlying dopamine receptor antagonist-mediated activation of autophagy pathways and the identity of dopamine receptors that mediate this function remain unclear. In this study, we investigated the identity of dopamine receptor involved in autophagy activation. We also explored the mechanisms underlying induction of autophagy by raclopride, a well-studied D2 dopamine receptor antagonist.

METHODS

PREPARATION AND CULTURE OF CARDIAC MYOCYTES

This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996). The study was approved by the Second Affiliated Hospital of University of Nanchang's Institutional Animal Care and Use Committee.

Sprague-Dawley rats (1–2 days old) were obtained from the Animal Science Center, Nanchang University. Primary cultures of neonatal ventricular cardiac myocytes were prepared by enzymatic digestion of ventricle tissue with 0.25% trypsin (Invitrogen, USA). The cardiomyocytes were first plated for 2 h to reduce non-myocyte contamination. The cells were then washed to remove erythrocytes, replated at a density of 2.0×10^6 cells in culture flasks and incubated at 37°C in the presence of 5% CO_2 in a humidified incubator. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, St Louis, MI) containing antibiotics (streptomycin 100 μg/ml and penicillin 100 U/ml). Most primary cultures of ventricular cardiac myocytes contract spontaneously in a confluent monolayer 48–72 h after plating.

CARDIOMYOCYTE TREATMENT REGIMENS

Based on preliminary results (unpublished data), we treated 2×10^6 neonatal rat ventricular myocytes with 40 μM of SKF-38393, Bromocriptine, SCH2390, raclopride, 0.1 μM rapamycin (Sigma-Aldrich) for 24 h. For Bafilomycin A1 treatment, cells were incubated in the presence of 0.2 μM Bafilomycin A1 (Sigma-Aldrich) at 37°C for 2 h.

IMMUNOBLOTTING ANALYSES

For the purpose of immunoblot analysis, neonatal rat cardiomyocytes were lysed in RIPA buffer (Beyotime Institute of Biotechnology, China), and protein extracts were prepared according to the manufacturer's instructions. Protein concentrations were determined using the BCA protein assay (Beyotime Institute of Biotechnology). Equal protein amounts (40–60 μg) were loaded and separated in 8–15% sodium dodecyl sulfate polyacrylamide gradient gels and transferred to a polyvinylidene fluoride membranes. Membranes were blocked with 5% albumin bovine V (Sigma-Aldrich) at room temperature for 30 min and then incubated overnight at 4°C with an 1:500–1,000 dilution of rabbit anti-LC3 (Abcam, USA), Atg7, Atg5, p62 (Sigma-Aldrich), phospho-4E-BP1, phospho-p70S6K, Rab9 (Cell Signaling, USA), or rabbit anti-β-actin antibody (Santa Cruz Biotech, USA). Membranes were then washed extensively and incubated with a 1:5,000 dilution of HRP-conjugated anti-rabbit IgG (Sigma-Aldrich) for 1 h at room temperature. Protein was visualized using an ECL kit (Thermo, USA).

ULTRASTRUCTURAL ASSESSMENT OF CARDIAC MYOCYTES BY ELECTRON MICROSCOPY (EM)

Cardiac myocytes were incubated with the specified drug for 24 h and collected by centrifugation. The cells were fixed with 4% glutaraldehyde, post-fixed with 1% osmium tetroxide and immersed in 0.1 M of sodium cacodylate buffer (pH 7.3) containing 2.5% glutaraldehyde for 4 h. The cells were then rinsed with the cacodylate buffer alone, followed with post-fixation with 1% osmium tetroxide in cacodylate buffer for 1 h. The cells were embedded in epoxy resin and cut into 600-nm thick slices using an ultra-microtome. The slices were stained with uranyl acetate and lead citrate and evaluated by EM (Hitachi, H7500, Japan) at 75 kV.

EVALUATION OF ACIDIC VACUOLE (AVO) ACCUMULATION USING FLUORESCENCE MICROSCOPY

AVO accumulation was assessed as previously described [Malagoli et al., 2009]. We quantitated AVO accumulation using acridine

orange (AO). For AO staining, cells were cultured on slides in medium containing drugs as described. The cells were washed twice with PBS and stained for 15 min with medium containing 1 μg/ml AO (Sigma-Aldrich). The slides were examined using fluorescence microscopy using 490 nm band-pass blue excitation filters and a 515 nm long-pass barrier filter.

IMMUNOFLUORESCENCE ANALYSIS

Cardiac myocytes were incubated with the specified drug for 24 h and then fixed with 4.0% paraformaldehyde for 30 min followed by 10 min incubation with acetone. Cells were treated with 0.2% Triton X-100 for permeation then blocked in 2% albumin bovine V (Sigma-Aldrich) phosphate-buffered saline immunostaining blocking buffer for 1 h at room temperature. For dual labeling studies, the fixed cells were incubated overnight at 4°C with a 1:50 dilution of rabbit anti-LC3 antibody (Abcam) and mouse anti-α-actin antibody (Abcam). The cells were then washed and incubated for 1 h at room temperature with a 1:50 dilution of FITC-conjugated anti-rabbit IgG or TRITC-conjugated anti-mouse IgG (Jackson Lab, USA). Nuclei were stained with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI; Roche, USA). Cells were observed using confocal microscopy (Leica SP5, Mannheim, Germany).

MEASUREMENT OF INTRACELLULAR CALCIUM CONCENTRATIONS

Free intracellular calcium concentrations (Ca^{2+}) in myocardial cells were determined using the Fluo-3/AM probe (Sigma-Aldrich) as previously described [Li et al., 2009]. Neonatal rat cardiomyocytes were treated with the specified drugs for 30 min and then incubated for 40 min at 37°C with 5 μmol/l Fluo-3/AM in the presence of 5% CO_2 in a humidified incubator. The cells were washed three times with phosphate-buffered saline and further incubated for 20 min in DMEM. All procedures were performed in the dark.

Dynamic changes in (Ca^{2+}) in myocardial cells were measured by determining Fluo-3 fluorescence. The images were captured using the 40× oil immersion objective lens (488 nm excitation, 522 nm emission). The change in fluorescence intensity in the cells represented the change of intracellular Ca^{2+} concentration. The fluorescence intensity was observed in eight randomly chosen cells using laser scanning confocal microscopy (Leica SP5) and the average fluorescence intensity was calculated using LAS AF Lite (Leica SP5).

GENE SILENCING WITH siRNA-RAB7 AND siRNA-RAB9

We obtained Rab9 (Cat #: 1734883, 1734884, and 1734885), Atg7 (Cat #: 1609821, 1609822, and 1609823) and silencer AccuTarget™ control (Cat #: SN-1004) siRNAs from BioNEER (Daejeon, South Korea). Cells were plated in 35-mm tissue culture dishes at a density of 0.7×10^6 cells per dish and cultured for 24 h. The cells were then transfected with siRNA (180 pmol/dish), using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. Three days after transfection (48 h in siRNA followed by 24 h in drugs), cells were harvested and subjected to western blot analysis as described above. siRNA-transfected cells were also treated with the various dopamine agonists and antagonists as described above.

RESULTS

DOPAMINE ANTAGONIST RACLOPRIDE UPREGULATES THE EXPRESSION OF AUTOPHAGY MARKERS IN NEONATAL RAT VENTRICULAR MYOCYTES

We evaluated the expression of autophagy markers in neonatal rat ventricular myocytes treated for 24 h with 40 μM of dopamine D2 receptor antagonist, raclopride, D1 receptor antagonist, SCH-23390, D1 receptor agonists, SKF-38393, D2 receptor agonists, and bromocriptine. We also treated cells with 0.1 μM rapamycin, which is known as an inducer of autophagy. We used western blotting to evaluate the levels of p62, LC3, and downstream targets of mTOR such as P-4EBP1 and P-p70S6K (Fig. 1A). Immunoblotting data demonstrated that raclopride and rapamycin downregulated the levels of p62, while SKF-38393, bromocriptine and SCH-23390 caused accumulation of p62. We also showed no significant difference in the levels of P-4EBP1 and P-p70S6K in raclopride-treated cells compared with untreated cells. There was a significant conversion of LC3-I to LC3-II in raclopride- and rapamycin-treated cells. However, there was no difference in the ratio between LC3-I and LC3-II in cells treated with SKF-38393, bromocriptine (Fig. 1A). We used AO staining to show the accumulation of acidic lysosome and showed when compared to DMSO treatment (Fig. 1B), the addition of either rapamycin (Fig. 1C) or raclopride (Fig. 1D) increases the amount of acidic lysosomes in the cytosol in the primary neonatal ventricular cardiomyocytes.

EFFECT OF DOPAMINE ANTAGONISTS AND AGONISTS ON INTRACELLULAR CALCIUM CONCENTRATIONS IN NEONATAL VENTRICULAR MYOCYTES

We evaluated the effect of raclopride, SCH-23390, bromocriptine, and SKF-38393 on the regulation of intracellular calcium homeostasis. We measured Fluo-3 fluorescence levels to evaluate intracellular calcium levels in treated cells (Fig. 2A–D) and showed a significant upregulation of Ca^{2+} in cells treated with dopamine receptor agonists, SKF-38393, and bromocriptine, compared with untreated cells (Fig. 2E). However, there was a modest decrease of Ca^{2+} in raclopride-treated cells, and only a modest increase in Ca^{2+} levels in SCH-23390-treated cells (Fig. 2E).

CHARACTERIZATION OF MORPHOLOGICAL CHANGES DURING RACLOPRIDE-INDUCED AUTOPHAGY BY EM

We used EM to evaluate whether raclopride treatment induced the formation of autophagosomes in cardiac myocytes. Compared to control cardiac myocytes (Fig. 3A), the rapamycin-treated (Fig. 3B) and raclopride-treated cardiac myocytes (Fig. 3C) showed the formation of autophagosomes containing heterogeneous organelles (ranging from mitochondria to multivesicular bodies). After quantification, raclopride-treated cells had a significantly higher number of autophagosomes per field compared with untreated cells and the level was comparable to that of rapamycin-treated cells (Fig. 3D).

RACLOPRIDE INDUCES AUTOPHAGY FLUX IN CARDIAC MYOCYTES

We used Bafilomycin A1 treatment to investigate if the accumulation of LC3-II was the result of inhibition of lysosomal fusion or due

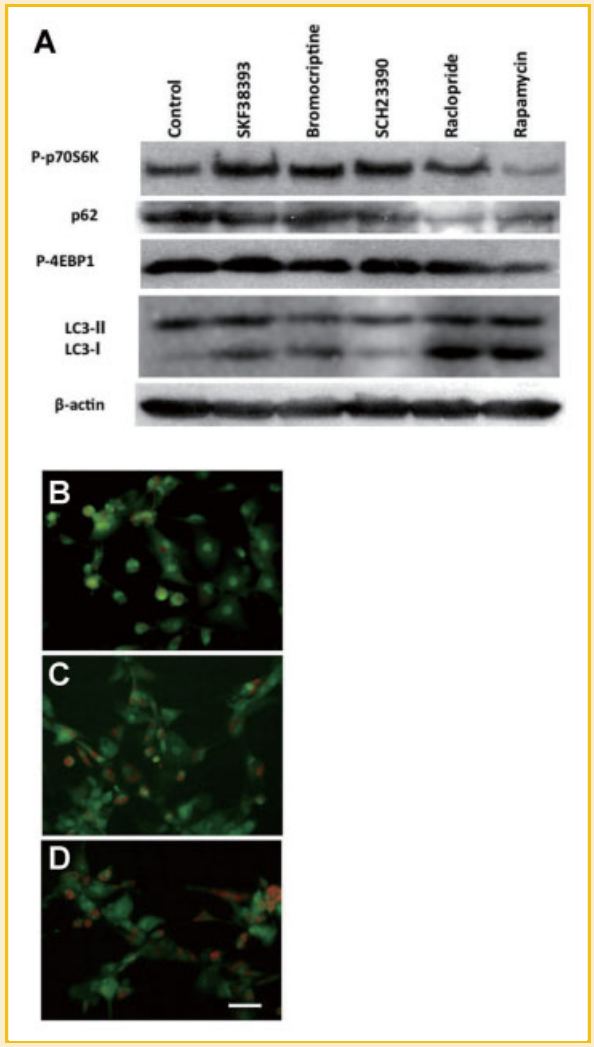

Fig. 1. A: Examination of autophagy markers expression in neonatal cardiac myocytes treated with dopamine receptor modulators. Neonatal rat cardiac myocytes were incubated with 40 μM of dopamine receptor D1 antagonists (SCH-23390), D1 agonist (SKF-38393), D2 agonists (bromocriptine), and D2 antagonist (raclopride) for 24 h or 0.1 μM rapamycin for 24 h. Conversion of LC3-I to LC3-II and p62 degradation was analyzed by immunoblotting. Phospho-4EBP1 and phospho-70S6K levels were also analyzed by immunoblotting. β-actin expression level was used as the loading control. Lane 1, control; lane 2, SKF-38393; lane 3, bromocriptine; lane 4, SCH-23390; lane 5, raclopride; and lane 6, rapamycin. Cultured neonatal cardiomyocytes were first treated with (B) DMSO for 18 h; (C) 0.1 μM rapamycin for 18 h; and (D) 40 μM raclopride for 18 h. After treatment, cells were washed twice with PBS, stained with medium containing 1 μg/ml AO for 15 min, and examined immediately by fluorescence microscopy. Scale bar = 20 μm.

to increased autophagy. We evaluated the accumulation of raclopride-induced autophagosomes in the presence of bafilomycin A1. Bafilomycin A1 has been reported to inhibit autophagosome-lysosome fusion and lysosomal activity by raising lysosomal pH. By inhibiting the autophagy flux in neonatal rat ventricular myocytes, the amount of autophagosomes should be increased. We used immunoblotting to show increased LC3-II levels in cells treated with raclopride for either 8 or 16 h compared with control cells. LC3-II levels increased further after treating the cardiac myocytes with 0.2 μM bafilomycin A1 for 2 h (Fig. 4A). We also examined the increase of autophagosome formation by confocal microscopy, using an anti-LC3 antibody to determine autophagosome formation in primary cardiac myocytes (Fig. 4B). We showed a significant increase in autophagosome formation in raclopride-treated cardiac myocytes in the presence of bafilomycin A1.

Rab9 siRNA AND Atg7 siRNA INHIBIT RACLOPRIDE-INDUCED AUTOPHAGY IN CARDIAC MYOCYTES

Previous results showed that raclopride-induced autophagy was independent of mTOR activity, suggesting raclopride may trigger autophagy through a non-canonical pathway. To test this hypothesis, we tested whether a known, Rab9-mediated non-canonical pathway played a role in raclopride-induced autophagy. First, we tested the expression of autophagy markers in a 24-h window after raclopride treatment. Our data showed that cardiac myocytes treated with raclopride exhibited a time dependent (1) inhibition of p62 levels, (2) conversion of LC3-I to LC3-II between 12 and 24 h, (3) an increase of Rab9 protein expression at the 12 h time point, and (4) an increase of Atg5-Atg12 complex level at the 18 h time point (Fig. 5). There was no change in Atg7 protein levels through the 24-h window. The results suggested the involvement of Rab9 in the induction of autophagy by the raclopride treatment.

To test if raclopride induced the non-canonical autophagy pathway, we used siRNA to downregulate Atg7 or Rab9 in neonatal rat ventricular myocytes. First, we tested the efficiency of siRNAs in silencing the expression of target proteins by transient transfections. We showed that siRNA Atg7-c and siRNA Rab9-b downregulated the protein levels of Atg7 and Rab9 most efficiently (Fig. 6A, B). We therefore used these two siRNAs in subsequent experiments. We treated siRNA-transfected cardiac myocytes with raclopride as described and showed that downregulation of Atg7 inhibited rapamycin-mediated conversion of LC3-I to LC3-II as well as p62 clearance (Fig. 6C). Interestingly, downregulation of Atg7 in raclopride-treated cells had no effect on either LC3-I to LC3-II conversion or p62 clearance. But the downregulation of Rab9 caused a reduction in p62 clearance and the conversion of LC3-I to LC3-II (Fig. 6C). These results suggested that raclopride-induced autophagy is mediated via a non-canonical pathway that required Rab9.

DISCUSSION

The mechanism of dopamine function in the cardiovascular system is rather complicated. Cardiac dopamine D2 receptors are thought to play a role in cardiac aging [Cavallotti et al., 2002; Gomez Mde et al., 2002]. The dopamine receptor agonist, bromocriptine, may be involved in cardiac hypertrophy and in the aggravation of heart valve diseases [Stephens et al., 2007; Li et al., 2009; Tan et al., 2009]. In this study, we evaluated the role of dopamine receptor antagonists (raclopride and SCH-23390) and agonists (bromocriptine and SKF-38393) on induction of autophagy in neonatal rat ventricular myocytes.

A number of studies previously showed the correlation between the dopamine D2 receptor and neural pathology [Tinsley et al.,

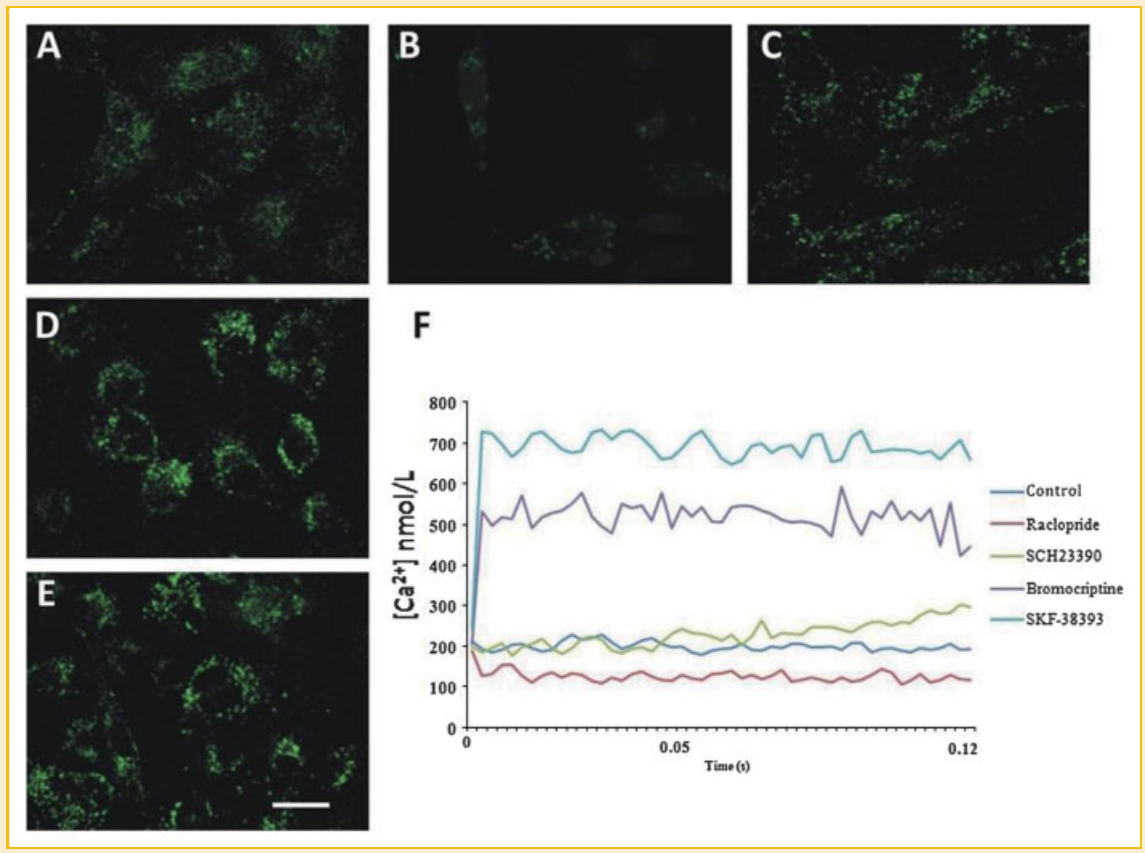

Fig. 2. Effect of dopamine antagonists and agonists on intracellular calcium concentrations (Ca^{2+}) in neonatal ventricular myocytes. A: The change of (Ca^{2+}) levels as shown by Fluo-3 fluorescence were recorded under confocal microscopy of cells treated with (A) control medium, (B) 40 μM raclopride, (C) 40 μM SCH-23390, (D) 40 μM bromocriptine, and (E) 40 μM SKF-38393. F: Dynamic changes in (Ca^{2+}) in myocardial cells were measured 1 h after stimulation in the different treatment groups. Scale bar = 5 μm.

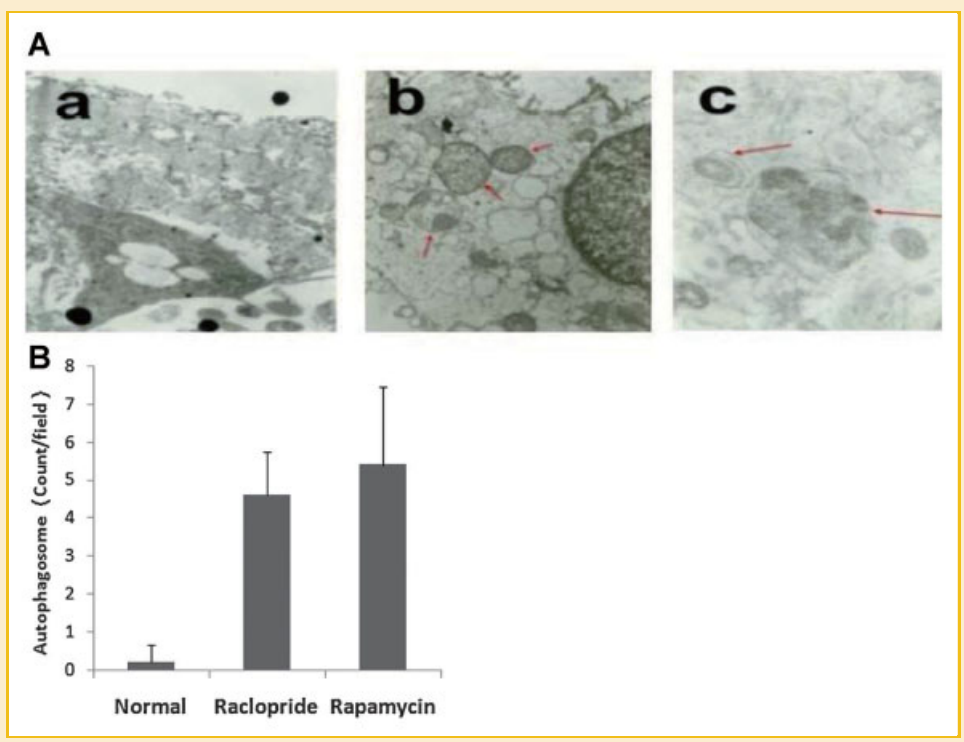

Fig. 3. Representative electron micrograph images of raclopride-induced autophagy in cardiac myocytes. Neonatal rat cardiac myocytes were cultured in (A) untreated medium, (B) 0.1 μM rapamycin for 24 h or (C) 40 μM raclopride for 24 h and then processed for observation under transmission electron microscope (×25,000). D: Quantification of autophagosomes in a single field in three different treatment groups.

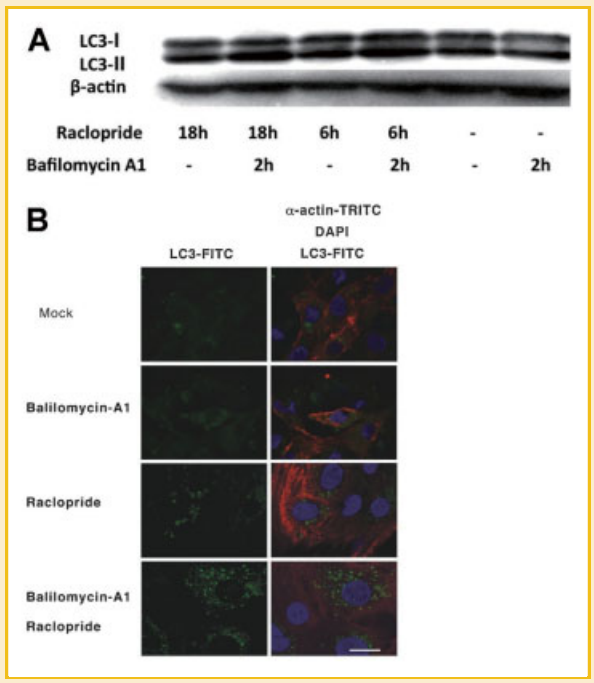

Fig. 4. Raclopride-induced autophagy flux was examined by immunoblotting and immunofluorescence. Cardiac myocytes were cultured in CM or raclopride (40 μM) in presence or absence of Bafilomycin A1 (0.2 μM). A: Cardiac myocytes were cultured in CM or raclopride (40 μM) for 0, 6, or 18 h in presence or absence of Bafilomycin A1 (0.2 μM) for 2 h by Western blotting. B: Cardiac myocytes were cultured in CM or raclopride (40 μM) for 24 h in presence or absence of Bafilomycin A1 (0.2 μM) for 2 h. Expression of endogenous LC3 and α-actin was detected using FITC-conjugated or TRITC-conjugated antibody, respectively. Cell nuclei were shown by DAPI (4′,6-diamidino-2-phenylindole) staining. Scale bar = 5 μm.

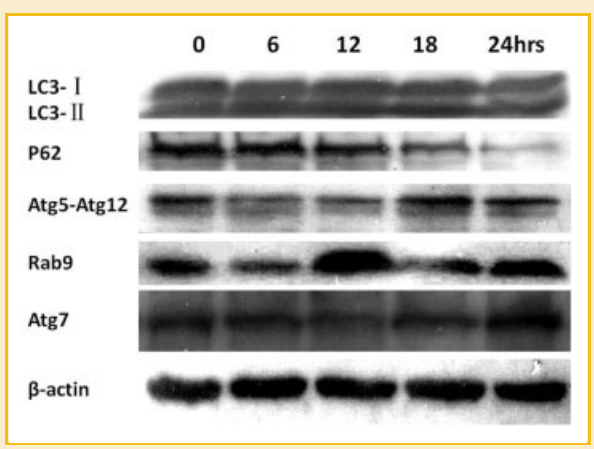

Fig. 5. Time course of autophagy induction by raclopride. Cardiac myocytes were incubated with 40 μM raclopride for 0, 6, 12, 18, or 24 h and harvested for immunoblotting. Conversion of LC3-I to LC3-II, p62 clearance and expression levels of Atg7, Rab9, and Atg5–Atg12 complex were analyzed by immunoblotting. β-actin expression level was used as the loading control.

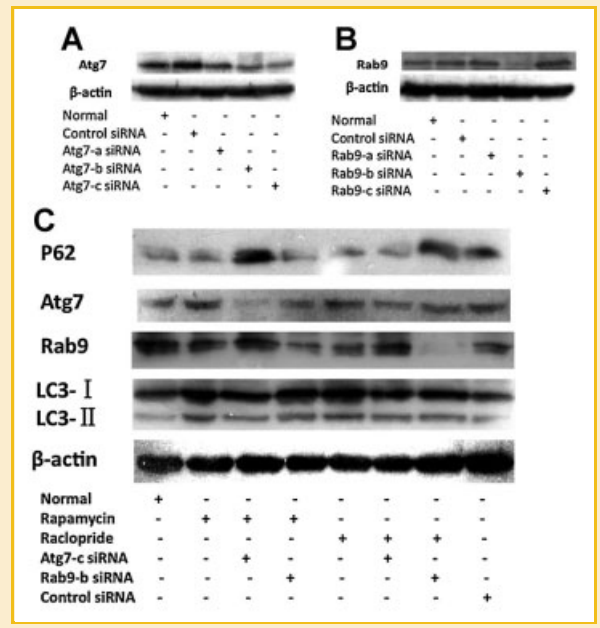

Fig. 6. Identifying the pathway of raclopride-mediated autophagy. Cardiac myocytes transfected with indicated siRNAs targeting (A) Atg7 or (B) Rab9, incubated for 48 h and harvested for the examination of siRNA efficacy using immunoblotting. C: Cardiac myocytes transfected for 48 h with the indicated siRNA sequences were treated with 40 μM raclopride and 0.1 μM rapamycin for another 24 h and harvested for immunoblotting. Conversion of LC3-I to LC3-II, p62 clearance, and expression of Atg7 and Rab9 were analyzed by immunoblotting. β-actin expression level was used as the loading control.

2009], cardiovascular aging [Cavallotti et al., 2002], and cardiomyocyte hypertrophy [Li et al., 2009]. Cardiomyocytes were also protected against hypoxia by a dopamine receptor antagonist [Xiao et al., 2011]. We used dopamine receptor antagonist, raclopride, because the specific inhibitory effects of raclopride towards the dopamine D2 receptor have been extensively documented. We showed a significant conversion of LC3-I to LC3-II in raclopride-treated cells, suggesting induction of autophagy. We also showed that, while there was a significant upregulation of Ca^{2+} in cells treated with dopamine agonists, SKF-38393, and bromocriptine, compared to untreated cells, there was no significant difference in raclopride- and SCH-23390 treated cells. We dissected the molecular mechanisms underlying raclopride-induced autophagy and showed that raclopride induced a Rab9-mediated non-canonical autophagy pathway in neonatal rat ventricular myocytes.

The cardioprotective effects of autophagy in the heart suggest that modulation of autophagy pathways may be a potential therapeutic strategy in the treatment of heart disease. Cardiac-specific knockdown of Atg5 was recently shown to result in cardiomyopathy in adult mice [Nakai et al., 2007]. The canonical autophagy pathway is mediated via a number of key molecules including Atg proteins, p62 and LC3. P62, a molecule with binding sites for ubiquitin as well as LC3, has been identified as bridging molecule that links ubiquitin-linked protein aggregates and LC3-decorated autophagosomes during autophagy [Bjorkoy et al., 2005; Moscat and Diaz-Meco, 2009]. However, a number of dopamine

receptor blockers have been recently shown to induce non-canonical autophagy pathways in nerve cells [Zhang et al., 2007]. Non-canonical autophagy pathways are of particular relevance in cardiac myocytes [Dong et al., 2010]. Our data demonstrated no significant difference in the levels of mTOR downstream targets, p70S6K and 4EBP1 phosphorylation in raclopride-treated cells compared to control cells, suggesting that raclopride-induced autophagy in cardiac myocytes occurs via a non-canonical, mTOR-independent pathway. Our data showing downregulation of p62 in raclopride-treated cells suggested that raclopride-induced macroautophagy, since p62 degradation is a characteristic of macroautophagy. Increased levels of LC3-II in raclopride-treated cells which were exposed to bafilomycin A1 also suggested raclopride enhance autophagy flux.

We showed that the upregulation of autophagy markers in cardiac myocytes in raclopride-treated cells was accompanied by decreased levels of intracellular calcium. Calcium signaling pathways have been shown to play an important role in a number of physiological processes including starvation-induced autophagy [Decuypere et al., 2011]. Inositol 1,4,5-triphosphate receptors release Ca^{2+} from the ER to the cytosol in response to IP3. Inhibition of IP3R or the presence of calcium-chelating agents resulted in stimulation of autophagy via activation of AMP-activated protein kinase under conditions of starvation [Decuypere et al., 2011]. Upregulation of intracellular calcium by thapsigargin, ionomycin, ATP, and vitamin D3 have been reported to induce autophagy via mTOR inhibition in non-starved cells [Hoyer-Hansen et al., 2007]. However, recent studies suggested that thapsigargin-mediated increase in calcium concentrations stimulated the endoplasmic reticulum and inhibited the degradation of the phagosome–lysosome complex and the binding of Rab9 with LC3, thereby inhibiting autophagy [Ganley et al., 2011]. These results were consistent with our data, which showed upregulation of Rab9 expression in raclopride-treated cells, suggesting a role for intracellular calcium transport in autophagy.

Interestingly, inhibition of calpain 1 and calcium flux were also shown to induce autophagy in non-starved cells, via inhibition of Atg5 cleavage [Norman et al., 2010; Xia et al., 2010]. These results were consistent with our data, which showed high levels of the myocardial Atg5–Atg12 complex in raclopride-treated cells. It will be interesting to dissect the mechanism underlying the more potent induction of autophagy by the D2 receptor antagonist, raclopride, compared to the D1 receptor antagonist, SCH23390, which we observed in this study.

Mouse cells deficient in Atg5 and Atg7 were shown to be capable of forming autophagosomes in a Rab9-dependent manner [Nishida et al., 2009]. In this study, we showed an upregulation in myocardial Rab9 expression in raclopride-treated cells. Interestingly, siRNA-mediated knockdown of Atg7 resulted in a significant increase in the expression of Rab9, suggesting that blocking the classical autophagy pathway results in activation of non-canonical autophagy pathway. This observation is supported by the previous finding that knockout of Atg7, which plays a role in the classical autophagy pathway, resulted in a significant increase in the expression of Rab9 [Zafar et al., 2011]. It will be interesting to investigate if intracellular Ca^{2+} levels affect the switch from the classical to the alternate pathway. Our future goals also include an investigation of upstream activators of Rab9.

The limitation of this current study lies in the fact that (1) autophagy has been shown to be susceptible to the external environment and (2) the primary neonatal rat cardiomyocytes used in this study differ from adult cardiomyocytes.

To the best of our knowledge, this is the first report describing the role of the D2 dopamine receptor on autophagy. The significant role of autophagy in cardio-protection makes it important to further dissect mechanisms for dopamine-induced autophagic pathways in cardiac myocytes.

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