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Dopamine D_5 receptor-mediated decreases in mitochondrial reactive oxygen species production are cAMP and autophagy dependent

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Abstract

Overproduction of reactive oxygen species (ROS) plays an important role in the pathogenesis of hypertension. The dopamine D_5 receptor (D_5R) is known to decrease ROS production, but the mechanism is not completely understood. In HEK293 cells overexpressing D_5R, fenoldopam, an agonist of the two D_1-like receptors, D_1R and D_5R, decreased the production of mitochondria-derived ROS (mito-ROS). The fenoldopam-mediated decrease in mito-ROS production was mimicked by Sp-cAMPS but blocked by Rp-cAMPS. In human renal proximal tubule cells with *DRD1* gene silencing to eliminate the confounding effect of D_1R, fenoldopam still decreased mito-ROS production. By contrast, Sch23390, a D_1R and D_5R antagonist, increased mito-ROS production in the absence of D_1R, D_5R is constitutively active. The fenoldopam-mediated inhibition of mito-ROS production may have been related to autophagy because fenoldopam increased the expression of the autophagy hallmark proteins, autophagy protein 5 (ATG5), and the microtubule-associated protein 1 light chain (LC)3-II. In the presence of chloroquine or spautin-1, inhibitors of autophagy, fenoldopam further increased ATG5 and LC3-II expression, indicating an important role of D_5R in the positive regulation of autophagy. However, when autophagy was inhibited, fenoldopam was unable to inhibit ROS production. Indeed, the levels of these autophagy hallmark proteins were decreased in the kidney cortices of $Drd5^{-/-}$ mice. Moreover, ROS production was increased in mitochondria isolated from the kidney cortices of $Drd5^{-/-}$ mice, relative to $Drd5^{+/+}$ littermates. In conclusion, D_5R-mediated activation of autophagy plays a role in the D_5R-mediated inhibition of mito-ROS production in the kidneys.

Keywords

Autophagy; Dopamine D_5 receptor; Hypertension; Mitochondria; Reactive oxygen species

Introduction

Reactive oxygen species (ROS) are a family of chemically derived reactive molecules from oxygen [1–3]. In addition to their established role in killing invading microorganisms, ROS, via direct oxidative damage or activation of cellular signaling pathways, are implicated in the pathogenesis of a wide variety of disorders, including hypertension [1–3]. There are many sources of ROS, e.g., NADPH oxidase [1–3], and mitochondria-derived ROS (mito-ROS) play an important role in the pathophysiology of hypertension [1–4].

Dopamine, produced in the kidney and via activation of dopamine receptors, regulates sodium balance and blood pressure [5–7]. Dopamine receptors are classified into D_1-like (D_1R and D_5R) receptors that stimulate adenylyl cyclases and D_2-like (D_2R, D_3R, and D_4R) receptors that inhibit adenylyl cyclases [5–7]. Abnormalities in renal dopamine production and D_1-like and D_2-like receptors are associated with hypertension and/or salt sensitivity in several ethnic groups [5–7]. The loci of human D_5R (4p15.1–16.1) and its pseudogenes (1q21.1 and 2p11.1–p11.2) are linked to human hypertension [8, 9]. Germline deletion of the *Drd5* gene ($Drd5^{-/-}$) in mice causes hypertension [10]. However, the mechanisms responsible for the increased blood pressure in $Drd5^{-/-}$ mice are not completely understood.

In addition to genetic factors, many cellular factors, including ROS, are involved in the pathogenesis and maintenance of hypertension [1–4]. We have reported that ROS production in the kidneys and brain is greater in *Drd5*$^{-/-}$ mice than in their *Drd5*$^{+/+}$ littermates and that D_5R exerts its anti-oxidant properties in part by inhibiting NADPH oxidase activity [10]. NADPH oxidase is an important source of ROS production in renal tubule cells [1–3, 10–12], and the results of experiments inhibiting this enzyme complex with specific or nonspecific inhibitors support its importance in the regulation of blood pressure [1–3, 12, 13]. Recently, mitochondria-generated ROS have been reported to contribute to oxidative stress-mediated hypertension [1–4], but the mechanism is not known.

There is accumulating evidence linking autophagy and hypertension in response to oxidative stress [14]. Autophagy, an intracellular self-degradative process that delivers cytoplasmic constituents to lysosomes, has long been considered a cellular physiological process involved in the turnover of subcellular organelles and proteins [15, 16]. However, recent studies have indicated that autophagy plays important roles in the pathogeneses of human diseases, including those caused by oxidative stress [17]. Furthermore, autophagy can mediate inflammatory responses that induce oxidative stress [18, 19]. Oxidative stress increases the activity of both autophagosomes and auto-lysosomes; inhibition of autophagy attenuates autophagic flux [14], but autophagy protects against oxidative injury [20]. Moreover, autophagy is important in the removal of damaged mitochondria and polyubiquitinated proteins and in protection from damage caused by oxidative stress [14, 17]. D_5R can regulate the expression and activity of proteins that increase oxidative stress, for example, by triggering the ubiquitination and subsequent degradation of angiotensin II (Ang II) type 1 receptor (AT_1R) [10, 21–23]. However, investigations linking D_5R to autophagy and mitochondrial oxidative stress are sparse.

We investigated the potential role(s) of D_5R in mito-ROS production by determining whether the D_5R-mediated inhibition of renal cellular ROS production is caused in part by a decrease in mito-ROS production through an increase in autophagy.

Methods

Reagents

MitoTracker Green, MitoSOX Red (Mito-HE), and Amplex Red were purchased from Molecular Probes (Eugene, OR), and anti-autophagy protein 5 (ATG5) (Cat. No. 12994) and anti-LC3-II (Cat. No. 3868) antibodies were purchased from Cell Signaling Technology (Danvers, MA). The origins of the anti-D_1R and anti-D_5R antibodies in renal proximal tubule (RPT) cells and rat kidneys have been reported previously, and the antibodies have been verified in $D_1R^{-/-}$ and $D_5R^{-/-}$ mice [24–28]. Anti-Bax and anti-Bcl-xL antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); anti-Mn-SOD and anti-Cu/Zn-SOD antibodies were purchased from Abcam (Cambridge, MA); an anti-prohibitin antibody was purchased from GeneTex (Irvine, CA); and anti-calnexin, anti-histone B4, and anti-GM130 antibodies were purchased from BD Transduction Laboratories (Lexington, KY). Culture media, fetal bovine serum (FBS), glutamine, and Lipofectamine transfection reagent were purchased from Invitrogen (Gaithersburg, MD). Percoll was purchased from GE Healthcare (Piscataway, NJ). NADPH was purchased from MP Biomedicals (Solon,

OH). An adenosine triphosphate (ATP) bioluminescence assay kit was purchased from Roche Applied Science (Indianapolis, IN). Spautin-1 was purchased from Griffin Biotech (Hong Kong, China). Fenoldopam, Sch23390, Rp-cAMPS, Sp-cAMPS, chloroquine, antimycin A, and other reagents were purchased from Sigma (St. Louis, MO).

Cell culture, siRNA, and transfection

Human embryonic kidney 293 (HEK293) cells expressing D_5R (D_5R-HEK293) and human RPT cells were cultured as previously described [23, 25]. Empty vector (EV)-transfected HEK293 and D_5R expression vector-transfected HEK293 cells (D_5R-HEK293 cells) were maintained in culture with 10 μg/mL blasticidin. The stable protein expression of D_5R in D_5R-HEK293 cells was confirmed before the actual experiments were performed (Supplementary Fig. 1). The RPT origins of human RPT cells were verified by staining with antibodies against γ-glutamyl transpeptidase, as previously described [25]. The cells were cultured in a 1:1 mixture of DMEM and Ham's F-12 medium supplemented with 5% FBS, selenium (5 ng/mL), insulin (5 μg/mL), transferrin (5 μg/mL), hydrocortisone (36 ng/mL), triiodothyronine (4 pg/mL), and epidermal growth factor (10 ng/mL).

Specific *DRD1*, *DRD5* (Santa Cruz Biotechnology), *ATG5*, and *LC3-II* (Cell Signaling Technology) siRNAs and their control siRNA constructs were transfected into human RPT cells or D_5R-HEK293 cells using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions and our published procedure [29].

NADPH oxidase activity

NADPH oxidase activity was measured as previously described [10]. Equal amounts of cell membranes were incubated with lucigenin (5 μmol/L) for 10 min at 37 °C in a final volume of 1 mL of assay buffer. Recording of the dynamic chemiluminescence traces was started upon injection of NADPH (final concentration: 100 μmol/L) and continued for 180 s (Autolumet Plus LB953, EG&G Berthold, Bad Wildbad, Germany). The activity levels are expressed as arbitrary light units (ALUs) and were corrected for the protein concentration and duration of the experiment (ALU/s/mg protein).

Superoxide anion production

Superoxide anion production was measured using a cytochrome c assay, as previously described [10]. Cell homogenates in Hank's balanced salt solution (final concentration 1 mg/mL) were distributed in 96-well plates (final volume 200 μL/well). Cytochrome c (500 μmol/L) was added, and the homogenates were incubated in the presence or absence of superoxide dismutase (SOD, 200 U/mL) at room temperature for 30 min. Cytochrome c reduction was measured by reading the absorbance at 550 nm on a microplate reader.

Confocal fluorescence microscopy

Confocal fluorescence microscopy was performed as previously described [29, 30]. The mito-ROS signals in live cells stained with MitoSOX Red were monitored by laser scanning confocal microscopy (LSM 510, Carl Zeiss). The cells were treated with vehicle or the D_1-like receptor agonist fenoldopam (1 μM, 15 min) in the presence or absence of the D_1-like receptor antagonist Sch23390 (1 μM, 45 min). After washing the cells with

Hank's balanced salt solution, the cells were incubated with 5 μM MitoSOX Red for 10 min. Images were captured with excitation at 405 nm and emission at 590 nm under a Plan-Apochromat 63×/1.4 Oil NA objective. The MitoSOX Red fluorescence density was semiquantified in 3–5 random fields from 25–40 cells [30]. MitoTracker Green was used to identify mitochondria. Endogenous light chain (LC)3-II was immunostained with an anti-LC3-II antibody (Cell Signaling Technology). The fluorescence intensity was estimated from the range within the initial linear phase to minimize potential problems arising from mitochondrial dye saturation and leakage.

Western blotting

Western blotting was performed as previously described [10, 25]. Samples (D_5R-HEK293, human RPT cells, kidney cortices, and isolated mitochondria) were adjusted to the same protein concentration. The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto a nitrocellulose membrane, and then probed with primary antibodies and appropriate horseradish peroxidase-conjugated secondary antibodies. The images were visualized by chemiluminescence.

ATP assay

ATP concentrations were quantified as previously described [30, 31]. In brief, human RPT cells were grown in 25-cm tissue culture dishes. After harvesting the cells by centrifugation, the cell pellets were sonicated in 800 μL of lysis buffer. The ATP released in 300 μL of buffer (100 mM Tris-Cl, pH 7.75; 4 mM EDTA) was boiled for 2 min. ATP bioluminescence was monitored by a microplate lumen-ometer (Centro LB 960, Berthold Technologies, Bad Wildbad, Germany).

Generation of *Drd5*$^{-/-}$ mice

The method used for generation of *Drd5*$^{-/-}$ mice has been reported [10, 23]. F6 generation *Drd5*$^{-/-}$ mice on a C57Bl/6 (>98% congenic) background and their sex-matched wild-type littermates were used in this study. Fenoldopam (1 mg/kg body weight, 0.5 mL) or vehicle (saline, for control) was injected intraperitoneally daily for 7 days. Subsequently, the kidneys were collected, and their cortices were excised and homogenized for immunoblotting of autophagy marker proteins. Mitochondria were isolated and purified for H_2O_2 production assays.

The animal protocols were reviewed and approved by the Georgetown University, Children's National Medical Center, and University of Maryland School of Medicine Animal Care and Use Committees.

Mitochondria isolation and H_2O_2 measurement

Mitochondria were isolated from D_5R-HEK293 cells, human RPT cells, and mouse kidney cortices by Percoll density gradient centrifugation as previously described [32] with modifications. In brief, cell pellets or minced kidney cortices were homogenized. The homogenates were suspended in a buffer (pH 7.4) containing 225 mM mannitol, 75 mM sucrose, 5 mM HEPES, 1 mM ethylene glycol-bis (2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), and 1 mg/mL fatty acid-free bovine serum albumin and centrifuged

at 1300 × *g* for 3 min The pelleted material was resuspended in the above buffer and centrifuged at 1300 × *g* for 3 min. The combined supernatants were then centrifuged at 21,000 × *g* for 10 min. The pellets were suspended in 15% Percoll and gently transferred onto the surface of 23% Percoll without touching the 40% Percoll. The gradients were centrifuged at 29,000 × *g* for 8 min The mitochondria were isolated from the interface of the 23 and 40% Percoll. The isolated mitochondria, which had no measurable NADPH oxidase activity and were characterized with pyruvate/malate (5 mM) and succinate (4 mM) in a medium containing 125 mM KCl, 2 mM K_2HPO_4, 3 mM HEPES, 4 mM $MgCl_2$, 3 mM ATP, and 0.2% fatty acid-free bovine serum albumin (pH 7.2), showed coupling of oxidative phosphorylation and normal respiration. The purity of the mitochondrial fraction was confirmed by immunoblotting markers of organelles.

Mito-ROS production by isolated mitochondria was measured with Amplex Red in the presence of exogenous superoxide dismutase (40 U/mL) and horseradish peroxidase (10 U/mL), as described previously [33] with modifications. The fluorescence intensity was measured with a microplate reader in 96-well plates at an excitation wavelength of 530 nm and an emission wavelength of 590 nm. The rate of H_2O_2 production was linear for a fluorescence density up to 3 μmol/L in the standard curve. ROS production is expressed in picomoles of H_2O_2 per mg of mitochondrial protein/min.

Statistical analysis

The results are expressed as the mean ± standard deviation, as indicated. Significant differences among groups ($n > 2$) were determined by one-way factorial ANOVA and the Newman–Keuls test, and significant differences between two groups were determined by Student's *t* test. $P < 0.05$ was considered to indicate statistical significance (SigmaStat 3.0, SPSS Inc, Chicago, IL).

Results

Activation of D_5R decreased ROS production in mitochondria from D_5R-HEK293 cells

Consistent with our previous report [10], stimulation of D_5R with fenoldopam decreased both cellular superoxide ($O_2^{-\bullet}$) production (0.296 ± 0.033 ΔOD_{550}/min/mg vs vehicle 0.493 ± 0.024 ΔOD_{550}/min/mg, $P < 0.05$), as quantified by cytochrome *c* reduction (Supplementary Fig. 2A), and NADPH oxidase activity (2183 ± 123 ALU/min/mg vs vehicle 2449 ± 52 ALU/min/mg, $P < 0.01$), as quantified by lucigenin assay (Supplementary Fig. 2B), in D_5R-HEK293 cells. However, O_2^- production was lower in the presence of both fenoldopam and apocynin (10 μM), an NADPH oxidase inhibitor, than in the presence of apocynin alone (0.187 ± 0.012 ΔOD_{550}/min/mg vs apocynin alone 0.358 ± 0.045 ΔOD_{550}/min/mg, $P < 0.01$) (Supplementary Fig. 2A). By contrast, under the same experimental conditions, fenoldopam did not enhance the apocynin-mediated inhibition of NADPH oxidase activity (1890 ± 147 ALU/min/mg vs apocynin alone 1906 ± 77 ALU/min/mg, $P > 0.05$) (Supplementary Fig. 2B). These results indicate that fenoldopam decreased ROS production by an additional mechanism or mechanisms other than inhibition of NADPH oxidase activity.

Because mitochondria are important sources of cellular ROS production in the kidneys [1, 3, 4], we next determined whether mitochondria are the additional sources of ROS targeted by D_5R, as observed in Supplementary Fig. 2A. Mitochondria isolated from D_5R-HEK293 cells (Fig. 1A) produced less hydrogen peroxide (H_2O_2) than those from EV-transfected HEK293 cells (Fig. 1B). Fenoldopam treatment further decreased H_2O_2 production in D_5R-HEK293 cells but had no effect on EV-transfected HEK293 cells (Fig. 1B). In intact D_5R-HEK293 cells, fenoldopam decreased mito-ROS production, as detected with MitoSOX Red, a hydroethidine (HE)-derived fluorescent dye (Fig. 1C). The colocalization of MitoSOX Red staining with MitoTracker Green staining indicated the mitochondrial origin of the generated ROS (Fig. 1C). The inhibitory effect of fenoldopam on ROS production was prevented by Sch23390, a D_1-like receptor antagonist, in both isolated mitochondria (Fig. 1B) and intact cells (Fig. 1C, D). These findings indicate that fenoldopam, a D_1R/D_5R agonist, decreased ROS production by stimulating D_5R (D_1R is not expressed in D_5R-HEK293 cells). Sch23390, a D_1R/D_5R antagonist but a D_5R antagonist in the absence of D_1R, itself increased ROS production (Fig. 1B–D), which could be due to its blockade of D_5R's constitutive activity [10] because, as stated earlier, HEK293 cells do not express D_1R. H_2O_2 production was increased by antimycin A, a Qi site inhibitor of mitochondrial electron transport chain (ETC) Complex III and prevented the inhibitory effect of fenoldopam (Fig. 1B). By contrast, rotenone, a Complex I inhibitor (Supplementary Fig. 3), had no effect on the inhibitory effect of fenoldopam, indicating that Complex III, not Complex I, is the target of the D_5R-mediated decrease in mito-ROS production. H_2O_2 production, as expected, was decreased by catalase despite the presence of antimycin A (Fig. 1B).

Activation of D_5R, similar to activation of D_1R, stimulates cAMP and protein kinase A (PKA) [5–7]. To determine whether the D_5R-mediated inhibition of mito-ROS is cAMP-dependent, D_5R-HEK293 cells were pretreated with Rp-cAMPS (Fig. 2), a selective inhibitor of PKA [34]. Rp-cAMPS alone did not affect H_2O_2 production but prevented the inhibitory effect of fenoldopam. Sch23390 itself increased H_2O_2 production; as indicated above, this effect was probably mediated by inhibition of the constitutive activity of D_5R [10]. This stimulatory effect of Sch23390 was also inhibited by Rp-cAMPS. Sp-cAMPS, a PKA activator, decreased mito-ROS production (Fig. 2), an effect that was prevented by Rp-cAMPS, indicating the specificity of the effect of Sp-cAMPS on the cAMP pathway. All these results indicate that the D_5R-mediated inhibition of mito-ROS production is cAMP-dependent.

Activation of D_5R decreased ROS production in mitochondria from human RPT cells

To study further the effect of D_5R on mito-ROS production in connection with its potential role in regulating renal sodium transport and ultimately blood pressure [10, 23], we studied mito-ROS production in human RPT cells [21]. Similar to the observations in D_5R-HEK293 cells (Fig. 1), fenoldopam decreased mito-ROS production in human RPT cells. This effect was inhibited by Sch23390, a D_1R/D_5R antagonist, which increased mito-ROS production when used alone (Fig. 3A).

Since no commercially available antagonist or agonist can distinguish the effect of D_5R from that of D_1R, both of which are expressed endogenously in human RPT cells, we

silenced the expression of D_1R or D_5R by transfection with either *DRD1*- or *DRD5*-specific siRNA (Supplementary Fig. 4). With *DRD1* silencing, fenoldopam still decreased mito-ROS production in both isolated mitochondria (Fig. 3B) and intact human RPT cells (Fig. 3C). There was no effect of fenoldopam when *DRD5* was silenced, indicating that D_5R, not D_1R, inhibits ROS production in the mitochondria. Similar to the D_5R-HEK293 results, the inhibitory effect of fenoldopam on ROS production was reversed by the D_1-like receptor antagonist Sch23390 in human RPT cells with intact D_1R (Fig. 3A) or silenced D_1R (Fig. 3B), affirming that the effect of fenoldopam is exerted via D_5R.

Similar to the findings in D_5R-HEK293 cells (Fig. 1), Sch23390 alone increased mito-ROS production in D_1R-intact (Fig. 3A) and D_1R-silenced (Fig. 3B) human RPT cells. *DRD5* silencing alone also increased H_2O_2 production (Fig. 3B), similar to the situation observed with Sch23390 in D_5R-HEK293 cells (Fig. 2). These results support the notion that D_5R is constitutively active [10]. By contrast, fenoldopam failed to decrease and Sch23390 failed to increase mito-ROS production in human RPT cells with silenced *DRD5* (Fig. 3B), indicating a minimal role of D_1R in the regulation of mito-ROS in human RPT cells.

Activation of D_5R inhibited mito-ROS through an increase in autophagy

Recent evidence suggests that oxidative stress regulates autophagy [14, 16, 17] and that products of dopamine oxidation stimulate autophagy in a neuroblastoma cell line [35]. Therefore, we investigated whether D_5R stimulation regulates autophagy. The D_1-like receptor agonist fenoldopam inhibited ROS production (Figs. 1–3) [10]. In D_5R-HEK293 cells, fenoldopam increased cellular autophagy, as shown by the increase in LC3-II immunostaining (Supplementary Fig. 5). Consistently, fenoldopam also increased the protein expression of ATG5 and LC3-II (Fig. 4A), both of which are involved in autophagy. In the presence of chloroquine, an inhibitor of autophagosome-lysosome fusion and subsequently lysosome-mediated proteolysis [36], the ATG5 and LC3-II protein expression levels were increased further by fenoldopam. These results indicate that the fenoldopam-induced increases in the expression of ATG5 and LC3-II protein were due to an increase in autophagic flux rather than to blockade of the degradation of these proteins. Consistent with this interpretation is the demonstration that Sch23390, an antagonist of D_5R (in the absence of D_1R), decreased the protein expression of ATG5 and LC3-II by blocking D_5R activation. This effect was blocked by chloroquine (Fig. 4B), probably because chloroquine prevented the degradation of ATG5 and LC3-II protein [36].

In *DRD1*-silenced human RPT cells, fenoldopam also increased the protein expression of ATG5 and LC3-II (Fig. 5A) but not the expression of the proapoptotic protein Bax or the antiapoptotic protein Bcl-xL (Fig. 5B). These results were associated with decreases in cellular ATP concentrations (Fig. 5C), indicating that D_5R activation increased autophagy but not apoptosis, which is related to a decrease in mitochondrial energy production in human RPT cells. We then studied whether autophagy involves D_5R-mediated inhibition of mito-ROS production using chloroquine. Chloroquine alone slightly but significantly increased mito-ROS production in D_1R-silenced human RPT cells but did not affect mito-ROS production in D_5R-silenced human RPT cells (Fig. 5D). Similar results were observed with spautin-1, another autophagy inhibitor (Supplementary Fig. 6). Indeed, when

autophagy was inhibited by either chloroquine (Fig. 5E) or spautin-1 (Supplementary Fig. 6), fenoldopam was unable to decrease mito-ROS in human RPT cells with intact D_5R when *DRD1* was silenced. These results indicate that stimulation of autophagy is involved in the D_5R-mediated decrease in mito-ROS production in human RPT cells. Moreover, in the presence of apocynin, an NADPH oxidase inhibitor, fenoldopam was still able to decrease mito-ROS production in *DRD5*-intact and *DRD1*-silenced (Fig. 5E) human RPT cells, confirming the involvement of a non-NADPH oxidase-mediated mechanism, presumably related to mito-ROS, similar to that observed in D_5R-HEK293 cells (Supplementary Fig. 2A).

Autophagy protein expression and mito-ROS production in *Drd5*$^{-/-}$ mouse kidneys

The protein expression levels of ATG5 and LC3-II in *Drd5*$^{-/-}$ mouse kidney cortices were lower than those in kidney cortices obtained from wild-type *Drd5*$^{+/+}$ littermates (Fig. 6A), indicating that the kidney cortices of *Drd5*$^{-/-}$ mice have decreased autophagy. Mitochondria from the kidney cortices of these mice were isolated (Supplementary Fig. 7) for determination of H_2O_2 production. As shown in Fig. 6B, H_2O_2 production in mitochondria isolated from the kidney cortices of *Drd5*$^{-/-}$ mice was significantly higher than in mitochondria isolated from the kidney cortices of *Drd5*$^{+/+}$ mice.

Treatment of *Drd5*$^{+/+}$ mice with fenoldopam significantly decreased mitochondrial H_2O_2 production (Fig. 6B) and increased ATG5 and LC3-II protein expression in kidney cortices (Supplementary Fig. 8A). By contrast, fenoldopam treatment of *Drd5*$^{-/-}$ mice did not change H_2O_2 production by isolated renal mitochondria (Fig. 6B) or the protein expression of ATG5 and LC3-II (Supplementary Fig. 8B). These findings indicated that the fenoldopam-mediated increase in autophagy and decrease in mito-ROS were D_5R- but not D_1R-dependent in vivo, which corroborated the in vitro observations.

As expected, antimycin A increased H_2O_2 production in mitochondria isolated from kidney cortices from both *Drd5*$^{-/-}$ and *Drd5*$^{+/+}$ mice, and catalase prevented the stimulatory effect of antimycin A in both *Drd5*$^{-/-}$ and *Drd5*$^{+/+}$ mice (Fig. 6B), consistent with the results of *DRD5* silencing studies in human RPT cells (Fig. 5D). Neither Mn-SOD nor Cu/Zn-SOD protein expression in kidney cortices differed between *Drd5*$^{-/-}$ and *Drd5*$^{+/+}$ mice (Fig. 6C).

Discussion

Oxidative stress is implicated in many pathological conditions, such as hypertension, atherosclerosis, and chronic kidney diseases [1–5, 10, 12–14, 19, 20, 37]. Mitochondria generate more than half of cellular ROS in the basal state [4]. Dopamine, through D_1-like (D_1R and D_5R) and D_2-like (D_2R, D_3R and D_4R) receptors, decreases ROS production in RPT cells and kidneys from humans and animals [1, 5–7, 10, 21, 23]. D_5R negatively regulates ROS production, in part through inhibition of NADPH oxidase expression and activity [10]. The current study demonstrates that fenoldopam, a D_5R agonist (in the absence of D_1R), decreases mito-ROS production in D_5R-HEK293 and human RPT cells and the kidneys of *Drd5*$^{+/+}$ mice but not in EV-HEK293 cells lacking D_5R, *DRD5*-silenced human RPT cells, or the kidneys of *Drd5*$^{-/-}$ mice.

Mitochondria have long been recognized as a major source of ROS in most eukaryotic cells [4]. However, the roles of mitochondrial dysfunction in the pathogeneses of cardiovascular disorders [38] and the importance of mito-ROS in the pathogenesis of hypertension are not as well appreciated as the roles of ROS produced by NADPH oxidase, partly due to the difficulty in accurately measuring mito-ROS production [37, 39–41]. Cytochrome *c* reduction assays, high-performance liquid chromatography, electron paramagnetic resonance spectroscopy, nitroblue tetrazolium assays, chemiluminescence-based assays, and immunospin trapping have all been used to detect cellular or extracellular superoxide [39–41]. However, due to cumbersome sample preparation methods, low sensitivity, or the need for expensive instruments, these available techniques are not widely used for measurement of mito-ROS in living cells [39–41]. Fluorescence microscopy with a fluorescence-based probe targeting mitochondria enables sensitive, real-time monitoring of mito-ROS signals in individual live cells. Mito-HE, a derivative of HE with a hexyl linker to triphenylphosphonium that accumulates preferentially in mitochondria by several hundred fold [42, 43], was used to monitor mito-ROS production in intact cells. Fluorescent 2-hydroxyethidium, which is generated by the reaction of Mito-HE with ROS, is a relatively reliable indicator of mito-ROS signaling in a biological context [43]. Using Mito-HE (which is also known as MitoSOX Red) as the probe for ROS, we found that activation of D_5R attenuated mitochondrial oxidative stress in live D_5R-HEK293 and human RPT cells. To avoid the potential technical limitations of Mito-HE [42] and cross-talk with NADPH oxidase-derived ROS in intact cells, additional experiments were performed in mitochondria isolated from D_5R-HEK293 and human RPT cells in which the other D_1-like receptor, D_1R, was silenced and in kidney cortices of *Drd5*$^{-/-}$ and *Drd5*$^{+/+}$ mice. All results of these studies consistently show that D_5R, in the absence of D_1R, negatively regulates mito-ROS production.

The production of ROS by mitochondria is caused by leakage of electrons from the ETC, with Complex I and Complex III conventionally recognized as the major sites [44, 45]. In vitro studies have demonstrated that Complex I is the major site for ROS production when cells do not produce ATP with high NADH/NAD^+ and $CoQH_2$/CoQ ratios and high proton motive force [44]. However, under most conditions, mitochondria in cells synthesize ATP. In this situation, Complex III and other sites are important sites for ROS production in mitochondria [44, 45]. Our in vitro data demonstrated that ROS production was significantly increased upon inhibition of the Complex III Qi site with antimycin A but not upon inhibition of Complex I with rotenone. Moreover, antimycin A, but not rotenone, prevented the inhibitory effect of fenoldopam on ROS production in intact D_5R-HEK cells, indicating that D_5R may directly or indirectly interact with Complex III rather than Complex I. The site(s) of ROS production within the mitochondria depend(s) on the cell bioenergetic status and the substrate being oxidized [44–46]. Whether D_5R targets Complex III in vivo under different bioenergetic conditions or mechanisms requires further investigation.

Mitochondria and NADPH oxidases are the two main sources of cellular ROS, and it is believed there is a vicious cycle between these two sources of ROS; NADPH oxidase-derived ROS increase mito-ROS formation, and mito-ROS activate NADPH oxidase [47]. We have reported that D_1-like receptor activation decreases renal ROS production in part through inhibition of NADPH oxidase in the kidneys [10, 48]. Yang et al. have also reported

that D_1-like receptors decrease NADPH oxidase-mediated ROS production in human RPT cells [49]. However, D_5R, not D_1R, decreases ROS production, in part by increasing paraoxonase 2 protein levels [49]. Our present study demonstrates that D_5R, not D_1R, decreases Mito-ROS production, which is consistent with a recent report that paraoxonase 2 decreases mitochondrial ROS production in cardiomyocytes [50]. The decrease in mito-ROS production mediated by the D_1R/D_5R agonist fenoldopam was not abrogated by the presence of the NADPH oxidase inhibitor apocynin in human RPT cells deficient in D_1R but with intact D_5R. This result indicates that the D_5R-mediated decrease in mito-ROS production is not downstream of its inhibition of NADPH oxidase activity.

Dopamine increases LC3-II expression and autophagy in neuroblastoma cells [35]. D_3R induces autophagy under hyperammonia [51]. By contrast, inhibition of D_2R activity induces autophagy in neuroblastoma cells [52] and cardiomyocytes [53]. However, inhibition of D_4R activity also disrupts autophagosome-lysosomal fusion [54]. In our study, we demonstrated that activation of D_5R using fenoldopam at concentrations that inhibit ROS production [10] increased autophagic protein expression and activation in human RPT cells, which was confirmed in the kidneys of *Drd5*$^{+/+}$ mice, indicating that D_5R, like D_3R and D_4R, is involved in the regulation of autophagy. Of note, all dopamine receptors are expressed in the kidneys, and their expression is different at different stages of development [25–27, 55–57]. Thus, it is possible that the role of D_5R in autophagy could be different from that of other dopamine receptors in different nephron segments at different stages of development.

Autophagy can be both a cause and consequence of oxidative stress [14, 17]. Mitochondria are both the major sources and targets of ROS; therefore, mitochondria could play critical roles in the cellular responses to oxidative stress. On the one hand, mito-ROS, via multiple signaling pathways, can lead to downregulation or upregulation of autophagy [14, 17, 58]. On the other hand, autophagy regulates ROS signaling or cellular consequences in response to oxidative stress [14]. Autophagic deficiency in mouse embryonic fibroblasts protects against H_2O_2-induced cell death [59]. However, hepatocyte-specific deletion of *Atg5* in mice induces oxidative stress in these cells [60]; pancreas-specific disruption of *Atg5* in mice also increases ROS production in this organ [61]. Intestinal epithelial cell-specific deletion of *Atg5* in mice decreases the basal intestinal epithelial cell mitochondrial membrane potential and increases ROS production [62], suggesting that deficiency of autophagy could lead to an increase in ROS production that may be related to mitochondrial dysfunction.

Mild oxidative stress induces autophagy, while severe oxidative stress inhibits autophagy [17, 58], and high concentrations of dopamine increase autophagy via oxidative products of dopamine [35]. High, cytotoxic concentrations of dopamine (>100 μM) increase oxidative stress, but low, noncytotoxic concentrations of dopamine decrease oxidative stress [63, 64]. Stimulation of D_1-like receptors by the D_1-like receptor agonist fenoldopam decreases ROS production [10], but the effect of fenoldopam on autophagy has not been reported. Ang II promotes autophagy, which is mediated by mito-ROS in podocytes [65, 66]. D_5R activation promotes the ubiquitination and proteasomal degradation of AT_1R [21–23], and germline deletion of *Drd5* in mice increases renal AT_1R expression [23]. By contrast, AT_1R blockade with losartan or germline deletion of *Agtr1* in mice increases D_5R protein expression

[7, 67]. Therefore, treatment with an AT_1R blocker should increase the inhibitory effect of fenoldopam on mito-ROS production but negate the stimulatory effect of fenoldopam on autophagy. However, fenoldopam decreases ROS production and stimulates autophagy, indicating that the effect of fenoldopam on autophagy is direct (vide infra) and not mediated through ROS. It is also known that enhancement of ubiquitination or inhibition of the proteasome pathway plays an important role in the induction of autophagy associated with mitochondrial oxidative stress [68–70]. However, D_5R activates both the ubiquitination and proteasome pathways in addition to inhibiting ROS production, as described previously [23]. Studies by others have shown that the increase in autophagy caused by ROS is mediated by AMPK [71], which can be stimulated by cAMP [71, 72], the levels of which are increased by D_5R [5–7]. The presence of soluble adenylyl cyclase both outside and inside the mitochondria suggests that phosphorylation of mitochondrial proteins may be a major regulatory mechanism for mitochondrial function [73]. cAMP-PKA signaling can regulate all steps of autophagy [74]. Therefore, cAMP signaling could act as a bridge between D_5R in the cell membrane and mitochondria to regulate mitochondrial function. However, depending on the local microenvironments of tissues and cells, activation of cAMP signaling could induce [75, 76], reduce [77], or have no effect [78] on autophagy. Thus, while both D_1R and D_5R increase cAMP production and decrease ROS production [5–7, 10, 48], these two D_1-like receptors have different effects on autophagy and ROS production: D_5R, but not D_1R, stimulates autophagy and inhibits the production of ROS in mitochondria. D_1R increases adenylyl cyclase type 5/6 proteins in lipid rafts, while D_5R increases adenylyl cyclase type 5/6 proteins in nonlipid rafts [79]. We speculate that these differential effects of D_5R and D_1R could be due to their differential expression in lipid and nonlipid raft microenvironments, their coupling to different isoforms of adenylyl cyclases [79], or other unknown factors; these possibilities warrant further study. Nevertheless, our current study shows that D_5R-mediated decreases in ROS production are probably due to increases in cAMP production and subsequent induction of autophagy.

In summary, activation of D_5R, independent of D_1R decreased mito-ROS production in D_5R-HEK293 and RPT cells. This effect was mimicked by the PKA activator Sp-cAMPS and was attenuated by the PKA inhibitor Rp-cAMPS and the autophagy inhibitors chloroquine and spautin-1. ROS production was increased in mitochondria isolated from the kidney cortices of *Drd5*$^{-/-}$ mice relative to their wild-type littermates. Furthermore, the renal protein expression of autophagy hallmark proteins was increased by fenoldopam, a D_5R agonist (in the absence of D_1R), in vitro and in vivo. These results suggest that D_5R negatively regulates ROS production in a cAMP- and autophagy-dependent manner.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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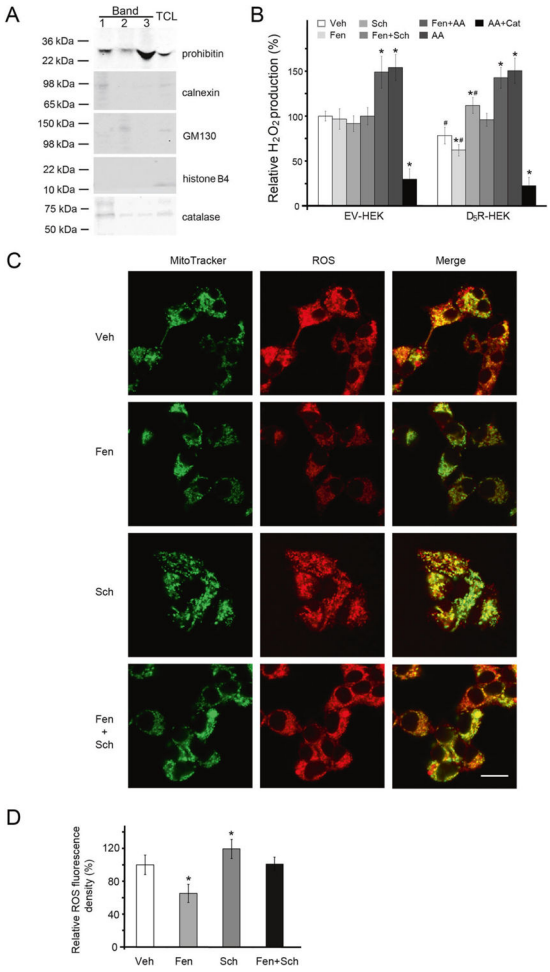

Fig. 1.

Mito-ROS production in D_5R-HEK293 cells. **A** Characterization of purified mitochondria (Band 3, as described in Supplementary Fig. 7) by immunoblotting with antibodies against markers for mitochondria (prohibitin), endoplasmic reticula (calnexin), Golgi bodies (GM130), nuclei (histone B4), and peroxisomes (catalase). TCL total cell lysate. **B** H_2O_2 production in isolated mitochondria, with pyruvate (5 mM) and malate (5 mM) as the substrates. Veh vehicle, Fen fenoldopam (D_1R/D_5R agonist, 1.0 μM, 12 h), Sch Sch23390 (D_1R/D_5R antagonist 1.0 μM, 12 h), AA antimycin A (Qi site inhibitor of mitochondrial ETC Complex III, 0.75 μM, 2 h), Cat catalase (10 μM, 30 min prior to AA treatment). Open rectangles, empty vector-transfected HEK293 (EV-HEK) cells; filled rectangles, D_5R-overexpressing HEK293 (D_5R-HEK) cells. $n = 4$/group, $^{*}P < 0.05$ vs Veh, $^{\#}P < 0.05$ vs EV-HEK. **C** D_5-RHEK293 cells were treated with Veh or Fen (1.0 μM, 12 h) in the absence or presence of Sch (1.0 μM, 12 h). Mitochondria were stained with MitoTracker Green; mito-ROS were monitored with MitoSOX Red. Bar, 20 μm. The images are from one of three independent experiments. **D** The MitoSOX Red fluorescence density was obtained in 3–5 random fields from 25–40 D_5R-HEK293 cells in three independent experiments, as described in **C**; $^{*}P < 0.05$ vs Veh

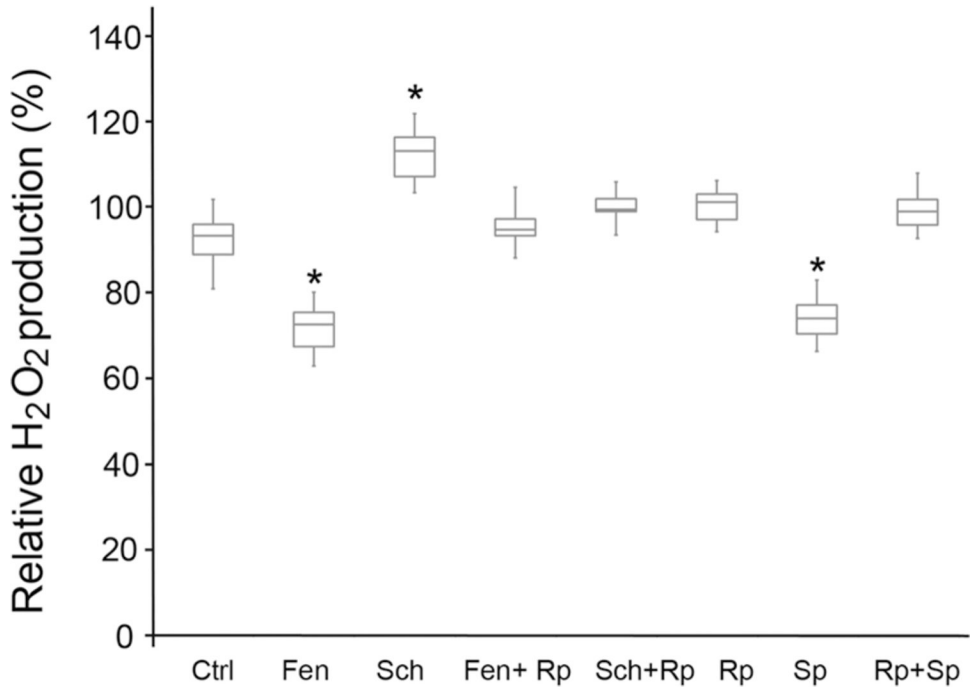

Fig. 2.

cAMP dependence of D_5R-mediated inhibition of mito-ROS production. D_5R-HEK293 cells were preincubated for 30 min with vehicle (Ctrl), Fen (fenoldopam, D_1R/D_5R agonist, 1.0 μM), Sch (Sch23390, D_1R/D_5R antagonist, 1.0 μM), Fen + Rp (Rp-cAMPS, PKA inhibitor, 50 μM), Sch + Rp, Rp alone, Sp (Sp-cAMPS, PKA activator, 10 μM) alone, or Rp + Sp. n = 6/group, *P < 0.05 vs Ctrl

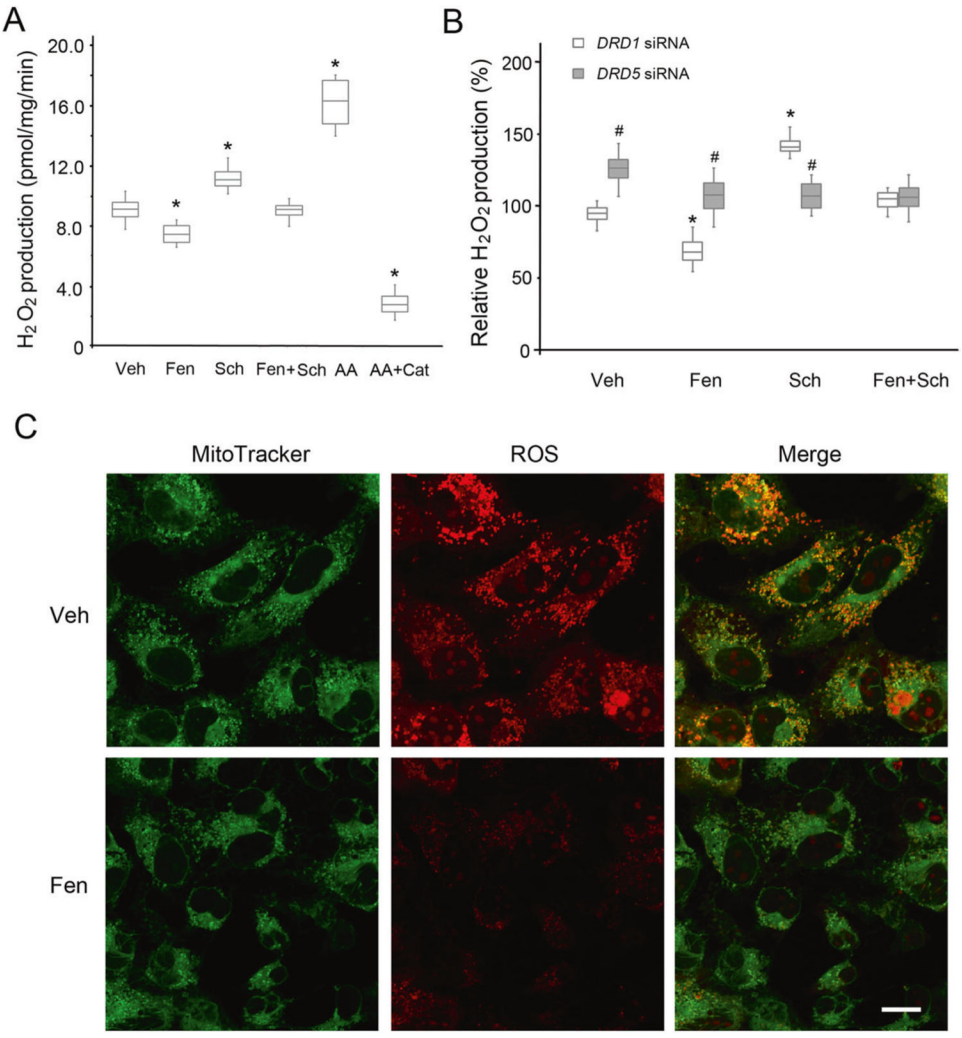

Fig. 3.

Mito-ROS production in human RPT cells. **A** H_2O_2 production from mitochondria isolated from human RPT cells, with pyruvate and malate as the substrates. Veh vehicle, Fen fenoldopam (D_1R/D_5R agonist, 1.0 μM, 12 h), Sch Sch23390 (D_1R/D_5R antagonist, 1.0 μM, 12 h), AA antimycin A (Qi site inhibitor of mitochondrial ETC Complex III, 0.75 μM, 2 h), Cat catalase (10 μM, 30 min prior to AA treatment). $n = 4$/group, $^*P < 0.05$ vs Veh. **B** H_2O_2 production from mitochondria isolated from *DRD1*- or *DRD5*-silenced human RPT cells. The cells were treated with vehicle (Veh), Fen (1.0 μM, 12 h), or Sch (1.0 μM, 12 h). $n = 4$/group, $^*P < 0.05$ vs Veh, $^{\#}P < 0.05$ vs *DRD1*-silenced RPT cells. **C** Mito-ROS staining in *DRD1*-silenced intact human RPT cells treated with Veh or Fen (1.0 μM, 12 h), as indicated. Bar, 10 μm. The images are from one of three independent experiments

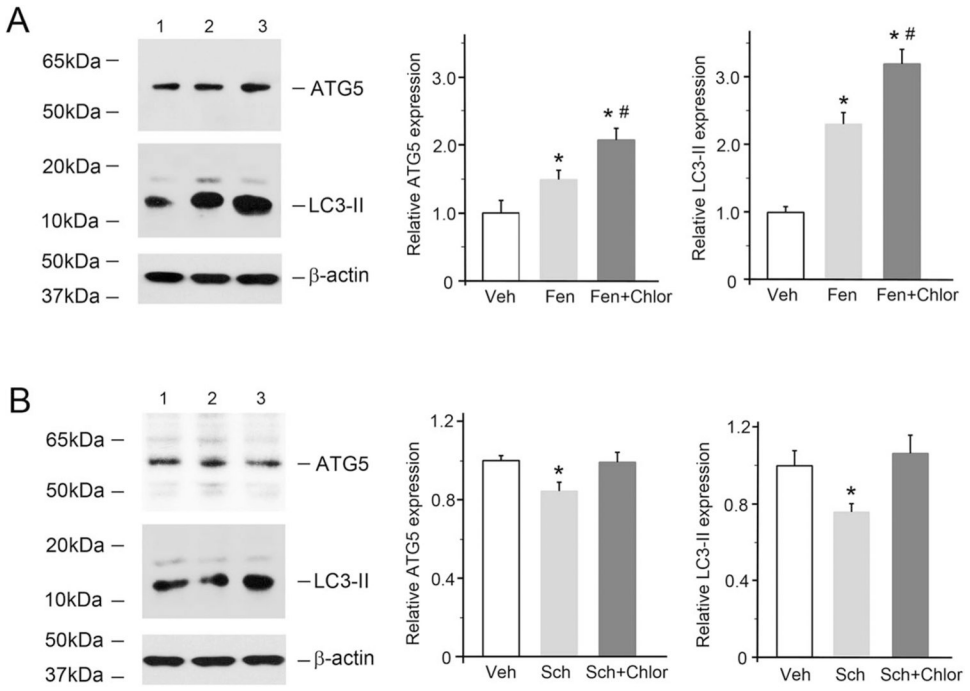

Fig. 4.

D_5R-mediated increases in the levels of autophagy-related proteins in D_5R-HEK293 cells. **A** The cells were treated with fenoldopam (Fen, 1.0 μM, 12 h) in the absence or presence of chloroquine (an inhibitor of autophagosome-lysosome fusion/lysosome-mediated proteolysis, 10 μM, 12 h). Immunoblots from one of five independent experiments are shown. Lane 1, vehicle (Veh); lane 2, fenoldopam (Fen); lane 3, fenoldopam plus chloroquine (Fen + Chlor). The β-actin protein level was used to measure the amount of sample loaded in each lane. $n = 5$/group, $^{*}P < 0.05$ vs Veh, $^{\#}P < 0.05$ vs Fen. **B** D_5R-HEK293 cells were treated with Sch (Sch23390, 1.0 μM, 12 h) in the absence or presence of chloroquine (Chlor). Immunoblots from one of five independent experiments are shown. Lane 1, Veh; lane 2, Sch; lane 3, Fen + Chlor. The β-actin protein level was used to measure the amount of sample loaded in each lane. $n = 5$/group, $^{*}P < 0.05$ vs others

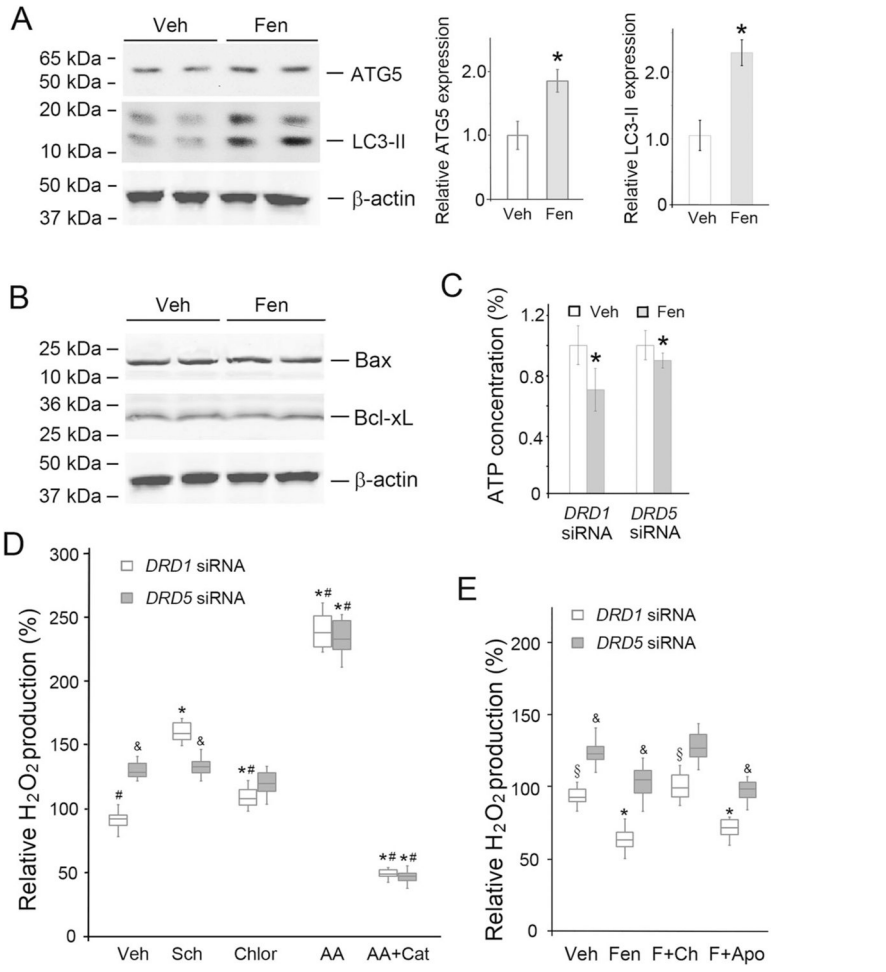

Fig. 5.

D_5R-mediated alteration of autophagy-related protein expression and function. **A** Autophagy marker protein expression in *DRD1*-silenced human RPT cells treated with Veh (vehicle) or Fen (fenoldopam, 1.0 μM, 12 h). The immunoblots show that the levels of the autophagy marker proteins ATG5 and LC3-II increased with Fen treatment. $n = 4$/group, $^{*}P < 0.05$ vs Veh. **B** Proapoptotic and antiapoptotic protein (Bax and Bcl-xL) expression in *DRD1*-silenced human RPT cells. The human RPT cells were treated, as described in **A**. **C** ATP concentrations were measured using a bioluminescence assay kit (Roche). The ATP concentration in human RPT cells treated with vehicle was set to 1. Veh vehicle, Fen fenoldopam (1.0 μM, 12 h). $n = 5$/group, $^{*}P < 0.05$ vs Veh. **D** H_2O_2 production in isolated mitochondria from *DRD1*- or *DRD5*-silenced human RPT cells treated as indicated. Veh vehicle, Sch Sch23390 (1.0 μM, 12 h), Chlor chloroquine (inhibitor of autophagosome-lysosome fusion/lysosome-mediated proteolysis, 10 μM, 12 h), AA antimycin A (Qi site inhibitor of mitochondrial ETC Complex III, 0.75 μM, 2 h), Cat catalase (10 μM, 30 min prior to AA treatment). $n = 5$–6/group, $^{*}P < 0.05$ vs Veh, $^{\#}P < 0.05$ vs Sch, $^{\&}P < 0.05$ vs *DRD1* siRNA. **E** H_2O_2 production in isolated mitochondria from human RPT cells, as described in **D**. Veh vehicle, Fen or F fenoldopam (1.0 μM, 12 h), Ch chloroquine (10 μM, 12 h), Apo apocynin A (10 μM, 12 h). $n = 8$/group, $^{*}P < 0.05$ vs Veh, $^{\S}P < 0.05$ vs Fen, $^{\&}P$

< 0.05 vs *DRD1* siRNA. There was no significant difference between the Fen and Fen + Apo groups in terms of human RPT cells lacking D_1R (*DRD1* siRNA) or D_5R (*DRD5* siRNA)

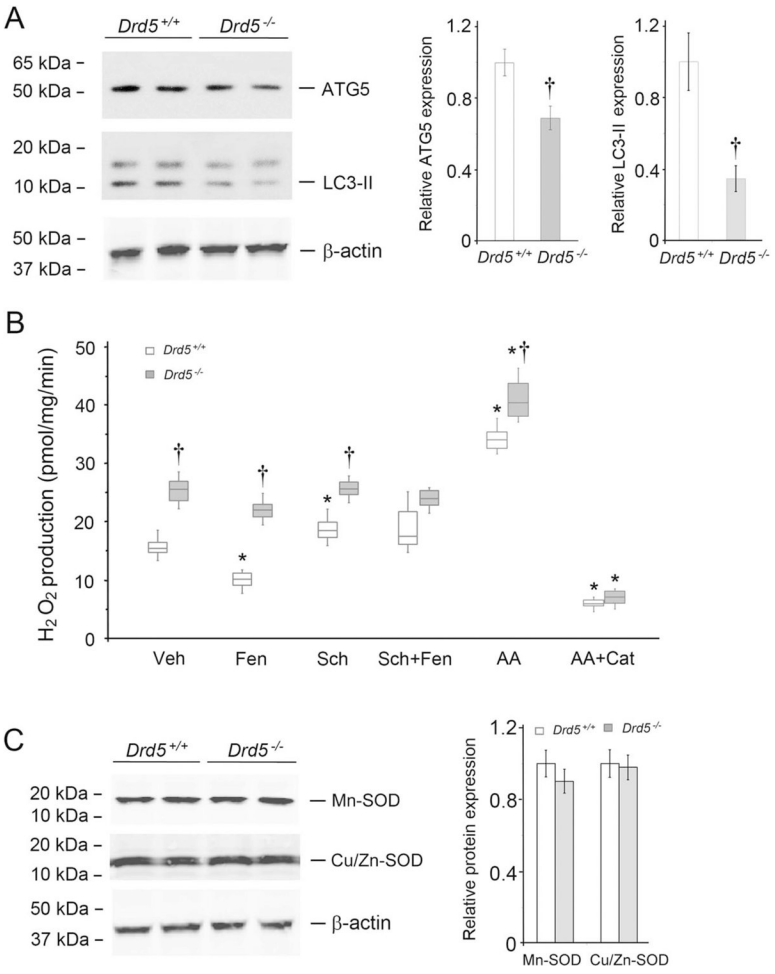

Fig. 6.

Autophagy protein expression and mito-ROS production in mouse kidney cortices. **A** Autophagy marker protein expression in kidney cortices of *Drd5*$^{-/-}$ mice and their wild-type (*Drd5*$^{+/+}$) littermates. The immunoblots show decreased protein expression of the autophagy marker proteins ATG5 and LC3-II in the kidney cortices of *Drd5*$^{-/-}$ mice relative to *Drd5*$^{+/+}$ littermates. $n = 4$/group, $^{\dagger}P < 0.05$ vs *Drd5*$^{+/+}$. **B** H_2O_2 production in isolated mitochondria from kidney cortices of *Drd5*$^{-/-}$ mice and their *Drd5*$^{+/+}$ littermates. Veh vehicle, Fen fenoldopam (1.0 μM, 12 h), Sch Sch23390 (1.0 μM, 12 h), AA antimycin A (0.75 μM, 2 h), Cat catalase (10 μM, 30 min prior to AA treatment). $n = 6$/group, $^{*}P < 0.05$ vs Veh, $^{\dagger}P < 0.05$ vs *Drd5*$^{+/+}$. **C** Mitochondrial superoxide dismutase (SOD) protein (Mn-SOD and Cu/Zn-SOD) expression in isolated mitochondria from kidney cortices of *Drd5*$^{-/-}$ mice and their *Drd5*$^{+/+}$ littermates. $n = 4$/group. There were no significant differences in SOD protein expression between *Drd5*$^{+/+}$ and *Drd5*$^{-/-}$ mice