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The α_{1D}-Adrenergic Receptor Induces Vascular Smooth Muscle Apoptosis via a p53-Dependent Mechanism[S]

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ABSTRACT

Activation of the endogenous α_1-adrenergic receptor (AR) associated with human aortic smooth muscle cells resulted in a dose- and time-dependent increase in the levels of mitochondrial reactive oxygen species (ROS). ROS increases were apparent within 10 min and maximal after 45 min. Prolonged activation (>4 h) of the α_1-AR resulted in smooth muscle cell apoptosis. Both the increase in ROS and apoptotic cell death were blocked by the nonselective α_1-AR antagonist prazosin as well as the selective α_{1D}-AR antagonist 8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4.5]decane-7,9-dione (BMY 7378). Increases in ROS and apoptosis produced by α_1-AR activation were also blocked by the p38 mitogen-activated protein kinase inhibitor 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1*H*-imidazole (SB 202190) and the NAPDH oxidase inhibitor apocynin. The extracellular signal-regulated kinase 1/2 inhibitor 2′-amino-3′-methoxyflavone (PD 98059) or the c-Jun NH_2-terminal kinase inhibitor 1,9-pyrazoloanthrone anthra(1,9-*cd*)pyrazol-6(2*H*)-one (SP 600125) was without effect on increases in ROS levels or apoptosis. Pifithrin-α, an inhibitor of the tumor suppressor protein p53, had no effect on ROS generation but did block α_{1D}-AR-induced apoptosis. Activation of the α_{1D}-AR resulted in translocation of p53 to the mitochondria. The mitochondrial translocation of p53 was blocked by prazosin, BMY 7378, apocynin, SB 202190, and pifithrin-α. Apoptosis was also blocked by small interfering RNA directed against p53. These data show that the α_{1D}-AR is coupled to the generation of mitochondrial ROS by a pathway involving p38 and NADPH oxidase. Sustained activation of the α_{1D}-AR results in smooth muscle cell apoptosis in a pathway that involves the tumor suppressor protein p53 and the mitochondrial translocation of p53. The data also provide evidence of a linkage between the α_{1D}-AR and p53.

Based on our current understanding, three subtypes of the α_1-AR, the α_{1A}-, α_{1B}-, and α_{1D}-ARs, have been cloned and characterized (for recent reviews, see Xiao et al., 2006; Hein and Michel, 2007; Koshimizu et al., 2007; Perez, 2007). Each of these subtypes transduces discrete actions of endogenous catecholamine neurotransmitters in the regulation of a variety of physiologic functions. Although it is known that the α_1-ARs are coupled to increases in inositol phosphates, the canonical pathway for G_q-coupled receptors (see above-cited references), recent evidence has shown that the α_1-ARs are also coupled to the generation of reactive oxygen species (ROS) through novel signaling pathways such as activation of the epidermal growth factor receptor and NADPH oxidase (Xiao et al., 2002; Bleeke et al., 2004; Zhang et al., 2004; Kuster et al., 2005; Hao et al., 2006; Javadov et al., 2006; Faber et al., 2007; Fernandez-Patron, 2007). Increases in vascular ROS have been associated with modulating smooth muscle contraction (Fernandez-Patron, 2007; Szasz et al., 2007) as well as maladaptive responses such as hypertension, hypertrophic growth, and apoptosis (Guo et al., 2006; Lyle and Griendling, 2006; Paravicini and Touyz, 2006; Szasz et al., 2007).

Critically important in cellular regulation, the tumor suppressor protein p53 is an important mediator of apoptotic cell death (Danial and Korsmeyer, 2004; Okada and Mak, 2004; Vousden and Lane, 2007). Although p53 is well known to induce the transcription of proapoptotic proteins, there is

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ABBREVIATIONS: AR, adrenergic receptor; ROS, reactive oxygen species; siRNA, small interfering RNA; BMY 7378, 8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4.5]decane-7,9-dione; WB 4101, 2-(2,6-dimethoxyphenoxyethyl)aminomethyl-1,4-benzodioxane hydrochloride; MAP, mitogen-activated protein; SB 202190, 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1*H*-imidazole; ERK, extracellular signal-regulated kinase; PD 98059, 2′-amino-3′-methoxyflavone; JNK, c-Jun NH_2-terminal kinase; SP 600125, 9-pyrazoloanthrone anthra(1,9-*cd*)-pyrazol-6(2*H*)-one; PBS, phosphate-buffered saline; BSA, bovine serum albumin; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; ANOVA, analysis of variance.

also evidence that p53 can activate the mitochondrial cell death pathway (Erster and Moll, 2005; Moll et al., 2006; Liu et al., 2008).

In this report, we show that activation of the α_1-AR (more specifically, the α_{1D}-AR) promotes increases of ROS levels in vascular smooth muscle. Prolonged activation of the α_{1D}-AR leads to apoptosis. Activation of the α_{1D}-AR promotes the translocation of p53 to the mitochondria. Pharmacologic inhibition of p53 or siRNA-mediated knockdown of its expression prevents α_{1D}-AR apoptosis. These are the first data to link an α_1-AR to pathways involving the multifunctional regulatory protein p53.

Materials and Methods

Cell Culture. Human aortic smooth muscle cells (Cascade Biologics, Portland, OR) were grown to confluence in medium 231 supplemented with smooth muscle growth supplement (Cascade Biologics) and a mixture of gentamicin and amphotericin (Cascade Biologics). Cells were grown in T-75 flasks in a 37°C cell culture incubator with a humidified atmosphere (95% air and 5% CO_2) and were fed every 2 to 3 days. After reaching confluence, the cells were plated on plain, untreated coverslips in 35-mm tissue culture dishes.

Effect of Activation of the α_1-AR on the Levels of Reactive Oxygen Species in Human Aortic Smooth Muscle Cells. The generation of mitochondrial ROS was measured using MitoTracker ROS (Invitrogen, Carlsbad, CA) diluted in dimethyl sulfoxide. Human aortic smooth muscle cells attached to glass coverslips were incubated for 20 min at 37°C with 5 nM MitoTracker ROS diluted in serum-free medium. These incubation conditions were established after preliminary studies of time and MitoTracker concentration. Cells were washed twice with medium, fresh medium was applied, and the effect of phenylephrine (Sigma-Aldrich, St. Louis, MO) was studied. We examined both the time and concentration dependence of phenylephrine-induced ROS accumulation. In inhibitor studies, cells were pretreated for 45 min with the following: prazosin (1 nM; Sigma-Aldrich), BMY 7378 (30 nM; Sigma-Aldrich), WB 4101 (1 nM; Sigma-Aldrich), or 5-methylurapidil (1 nM; Sigma-Aldrich); the p38 MAP kinase inhibitor SB 202190 (30 μM; Sigma-Aldrich); the ERK1/2 inhibitor PD 98059 (30 μM; BIOMOL Research Laboratories, Plymouth Meeting, PA); the JNK inhibitor SP 600125 (30 μM; BIOMOL Research Laboratories); the NADPH oxidase inhibitor apocynin (100 μM; Calbiochem, Darmstadt, Germany); or the p53 antagonist pifithrin-α (10 μM; Sigma-Aldrich). After preincubation with the antagonist, phenylephrine (10 μM) was added and incubated for 20 min. The cells were fixed with 1.0% formaldehyde in PBS for 10 min, washed with PBS, and mounted on slides with Prolong Gold antifade reagent (Invitrogen). The slides were viewed under a 63× oil immersion objective attached to an AOBS TCS SP5 inverted laser scanning confocal microscope using Application Suite Advanced Fluorescence software (Leica Microsystems, Inc., Deerfield, IL). Images were prepared using Adobe Photoshop (version 7.0; Adobe Systems, Mountain View, CA) and quantitated with ImageJ, version 3.91 software (http://rsb.info.nih.gov/ij). The fluorescence intensity of six areas (20 × 20 pixels) within a cell was measured, and an intensity average was determined for each cell. Data were analyzed by one-way analysis of variance followed by Tukey's post hoc test using Prism (version 3.00 for Windows; GraphPad Software Inc., San Diego, CA).

Immunocytochemical Localization of p53 in Human Aortic Smooth Muscle Cells. Dual-label immunocytochemistry was used to assess the extent to which the tumor suppressor protein p53 was localized into the mitochondria of human aortic smooth muscle cells. An antibody to succinate dehydrogenase was used as a marker for mitochondria. In these studies, cells were grown on glass coverslips. The cellular localization of p53 and succinate dehydrogenase was assessed before and after a 12-h treatment with 10 μM phenylephrine. In certain experiments, cells were treated with the antagonist series described above before the addition of phenylephrine. After drug treatments, cells were washed in PBS and fixed with 3.7% formaldehyde in PBS for 10 min. Cells were then washed with 0.05% BSA in PBS and permeabilized with 0.1% Triton in PBS for 5 min. After permeabilization, the cells were washed and blocked with 10% serum for 1 h at room temperature. After washing, polyclonal antibodies against p53 (Calbiochem; diluted 1:50 in 1% BSA in PBS) or succinate dehydrogenase (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; diluted 1:100 in 1% BSA in PBS) were added either alone or in combination and incubated overnight at 4°C. After this incubation, the cells were washed with 0.05% BSA in PBS, and secondary antibodies for either p53 (fluorescein isothiocyanate; Jackson ImmunoResearch Laboratories, West Grove, PA; diluted 1:200 in PBS) or succinate dehydrogenase (Texas Red; Abcam Inc., Cambridge, MA; diluted 1:200 in PBS) were added and incubated in the dark at room temperature for 1 h. Cells were washed with PBS and mounted on glass slides with Prolong Gold antifade reagent (Invitrogen) and visualized under a 63× oil immersion objective with a confocal microscope as described above. Image analysis was performed with MetaMorph software (version 6.2r6; Molecular Devices, Sunnyvale, CA), using the colocalization plug-in software tool. Fluorescence intensity was measured in six areas (20 × 20 pixels) within the cell, and the extent of overlap of p53 over succinate dehydrogenase was calculated and averaged for each cell. Data were then analyzed by one-way analysis of variance with Tukey's post hoc test using GraphPad Prism.

Effect of Activation of α1-AR on Apoptosis in Human Aortic Smooth Muscle Cells. Human aortic smooth muscle cells were plated in 35-mm glass-bottomed culture dishes (MatTek, Ashland, MA). To assess the role of the α-AR on apoptosis, the cells were treated with phenylephrine for 12 h. In certain experiments, the cells were pretreated for 45 min with a series of antagonists (see above) before phenylephrine addition. Cells were washed with PBS and fixed in fresh 4.0% paraformaldehyde for 1 h. The cells were washed in PBS and permeabilized using freshly prepared 0.1% sodium citrate. After additional PBS washes, a TUNEL reaction mixture (In Situ Cell Death Detection kit, fluorescein; Roche Diagnostics, Indianapolis, IN), which results in the transfer of fluorescein dUTP to free 3′ OH groups of genomic DNA, was added and incubated in a humidified chamber at 37° for 1 h. The cells were washed with PBS and imaged with a confocal microscope as described above. Images were prepared using Adobe Photoshop, version 7.0 (Adobe Systems). The number of apoptotic versus nonapoptotic cells was determined, and a ratio of apoptotic versus total cells was calculated. To facilitate quantitation of large numbers of cells, the counting of apoptotic or nonapoptotic cells was done at a 20× magnification. However, the data presented in Figs. 4 and 6 are at a 40× magnification to clearly see the presence of individual cells with apoptotic nuclei. Data were analyzed by one-way analysis of variance with Tukey's post hoc test using GraphPad Prism.

Effect of p53 siRNA on α1-AR-Induced Apoptosis in Human Aortic Smooth Muscle Cells. siRNA against p53 was obtained from Cell Signaling Technology Inc. (Danvers, MA). The sense strand sequence for this siRNA was 5′-CUACUUCCUGAAAACAACGTT. A nonsense siRNA served as a control for nonspecific effects on p53 expression. Human aortic smooth muscle cells were transfected with p53 siRNA (50, 100, and 150 nM) or the nonsense control (100 nM) using the TransIT-TKO transfection reagent (Mirus, Madison, WI) in serum-free medium for 12 h. After this time, cells were washed with PBS. The efficacy of siRNA treatment on p53 expression was determined with immunocytochemistry with a p53 antibody as discussed above. When studying the effects of siRNA treatment on ROS and TUNEL assays, the cells were transfected with 100 nM p53 siRNA or the nonsense control as described above.

Results

Using the fluorescence indicator MitoTracker ROS, we examined the effect of α_1-AR activation on mitochondrial ROS levels in human aortic smooth muscle cells. Our previous studies have demonstrated that these cells express mRNA for all three α_1-AR subtypes (García-Cazarín et al., 2008). Phenylephrine treatment resulted in a time- and dose-dependent increase in ROS in these cells (Fig. 1, A and B). ROS increases were apparent after 10 min of treatment and maximal after 45 min. The ED_{50} value for phenylephrine was approximately 10 μM. Based on the data in Fig. 1, we used a 20-min treatment with 10 μM phenylephrine in all additional studies of ROS accumulation. The α_1-AR antagonist prazosin (1 nM) completely blocked these increases in ROS (Fig. 2). The selective α_{1D}-AR antagonist BMY 7378 (30 nM) also blocked ROS increases, whereas the selective α_{1A}-AR antagonists WB 4101 (1 nM) and 5-methylurapidil (1 nM) were without effect (Fig. 2). These results indicate that the α_{1D}-AR is specifically coupled to ROS generation in human aortic smooth muscle cells.

We next used a series of antagonists to investigate the pathways linking the α_1-AR to increases in ROS (Fig. 3). Previous work has shown that these concentrations of inhibitors are effective at inhibiting their respective targets (Bleeke et al., 2004; Zhang et al., 2004; Walton et al., 2005; Hamanoue et al., 2007). The p38 MAP kinase inhibitor SB

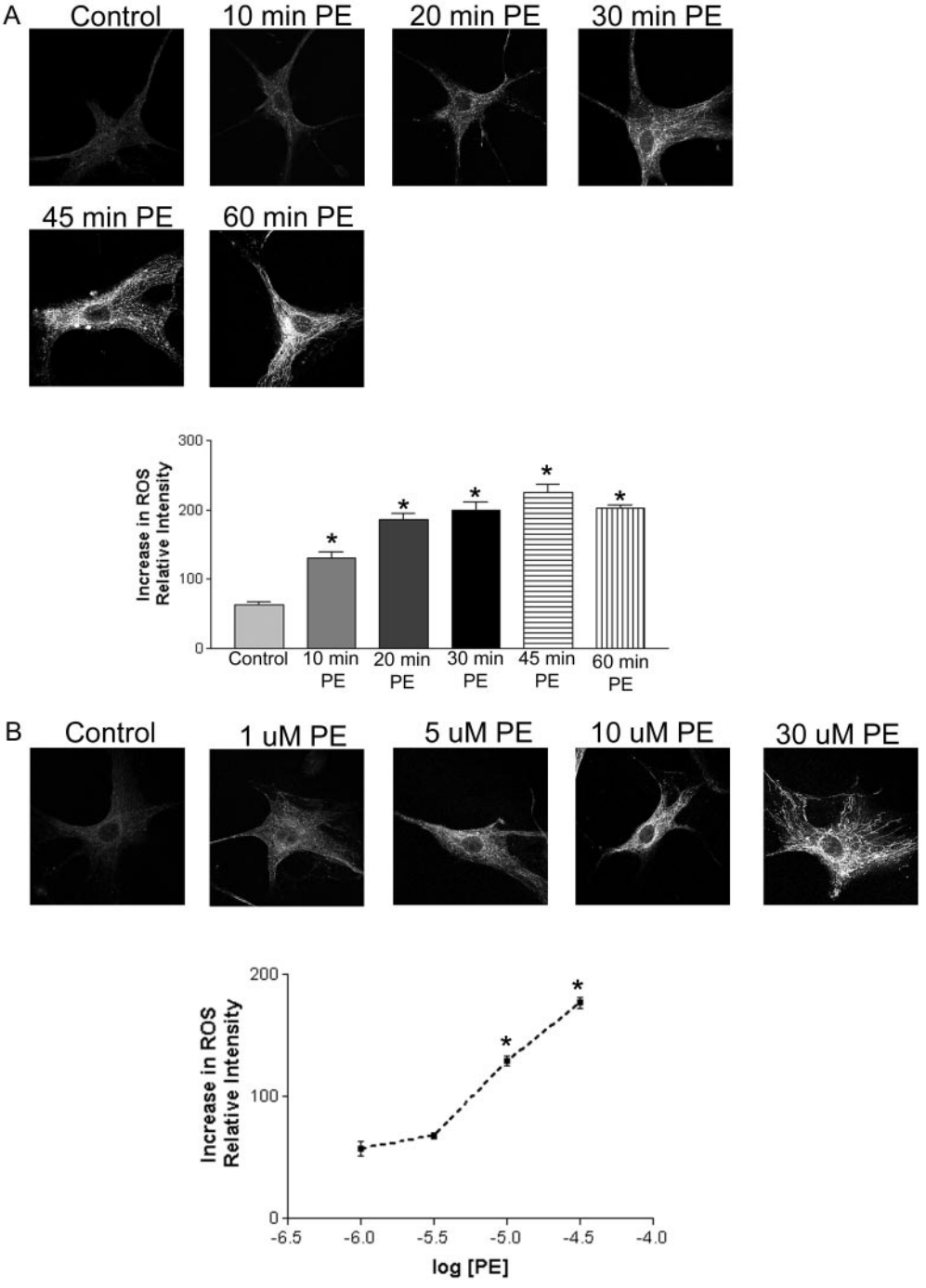

Fig. 1. A, effect of 10 μM phenylephrine on the time-dependent increase in ROS levels in human aortic smooth muscle cells. Experiments were carried out as described under *Materials and Methods*. Each point represents the mean ± S.E.M. of six experiments each on a different cell population. Statistical significance was determined by a one-way ANOVA followed by Tukey's post test. *, $p < 0.5$, statistically different from control. B, effect of phenylephrine on ROS levels in human aortic smooth muscle cells. Increasing amounts of phenylephrine were added to smooth muscle cells, and ROS levels were measured 20 min later. Each point represents the mean ± S.E.M. of four experiments each on a different cell population. Statistical significance was determined by a one-way ANOVA followed by Tukey's post test. *, $p < 0.5$, statistically different from control.

202190 (30 μM) significantly impaired the ability of phenylephrine to increase ROS levels in human aortic smooth muscle cells (Fig. 3). Apocynin (100 μM), an inhibitor of NADPH oxidase also blocked mitochondrial ROS generation. The inhibitory actions of both SB 202190 and apocynin occurred in a concentration-dependent manner (Supplemental Fig. 1). In contrast, neither the ERK1/2 inhibitor PD 98059 (30 μM) nor the JNK inhibitor SP 600125 (30 μM) had any effect on the phenylephrine-induced increase in ROS (Fig. 3). Pifithrin-α, an inhibitor of the tumor suppressor protein p53, also had no effect on the generation of ROS induced by phenylephrine (Fig. 3).

Cellular increases in ROS can trigger apoptotic cell death. A TUNEL assay was used to determine whether the α_1-AR-mediated increases in ROS resulted in smooth muscle cell apoptosis. Human aortic smooth muscle cells were exposed to 10 μM

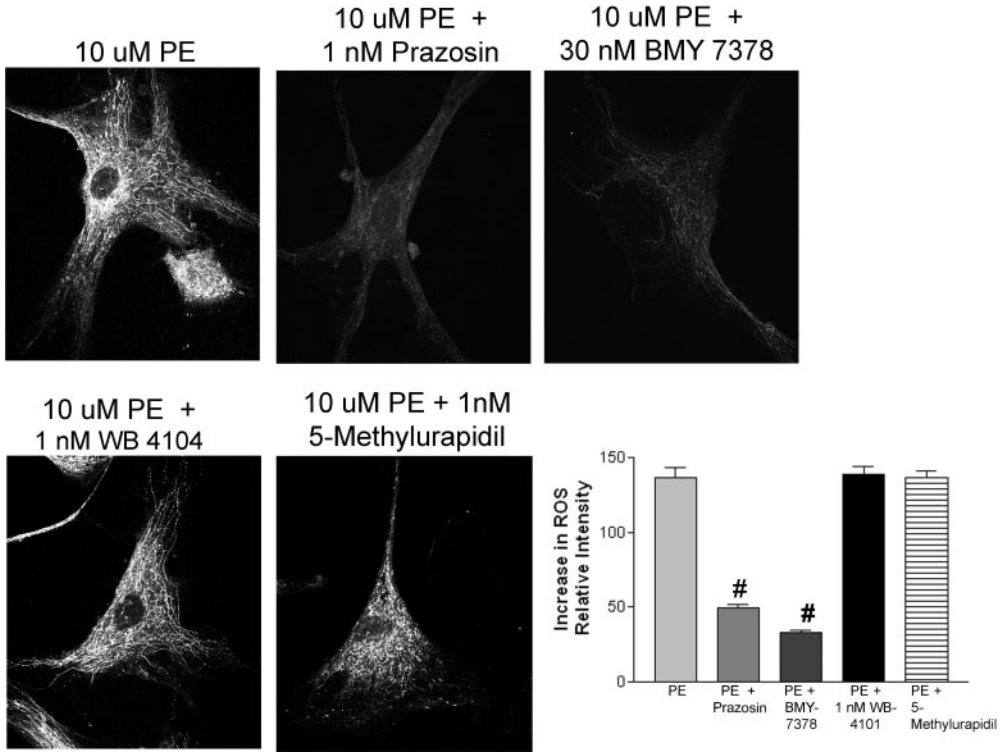

Fig. 2. Effect of α1-AR antagonists on phenylephrine-induced increases in human aortic smooth muscle cell levels of ROS. Smooth muscle cells were preincubated with either prazosin (1 nM), BMY 7378 (30 nM), WB 4101 (1 nM), or 5-methylurapidil (1 nM) for 45 min before the addition of 10 μM phenylephrine. ROS levels were measured 20 min after agonist addition. Each point represents the mean $\pm$ S.E.M. of seven experiments each on a different cell population. Statistical significance was determined by a one-way ANOVA followed by Tukey's post test. #, $p < 0.5$, statistically different from phenylephrine alone.

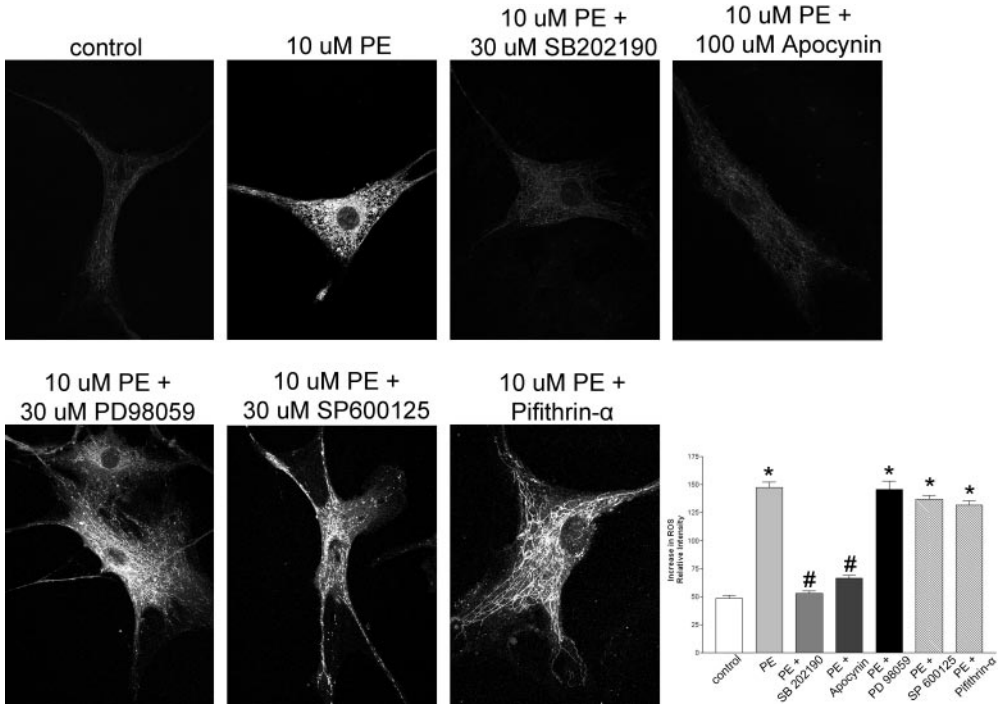

Fig. 3. Effect of antagonists of various cellular signaling pathways on phenylephrine-induced increases in ROS. Antagonists were incubated with human aortic smooth muscle cells for 45 min before the addition of phenylephrine. Each point represents the mean $\pm$ S.E.M. of six experiments each on a different cell population. Statistical significance was determined by a one-way ANOVA followed by Tukey's post test. *, $p < 0.5$, statistically different from control. #, $p < 0.5$, statistically different from phenylephrine alone.

phenylephrine, and the degree of apoptosis was determined at 4, 6, and 24 h. Phenylephrine produced a time-dependent apoptotic effect that was statistically significant at 6 h (Fig. 4A). The percentage of apoptotic cells was observed in untreated aortic smooth muscle cells was 1.1 ± 0.3. The percentage of apoptotic cells increased to 71.5 ± 3.3 after phenylephrine treatment (Fig. 4). This quantitation was done at a 20× magnification. However, to clearly show examples of apoptotic nuclei in individual cells, the data presented in Fig. 4 are shown at 40× magnification. The apoptosis of human aortic smooth muscle cells was blocked by pretreatment with prazosin or the α_{1D}-AR-selective antagonist BMY 7378 (Fig. 4B). Therefore, programmed cell death, like increases in ROS, was due to the activation of an α_1-AR, more specifically, the α_{1D}-AR. PD 98059 and SP 600125 were without effect on apoptosis (Fig. 4B). Inhibition of ROS generation with SB 202190 or apocynin blocked phenylephrine-induced apoptosis (Fig. 4B). The p53 antagonist pifithrin-α also blocked the apoptosis seen after phenylephrine treatment (Fig. 4B). The inhibitory actions of pifithrin-α occurred in a concentration-dependent manner (Supplemental Fig. 2). The results with pifithrin indicate that the multifunction tumor suppressor p53 mediates the apoptosis seen in response to the activation of the α_{1D}-AR. Furthermore, the data indicate that the activation of p53 occurs in response to the increase in the levels of ROS.

To assess the linkage between the α_1-AR and p53, we examined the effect of receptor activation on the cellular localization of p53 using dual immunofluorescence labeling. The antibodies used in these experiments were directed against p53 or the mitochondrial marker succinate dehydrogenase. In the absence of any intervention, p53 immunoreactivity (Fig. 5, green fluorescence) could be detected throughout the cell. Furthermore, there was no association with the mitochondrial localized succinate dehydrogenase (Fig. 5, red fluorescence). Phenylephrine treatment resulted in a time-dependent translocation of p53 (Fig. 5). The emergence of the yellow fluorescence signal indicates that the green (p53) and red (succinate dehydrogenase) now overlay one another. From these data we conclude that p53 is translocated to the mitochondria after activation of the α_{1D}-AR. This p53 fraction could be responsible for the apoptotic cell death seen after activation of the α_1-AR.

We used siRNA to further evaluate the role of p53 in α_1-AR-mediated apoptosis. As assessed by immunofluorescence, transfection of vascular smooth muscle cells with siRNA targeted against p53 resulted in a dose-dependent and statistically significant decrease in p53 expression. A nonsense siRNA (100 nM) had no effect on p53 expression (Fig. 6). The reduction in p53 levels with 100 nM siRNA had no effect on the ability of phenylephrine to promote increases in vascular ROS levels (Fig. 6B). In contrast, the same concentration of siRNA significantly decreased the ability of phenylephrine to stimulate apoptotic cell death (Fig. 6C).

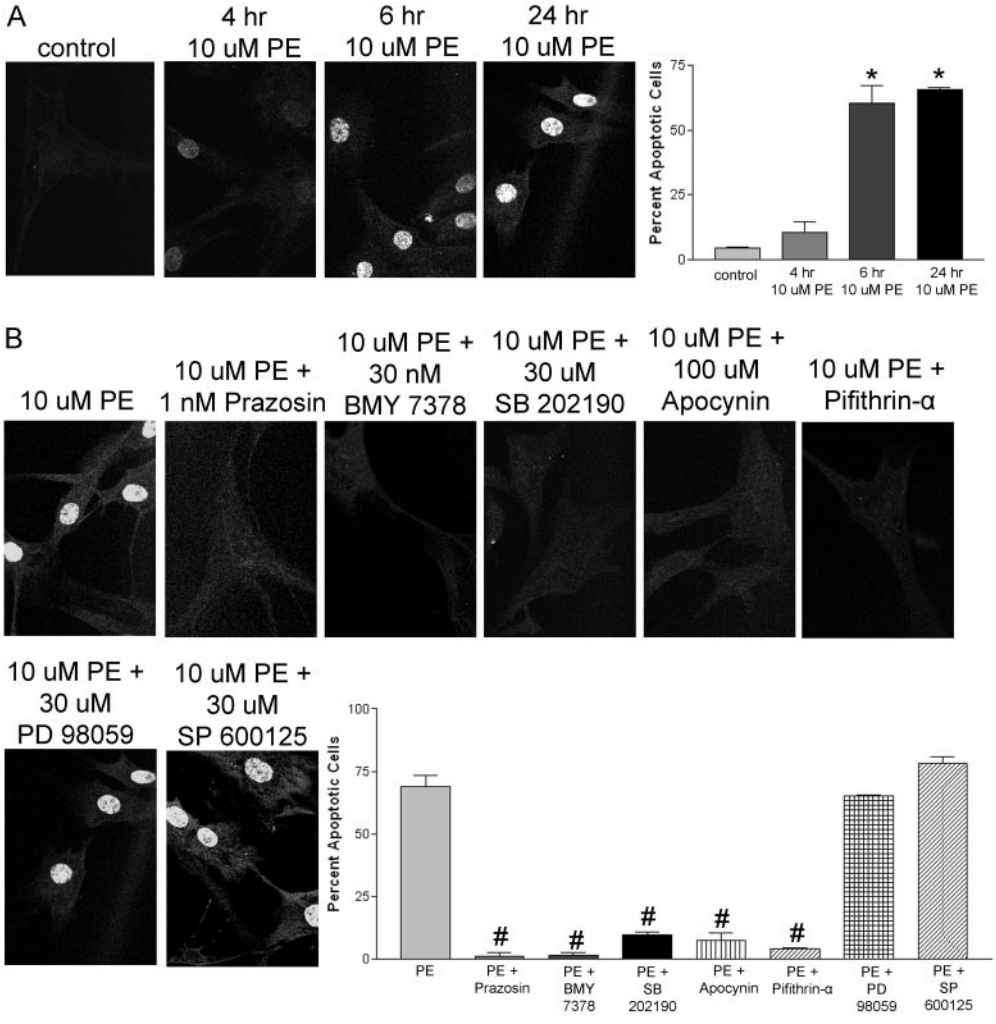

Fig. 4. A, effect of phenylephrine on vascular smooth muscle cell apoptosis. Phenylephrine (10 μM) was added for the indicated time. Apoptosis was quantitated by TUNEL assay as described under *Materials and Methods*. Each point represents the mean ± S.E.M. of four experiments each on a different cell population. Statistical significance was determined by a one-way ANOVA followed by Tukey's post test. *, $p < 0.5$, statistically different from control. B, effect of antagonists of various cellular signaling pathways on phenylephrine-induced apoptosis. Antagonists were incubated with human aortic smooth muscle cells for 45 min before the addition of 10 μM phenylephrine. Each point represents the mean ± S.E.M. of four experiments each on a different cell population. Statistical significance was determined by a one-way ANOVA followed by Tukey's post test. #, $p < 0.5$, statistically different from phenylephrine alone.

Discussion

The tonic effect of heightened sympathetic nervous system activity and circulating levels of catecholamines contributes to pathophysiologic conditions such as congestive heart failure, hypertension, and atherosclerosis. The sustained activation of the family of α_1-ARs plays a role in these maladaptive responses by the generation of ROS (Xiao et al., 2002; Bleeke et al., 2004; Zhang et al., 2004; Hao et al., 2006; Lyle and Griendling, 2006; Paravicini and Touyz, 2006; Fernandez-Patron, 2007), leading to vascular hypertrophy and apoptosis. The pathways linking the α_1-AR-stimulated increases in ROS to vascular pathological changes have been incompletely elucidated. In this report, we have probed the previously unrecognized link between this member of the G protein-coupled receptor family and the multifunctional tumor suppressor protein p53.

MitoTracker ROS was used as a specific indicator to detect mitochondrial increases in ROS. Activation of the α_1-AR resulted in a significant increase in MitoTracker fluorescence, indicating that this receptor is coupled to elevations in mitochondrial ROS levels. The α_1-AR-mediated increases in mitochondrial ROS were blocked by the nonselective α_1-AR blocker prazosin and by the selective α_{1D}-AR antagonist BMY 7378 (Fig. 2). The selective α_{1A}-AR blockers WB 4101 and 5-methylurapidil had no effect on phenylephrine-induced increases in ROS (Fig. 2). In our unpublished work, we show that BMY 7378 can block ROS generation in a dose-dependent manner. The dose of BMY 7378 used in these

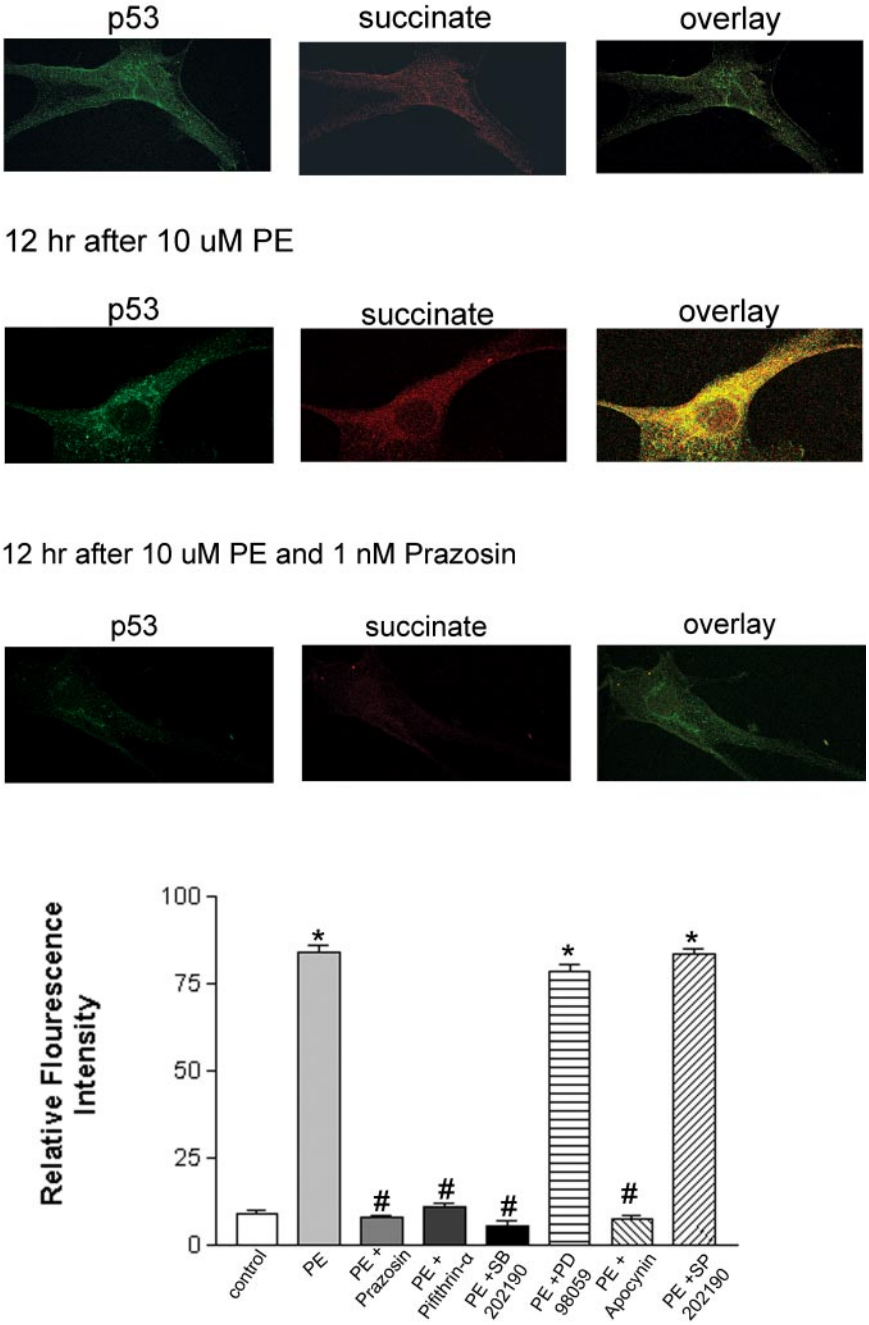

Fig. 5. Effect of phenylephrine treatment on the cellular localization of p53. Dual-label immunofluorescence was carried out as described under *Materials and Methods*. The localization of p53 and succinate dehydrogenase were determined alone and after treatment with 10 μM phenylephrine. The effect of pretreatment with a series of antagonists on the phenylephrine response was also determined. Although the effect of prazosin is illustrated with a photomicrograph, the data summaries are presented for all antagonists tested. Each point represents the mean ± S.E.M. of six experiments, each on a different cell population. Statistical significance was determined by a one-way ANOVA followed by Tukey's post test. *, $p < 0.5$, statistically different from control. #, $p < 0.5$, statistically different from phenylephrine alone.

studies (30 nM) would block 90% of the α_{1D}-AR population without effect on the other receptor subtypes (Piascik et al., 1997). Therefore, these data indicate that among members of the α_1-AR family, it is the α_{1D}-AR that is specifically coupled to mitochondrial increases in ROS.

We then examined potential signaling pathways linking the α_{1D}-AR to increases in ROS. Inhibition of ERK1/2 (with PD 98059; Fig. 3) or JNK (with SP 600125; Fig. 3) had no effect on α_{1D}-AR-mediated increases in ROS. Although the α_1-ARs are known to couple to these kinases (Xiao et al., 2006; Hein and Michel, 2007; Koshimizu et al., 2007; Perez, 2007), our data indicate that ERK1/2 or JNK do not play a role in the generation of ROS by the vascular α_{1D}-AR. The p53 antagonist pifithrin-α also had no effect on ROS generation (Fig. 3). These data argue that the tumor suppressor has no effect on the generation of ROS. In contrast to these results, the p38 MAP kinase inhibitor SB 202190 (30 μM) significantly impaired the ability of phenylephrine to increase ROS levels in human aortic smooth muscle cells (Fig. 3). These results provide evidence that the pathway leading to increases in ROS activated by the α_{1D}-AR includes p38 MAP kinase. Mitochondrial ROS increases were blocked by the NADPH oxidase inhibitor apocynin (Fig. 3). The α_1-ARs are known activators of NADPH oxidases (Xiao et al., 2002). These results indicate that for the α_{1D}-AR to increase mitochondrial ROS levels, NADPH must first be activated and generate cellular ROS. This observation is similar to the work of Doughan et al. (2008) who showed that angiotensin II-mediated increases in mitochondrial ROS were dependent on NADPH activation.

Sustained increases in ROS have been associated with apoptosis (Lyle and Griendling, 2006). We examined whether α_{1D}-AR-stimulated increases in mitochondrial ROS lead to apoptosis. Vascular smooth muscle cell apoptosis was ob-

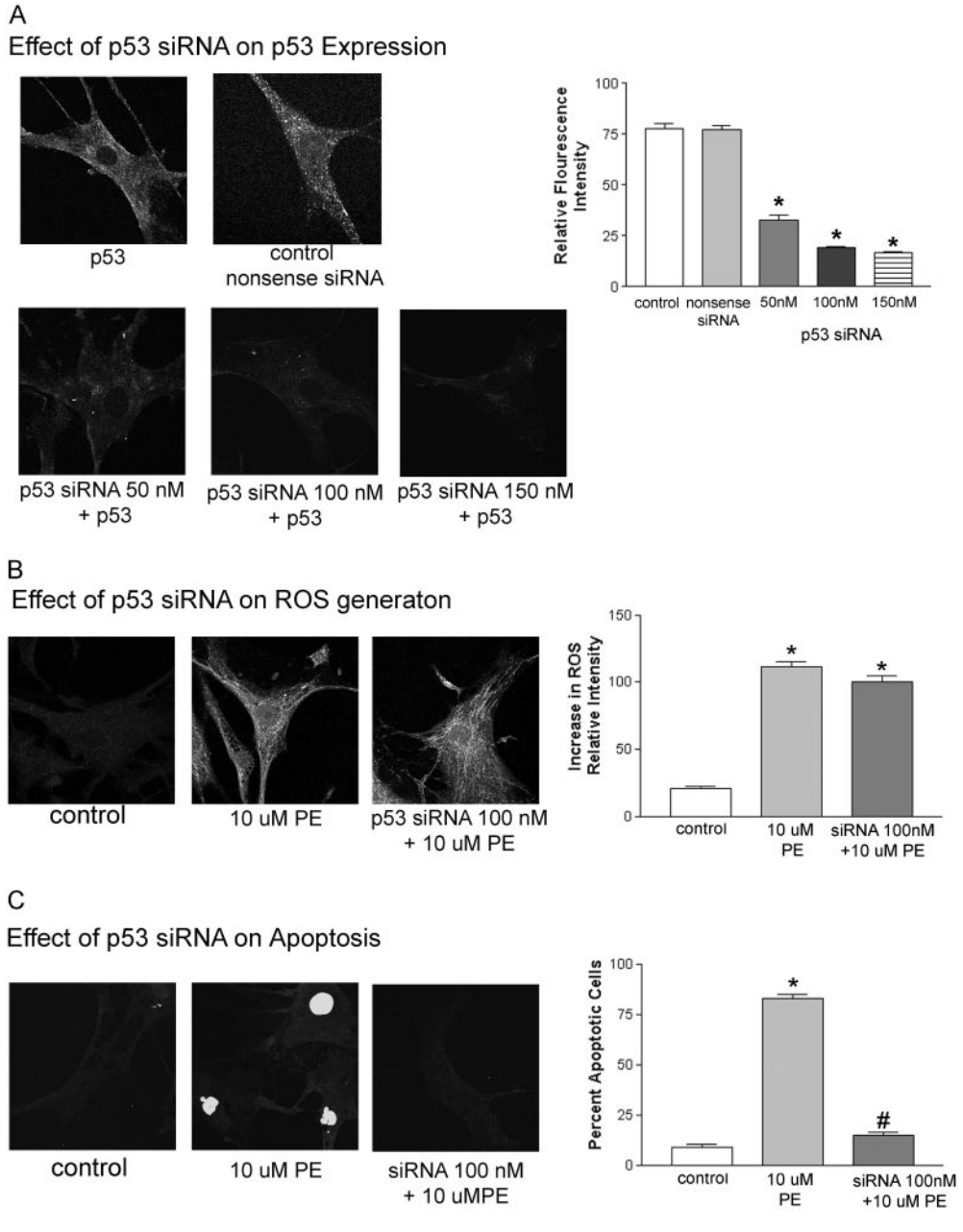

Fig. 6. Effect of increasing amounts of p53 siRNA on the expression of p53 in human aortic smooth muscle cells. A, experiments were carried out as described under *Materials and Methods*. Smooth muscle cells were treated with a p53 siRNA or a nonsense siRNA for 12 h. After this time, p53 expression was examined by immunocytochemistry. B, effect of p53 siRNA on phenylephrine-induced increases in ROS. C, effect of p53 siRNA on phenylephrine-induced apoptosis. Experiments were carried out as described under *Materials and Methods*. Each point represents the mean ± S.E.M. of four experiments each on a different cell population. Statistical significance was determined by a one-way ANOVA followed by Tukey's post test. *, $p < 0.5$, statistically different from control levels. #, $p < 0.5$, statistically different from phenylephrine alone.

served 6 h after activation of the α_{1D}-AR (Fig. 4A). These data argue that sustained increases in mitochondrial ROS ultimately trigger apoptotic cell death. Further evidence implicating ROS as the cause of the apoptosis shows that interventions that block ROS increases (prazosin, BMY 7478, SB 202190, or apocynin) also prevent apoptosis (Fig. 4B). Drug treatments (PD 98059 and SP 600125) that had no effect on ROS increases also had no effect on apoptosis (Fig. 4B). A well known mediator of apoptotic cell death is the tumor suppressor protein p53 (Nika et al., 2004; Okada and Mak, 2004; Erster and Moll, 2005; Moll et al., 2006; Vousden and Lane, 2007; Liu et al., 2008). p53 is known to trigger the mitochondrial cell death pathway (see above-cited references). We examined the effect of the specific p53 antagonist pifthrin-α on α_{1D}-AR-mediated responses in human aortic smooth muscle cells. In contrast to having no effect on ROS generation, pifthrin-α (10 μM) inhibited apoptosis induced by phenylephrine (Fig. 4B). The data indicate that p53 becomes apoptotically active in response to α_{1D}-AR-induced increases in ROS. Although p53 is a well known mediator of apoptosis, the α_1-ARs have not previously been shown to activate p53-dependent processes. Our results are the first to implicate p53 as a mediator of any process regulated by the α_1-ARs.

There is evidence that apoptosis is mediated in part by p53 translocation to the mitochondria and activation of the mitochondrial cell death pathway (Nika et al., 2004; Okada and Mak, 2004; Erster and Moll, 2005; Moll et al., 2006; Vousden and Lane, 2007; Liu et al., 2008). Our results indicate that the specific activation of the α_{1D}-AR promotes the mitochondrial translocation of p53. Any intervention that blocks either the α_{1D}-AR or the increase in ROS blocks p53 translocation (Fig. 5). Those treatments that are without effect on ROS levels fail to trigger translocation. These data argue that increases in mitochondrial ROS are the stimulus for p53 translocation.

To examine the hypothesis that p53 is involved in α_{1D}-AR-stimulated apoptosis in a different manner, we treated human aortic smooth muscle cells with siRNA directed against p53. Treatment with p53 siRNA resulted in a dose- and time-dependent decrease in the expression of smooth muscle cell p53 (Fig. 6A). These decreases in p53 expression were not associated with an inhibitory effect on α_{1D}-AR-mediated increases in ROS. Like the results obtained with pifithrin-α, these data argue that p53 is not involved in ROS increases. In contrast, siRNA treatment significantly impaired the ability of the α_{1D}-AR to trigger apoptosis. These data provide evidence that the α_{1D}-AR engages a pathway that ultimately results in p53 executed cell death. This conclusion is also supported by results with pifithrin-α.

In summary, we provide evidence for a role of the tumor suppressor protein p53 as a mediator of apoptosis induced by activation of the α_{1D}-AR. We also provide details of the pathway emanating from receptor activation to apoptosis. Agonist-mediated stimulation of the α_{1D}-AR leads to increases in mitochondrial ROS in a pathway that involved p38 MAP kinase and NADPH oxidase. The increase in ROS promotes the translocation of p53 to the mitochondria. Once assuming a mitochondrial location, p53 activates the mitochondrial cell death program, resulting in vascular smooth muscle apoptosis.

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