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The $Alpha_{1D}$-Adrenergic Receptor Induces Vascular Smooth Muscle Apoptosis via a p53-Dependent Mechanism

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Running Title:
alpha$_{1D}$-AR and p53

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Nonstandard abbreviations:

AR: adrenergic receptor
ROS: reactive oxygen species

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Abstract

Activation of the endogenous alpha$_1$-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 hr) of the alpha$_1$-AR resulted in smooth muscle cell apoptosis. Both the increase in ROS and apoptotic cell death were blocked by the non selective alpha$_1$-AR antagonist prazosin as well as the selective alpha$_{1D}$-AR antagonist BMY 7378. Increases in ROS and apoptosis produced by alpha$_1$-AR activation were also blocked by the p38 MAP kinase inhibitor SB 202190 and the NAPDH oxidase inhibitor apocynin. The ERK1/2 inhibitor PD 98059 or the JNK inhibitor SP 600125 were without effect on increases in ROS levels or apoptosis. Pifithrin alpha, an inhibitor of the tumor suppressor protein p53, had no effect on ROS generation but did block alpha$_{1D}$-AR-induced apoptosis. Activation of the alpha$_{1D}$-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 alpha. Apoptosis was also blocked by siRNA directed against p53. These data show that the alpha$_{1D}$-AR is coupled to the generation of mitochondrial ROS by a pathway involving p38 and NADPH oxidase. Sustained activation of the alpha$_{1D}$-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 alpha$_{1D}$-AR and p53.

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Introduction

Based on our current understanding, three subtypes of the alpha$_1$-AR; the alpha$_{1A}$-, alpha$_{1B}$-and alpha$_{1D}$-ARs have been cloned and characterized (see Hein and Michel, 2007, Koshimizu et al., 2007, Perez, 2007, Xiao et al., 2006 for recent reviews). Each of these subtypes transduce discrete actions of endogenous catecholamine neurotransmitters in the regulation of a variety of physiologic functions. While it is known that the alpha$_1$-ARs are coupled to increases in inositol phosphates, the canonical pathway for Gq coupled receptors (see above cited references), recent evidence has shown that the alpha$_1$-ARs are also coupled to the generation of reactive oxygen species (ROS) through novel signaling pathways such as activation of the EGF receptor and NADPH oxidase (Bleeke et al., 2004, Faber et al.,2007, Fernandez-Paton, 2007, Hao et al., 2006, Javadov et al., 2006, Kunster et al., 2005, Xiao et al., 2002, Zhang et al., 2004). Increases in vascular ROS have been associated with modulating smooth muscle contraction (Fernandez-Parton, 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). While p53 is well known to induce the transcription of pro-apoptotic proteins, there is also evidence that p53

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

In this report, we show that activation of the alpha$_1$-AR (more specifically the alpha$_{1D}$-AR) promotes increases of ROS levels in vascular smooth muscle. Prolonged activation of the alpha$_{1D}$-AR leads to apoptosis. Activation of the alpha$_{1D}$-AR promotes the translocation of p53 to the mitochondria. Pharmacologic inhibition of p53 or siRNA mediated knockdown of its expression prevents alpha$_{1D}$-AR apoptosis. These are the first data to link an alpha$_1$-AR to pathways involving the multifunctional regulatory protein p53.

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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 T75 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 Alpha$_1$-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 DMSO. 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 following preliminary studies of time and Mitotracker concentration. Cells were washed twice with medium, fresh medium applied and the effect of phenylephrine (Sigma-Aldrich, St. Louis, MO) 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(1nM Sigma-Aldrich) or 5-methylurapidil (1 nM Sigma-Aldrich), the p38

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MAP kinase inhibitor SB 202190 (30 uM, Sigma-Aldrich), the ERK1/2 inhibitor PD 98059 (30 uM Biomol, Plymouth Meeting, PA) the JNK inhibitor SP 600125 (30 uM, Biomol) the NADPH oxidase inhibitor apocynin (100 uM Calbiochem, Darmstadt, Germany) or the p53 antagonist pifithrin-alpha (10 uM Sigma-Aldrich). After preincubation with the antagonist, phenylephrine (10 uM) 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 63X oil immersion objective attached to a Leica AOBS TCS SP5 inverted Laser Scanning Confocal Microscope using Leica Application Suite Advanced Fluorescence software. Images were prepared using Adobe Photoshop version 7.0 (Adobe Systems, Mountain View, CA) and quantitated with ImageJ v3.91 software (http://rsb.info.nih.gov/ij). The fluorescence intensity of six areas (20x20 pixels) within a cell was measured and an intensity average determined for each cell. Data were analyzed by one-way analysis of variance followed by Tukey's post-hoc test using GraphPad Prism version 3.00 for Windows (GraphPad Software, 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 cover

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slips. The cellular localization of p53 and succinate dehydrogenase was assessed before and after a 12 hr treatment with 10 uM phenylephrine. In certain experiments cells were treated with the antagonist series described above prior to 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 hour at room temperature. After washing polyclonal antibodies against p53 (Calbiochem, diluted 1:50 in 1% BSA in PBS) or succinate dehydrogenase (Santa Cruz Biotechnology, Santa Cruz, CA, diluted 1:100 in 1% BSA in PBS) were added either alone or in combination and incubated overnight at 4°C. Following this incubation, the cells were washed with 0.05% BSA in PBS and secondary antibodies for either p53 (FITC, Jackson Laboratories, West Grove, PA, diluted 1:200 in PBS) or succinate dehydrogenase (Texas Red, Abcam, Cambridge, MA, diluted 1:200 in PBS) were added and incubated in the dark at room temperature for 1 hr. Cells were washed with PBS and mounted on glass slides with Prolong Gold™ antifade reagent (Invitrogen) and visualized under a 63X oil immersion objective with a confocal microscope as described above. Image analysis was performed with Metamorph software, (Version 6.2r6 Molecular Devices, Downingtown, PA), using the colocalization plug-in software tool. Fluorescence intensity was measured in six areas (20x20 pixels) within the cell and the extent of overlap of p53 over succinate dehydrogenase was

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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 the Alpha1-AR on the Apoptosis in Human Aortic Smooth Muscle Cells

Human aortic smooth muscle cells were plated in 35mm glass bottom culture dishes (MatTek Corporation, Ashland, MA). To assess the role of the alpha-AR on apoptosis, the cells were treated with phenylephrine for 12 hrs. In certain experiments the cells were pretreated for 45 min with a series of antagonists (see above) prior to phenylephrine addition. Cells were washed with PBS and fixed in fresh 4.0% paraformaldehyde for 1 hr. The cells were washed in PBS and permeabilized using freshly prepared 0.1% sodium citrate. After additional PBS washes, A TUNEL reaction mixture (*InSitu* Cell Death Detection Kit, Fluorescein from Roche Indianapolis, IN) which results in the transfer of fluoroscein dUTP to free 3' OH groups of genomic DNA, was added and incubated in a humidified chamber at 37° for 1 hr. 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, Mountain View, CA). 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 magnification of 20X. However, the data presented in Figures 4 and 6 are at a magnification of 40X to clearly see the presence of individual cells with apoptotic nuclei. Data

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were analyzed by one-way analysis of variance with Tukey's post-hoc test using GraphPad Prism.

Effect of p53 siRNA on Alpha1-AR-Induced Apoptosis in Human Aortic Smooth Muscle Cells

siRNA against p53 was obtained from Cell Signaling (Danvers, MA, Cat # 62305). The sense strand sequence for this siRNA was 5' – CUACUUCCUGAAAACAACGTT. A nonsense siRNA (Cat # 6201) 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 hours. 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.

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Results

Using the fluorescence indicator Mitotracker ROS, we examined the effect of alpha$_1$-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 alpha$_1$-AR subtypes (Garcia et al., 2008). Phenylephrine treatment resulted in a time and dose-dependent increase in ROS in these cell (see Figures 1A and 1B). ROS increases were apparent after 10 min of treatment and maximal after 45 min. The ED50 for phenylephrine was approximately 10 uM. Based on the data in Figure 1, we used a 20 minute treatment with 10 uM phenylephrine in all additional studies of ROS accumulation. The alpha$_1$-AR antagonist prazosin (1nM) completely blocked these increases in ROS (see Figure 2). The selective alpha$_{1D}$-AR antagonist BMY 7378 (30 nM) also blocked ROS increases while the selective alpha$_{1A}$-AR antagonists WB 4101 (1nM) and 5-methylurapidil (1 nM) were without effect (see Figure 2). These results indicate that the alpha$_{1D}$-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 alpha$_1$-AR to increases in ROS (see Figure 3). Previous work has shown that these concentrations of inhibitors are effective at inhibiting their respective targets (Bleeke et al., 2004, Hamanoue et al., 2007, Walton et al., 2005, Zhang et al., 2004). The p38 MAP kinase inhibitor SB 202190 (30uM) significantly impaired the ability of phenylephrine to increase ROS levels in human aortic smooth muscle cells (see Figure 3). Apocynin (100 uM), an inhibitor of NADPH

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oxidase also blocked mitochondrial ROS generation. The inhibitory actions of both SB 202190 and apocynin occurred in a concentration dependent fashion (see supplemental data, Figure 1). In contrast, neither the ERK1/2 inhibitor PD 98059 (30 uM) nor the JNK inhibitor SP 600125 (30 uM) had any effect on the phenylephrine-induced increase in ROS (see Figure 3). Pifithrin alpha, an inhibitor of the tumor suppressor protein p53 also had no effect on the generation of ROS induced by phenylephrine (see Figure 3).

Cellular increases in ROS can trigger apopotic cell death. A TUNEL assay was employed to determine if the alpha$_1$-AR-mediated increases in ROS resulted in smooth muscle cell apoptosis. Human aortic smooth muscle cells were exposed to 10 uM phenylephrine and the degree of apoptosis determined at 4, 6 and 24 hr. Phenylephrine produced a time-dependent apoptotic effect that was statistically significant at 6 hr (see Figure 4A). The percentage of apoptotic cells was observed in untreated aortic smooth muscle cells was 1.1 $\pm$ 0.3. The percentage of apoptotic cells increased to 71.5 $\pm$ 3.3 following phenylephrine treatment (see Figure 4). This quantitation was done at a magnification of 20X. However, to clearly show examples of apoptotic nuclei in individual cells, the data presented in Figure 4 are shown at 40X magnification. The apoptosis of human aortic smooth muscle cells was blocked by pretreatment with prazosin or the alpha$_{1D}$-AR selective antagonist BMY 7378 (see Figure 4B). Therefore, programmed cell death, like increases in ROS, was due to the activation of an alpha$_1$-AR, more specifically, the alpha$_{1D}$-AR. PD 98059 and SP 600125 were without effect on apoptosis (see Figure 4B). Inhibition of ROS

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generation with SB 202190 or apocynin blocked phenylephrine-induced apoptosis (see Figure 4B). The p53 antagonist, pifithrin alpha also blocked the apoptosis seen following phenylephrine treatment (see Figure 4B). The inhibitory actions of pifithrin alpha occurred in a concentration dependent fashion (see supplemental data, Figure 2). The results with pifithrin indicate that the multifunction tumor suppressor p53 mediates the apoptosis seen in response to the activation of the $alpha_{1D}$-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 $alpha_1$-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 (see Figure 5, green fluorescence) could be detected throughout the cell. Furthermore, there was no association with the mitochondrial localized succinate dehydrogenase (See Figure 5, red fluorescence). Phenylephrine treatment resulted in a time dependent translocation of p53 (see Figure 5). The emergence of the yellow fluorescence signal indicates that the green (p53) and red (succinate dehydogenase) now overlay one another. From these data we conclude that p53 is translocated to the mitochondria following activation of the $alpha_{1D}$-AR. This p53 fraction could be responsible for the apoptotic cell death seen following activation of the $alpha_1$-AR.

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We used siRNA to further evaluate the role of p53 in alpha$_1$-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 (see Figure 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 (see Figure 6B). In contrast, the same concentration of siRNA significantly decreased the ability of phenylephrine to stimulate apoptotic cell death (see Figure 6C).

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Discussion

The tonic effect of heightened sympathetic nervous system activity and circulating levels of catecholamines contribute to pathophysiologic conditions such as congestive heart failure, hypertension and atherosclerosis. The sustained activation of the family of alpha$_1$-ARs plays a role in these maladaptive responses by the generation of ROS (Bleeke et al., 2004, Fernandez-Paton, 2007, Hao et al., 2006, Lyle and Griendling 2006, Paravicini and Touyz 2006, Xiao et al., 2002, Zhang et al., 2004) leading to vascular hypertrophy and apoptosis. The pathways linking the alpha$_1$-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 GPCR 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 alpha$_1$-AR resulted in a significant increase in Mitotracker fluorescence indicating that this receptor is coupled to elevations in mitochondrial ROS levels. The alpha$_1$-AR mediated increases in mitochondrial ROS were blocked by the nonselective alpha$_1$-AR blocker prazosin and by the selective alpha$_{1D}$-AR antagonist BMY 7378 (see Figure 2). The selective alpha$_{1A}$-AR blockers WB 4101 and 5-methylurapidil had no effect on phenylephrine-induced increases in ROS (see Figure 2). In our unpublished work, we show that BMY 7378 can block ROS generation in a dose-dependent fashion. The dose of BMY 7378 used in these studies (30 nM) would block 90% of the alpha$_{1D}$-AR population without effect on the other receptor subtypes

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(Piascik et al., 1997). Therefore, these data indicate that among members of the alpha$_1$-AR family, it is the alpha$_{1D}$-AR that is specifically coupled to mitochondrial increases in ROS.

We then examined potential signaling pathways linking the alpha$_{1D}$-AR to increases in ROS. Inhibition of Erk1/2 (with PD 98059, see Figure 3) or JNK (with SP 600125, see Figure 3) had no effect on alpha$_{1D}$-AR mediated increases in ROS. While the alpha$_1$-ARs are known to couple to these kinases (Hein and Michel, 2007, Koshimizu et al., 2007, Perez, 2007, Xiao et al., 2006), our data indicate that Erk1/2 or JNK do not play a role in the generation of ROS by the vascular alpha$_{1D}$-AR. The p53 antagonist pifithrin alpha also had no effect on ROS generation (see Figure 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 (30uM) significantly impaired the ability of phenylephrine to increase ROS levels in human aortic smooth muscle cells (see Figure 3). These results provide evidence that the pathway leading to increases in ROS activated by the alpha$_{1D}$-AR includes p38 MAP kinase. Mitochondrial ROS increases were blocked by the NADPH oxidase inhibitor apocynin (see Figure 3). The alpha$_1$-ARs are known activators of NADPH oxidases (Xiao et al., 2002). These results indicate that for the alpha$_{1D}$-AR to increase mitochondrial ROS levels, NADPH must first be activated and generate cellular ROS. This observation is similar to the work of Doughhan et al (2008) who showed that angiotensin II mediated increases in mitochondrial ROS were dependent on NADPH activation.

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Sustained increases in ROS have been associated with apoptosis (Lyle and Griendling 2006). We examined whether alpha$_{1D}$-AR stimulated increases in mitochondrial ROS lead to apoptosis. Vascular smooth muscle cell apoptosis was observed 6 hrs after activation of the alpha$_{1D}$-AR (see Figure 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 show that interventions that block ROS increases (prazosin, BMY 7478, SB 202190 or apocynin) also prevent apoptosis (see Figure 4B). Drug treatments (PD 98059 and SP 600125) that had no effect on ROS increases also had no effect on apoptosis (see Figure 4B). A well known mediator of apoptotic cell death is the tumor suppressor protein p53 (Erster and Moll, 2005, Liu et al., 2008, Moll et al., 2006, Nika et al., 2004 Okada and Mack, 2004 Vousden and Lane 2007). p53 is known to trigger the mitochondrial cell death pathway (see cited references above). We examined the effect of the specific p53 antagonist, pifthrin alpha on alpha$_{1D}$-AR mediated responses in human aortic smooth muscle cells. In contrast to having no effect on ROS generation, pifthrin alpha (10 uM) inhibited apoptosis induced by phenylephrine (see Figure 4B). The data indicate that p53 becomes apoptotically active in response to alpha$_{1D}$-AR induced increases in ROS. While p53 is a well known mediator of apoptosis, the alpha$_1$-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 alpha$_1$-ARs.

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There is evidence that apoptosis is mediated in part by p53 translocation to the mitochondria and activation of the mitochondrial cell death pathway (Erster and Moll, 2005, Liu et al., 2008, Moll et al., 2006 Nika et al., 2004 Okada and Mack, 2004 Vousden and Lane 2007). Our results indicate that the specific activation of the alpha$_{1D}$-AR promotes the mitochondrial translocation of p53. Any intervention that blocks either the alpha$_{1D}$-AR or the increase in ROS blocks p53 translocation (see Figure 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 alpha$_{1D}$-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 (see Figure 6A). These decreases in p53 expression were not associated with an inhibitory effect on alpha$_{1D}$-AR mediated increases in ROS. Like the results obtained with pifithrin alpha, these data argue that p53 is not involved in ROS increases. In contrast, siRNA treatment significantly impaired the ability of the alpha$_{1D}$-AR to trigger apoptosis. These data provide evidence that the alpha$_{1D}$-AR engages a pathway that ultimately results in p53 executed cell death. This conclusion is also supported by results with pifithrin alpha.

In summary, we provide evidence for a role of the tumor suppressor protein p53 as a mediator of apoptosis induced by activation of the alpha$_{1D}$-AR. We also provide details of the pathway emanating from receptor activation to

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apoptosis. Agonist mediated stimulation of the alpha$_{1D}$-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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Footnotes

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Figure Legends

Figure 1

A) The effect of 10 uM phenylephrine on the time dependent increase in ROS levels in human aortic smooth muscle cells. Experiments were carried out as described in Methods. Each point represents the mean ± the standard error of the mean of 6 experiments each on a different cell population. Statistical significance was determined by a one way ANOVA followed by *Tukey Post test.* * indicates statistically different ($p < 0.5$) from control.

B) The 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 minutes later. Each point represents the mean ± the standard error of the mean of 4 experiments each on a different cell population. Statistical significance was determined by a one way ANOVA followed by Tukey Post test. * indicates statistically different ($p < 0.5$) from control.

Figure 2

The effect of alpha1-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 or 5-methylurapidil (1 nM) for 45 minutes prior to the addition of 10 um phenylephrine. ROS levels were measured 20 minutes following agonist addition. Each point represents the mean ± the standard error of the mean of 7 experiments each on a different cell population. Statistical significance was determined by a one way ANOVA followed by Tukey Post test. # indicates statistically different from phenylephrine alone.

Figure 3

The 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 minutes prior to the addition of phenylephrine. Each point represents the mean ± the standard error of the mean of 6 experiments each on a different cell population. Statistical significance was determined by a one way ANOVA followed by Tukey Post test. * indicates statistically different ($p < 0.5$) from control levels. # indicates statistically different from phenylephrine alone.

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Figure 4

A) Effect of phenylephrine on vascular smooth muscle cell apoptosis. 10 uM phenylephrine was added for the indicated time. Apoptosis was quantitated by TUNEL assay as described in Methods. Each point represents the mean ± the standard error of the mean of 4 experiments each on a different cell population. Statistical significance was determined by a one way ANOVA followed by Tukey Post test. * indicates statistically different ($p < 0.5$) from control.

B) The effect of antagonists of various cellular signaling pathways on phenylephrine-induced apoptosis. Antagonists were incubated with human aortic smooth muscle cells for 45 minutes prior to the addition of 10 uM phenylephrine. Each point represents the mean ± the standard error of the mean of 4 experiments each on a different cell population. Statistical significance was determined by a one way ANOVA followed by Tukey Post test. # indicates statistically different ($p < 0.5$) from phenylephrine alone.

Figure 5

The effect of phenylephrine treatment on the cellular localization of p53. Dual label immunofluorescence was carried out as described in Methods. The localization of p53 and succinate dehydrogenase were determined alone and after treatment with 10 uM phenylephrine. The effect of pretreatment with a series of antagonists on the phenylephrine response was also determined. While the effect of prazosin is illustrated with a photomicrograph, the data summaries are presented for all antagonists tested. Each point represents the mean ± the standard error of the mean of 6 experiments each on a different cell population. Statistical significance was determined by a one way ANOVA followed by Tukey Post test. * indicates statistically different ($p < 0.5$) from control levels. # indicates statistically different from phenylephrine alone.

Figure 6

The effect of increasing amounts of p53 siRNA on the expression of p53 in human aortic smooth muscle cells.
Panel A: Experiments were carried out as described in Methods. Smooth muscle cells were treated with a p53 siRNA or a nonsense siRNA for 12 hr. After this time, p53 expression was examined by immunocytochemistry. **Panel B:** The effect of p53 siRNA on phenylephrine-induced increases in ROS. **Panel C:** The effect of p53 siRNA on phenylephrine-induced apoptosis. Experiments were carried out as described in Methods. Each point represents the mean ± the standard error of the mean of 4 experiments each on a different cell population.

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Figure 1

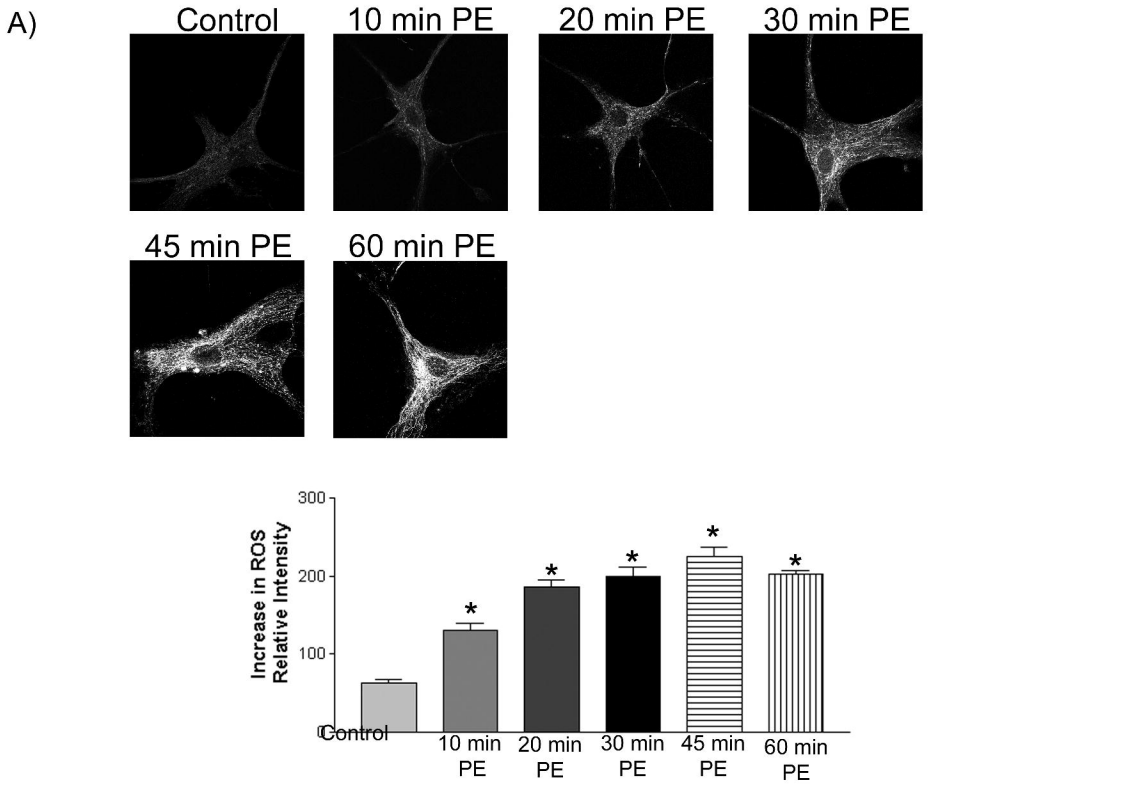

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Figure 2

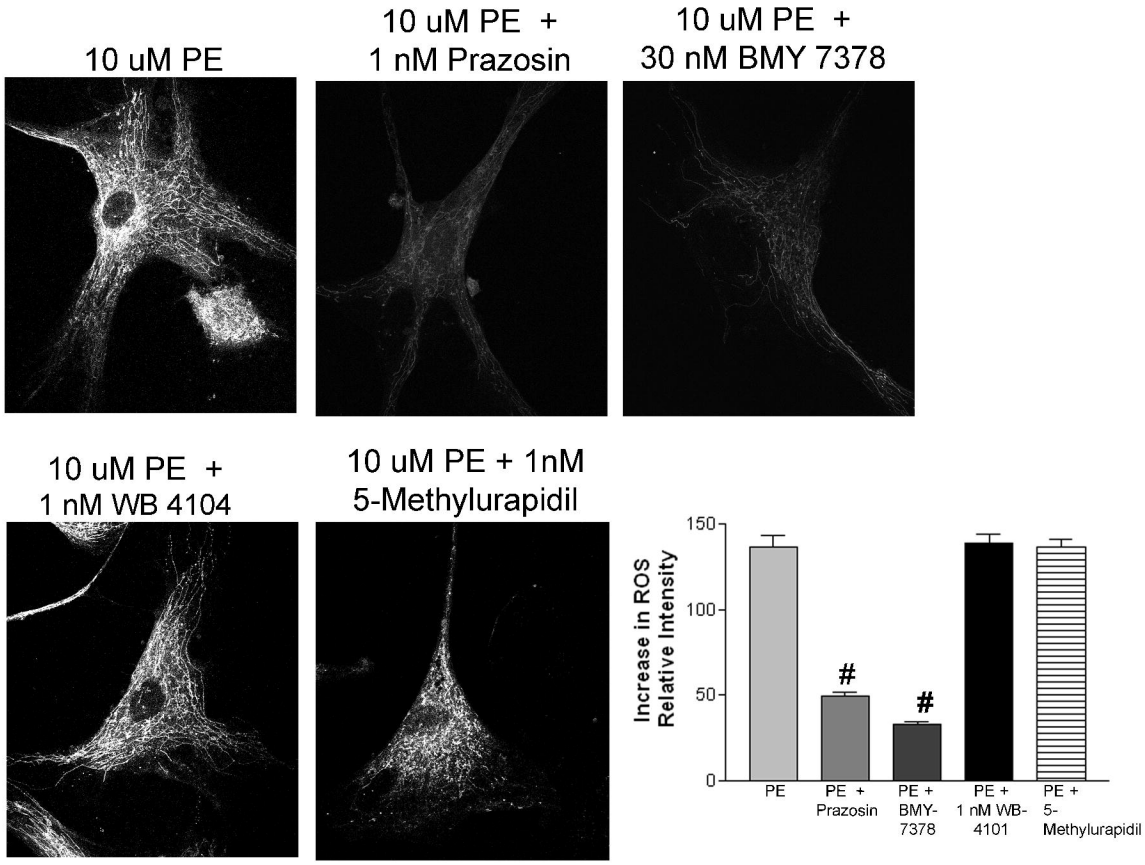

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Figure 3

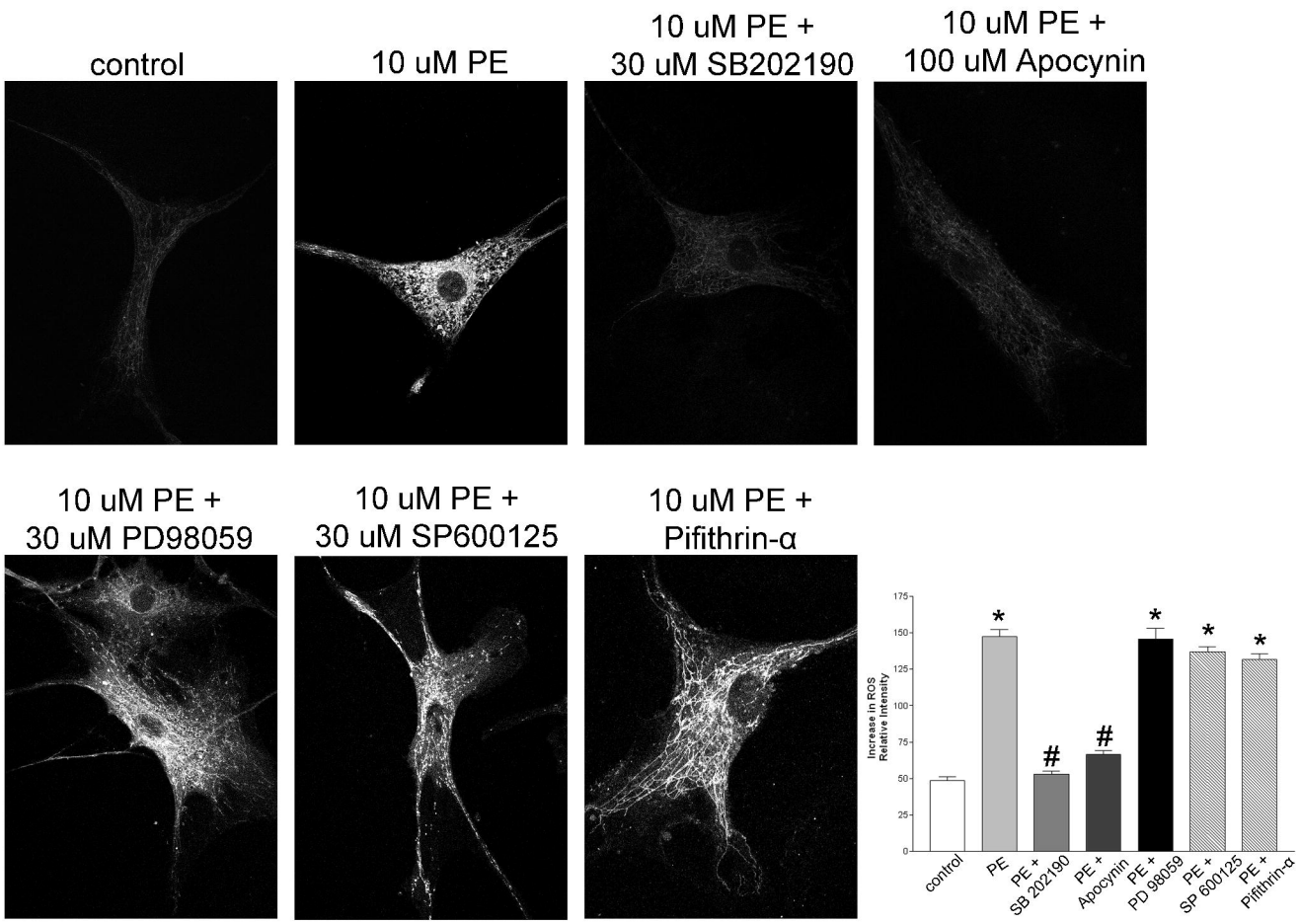

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Figure 4

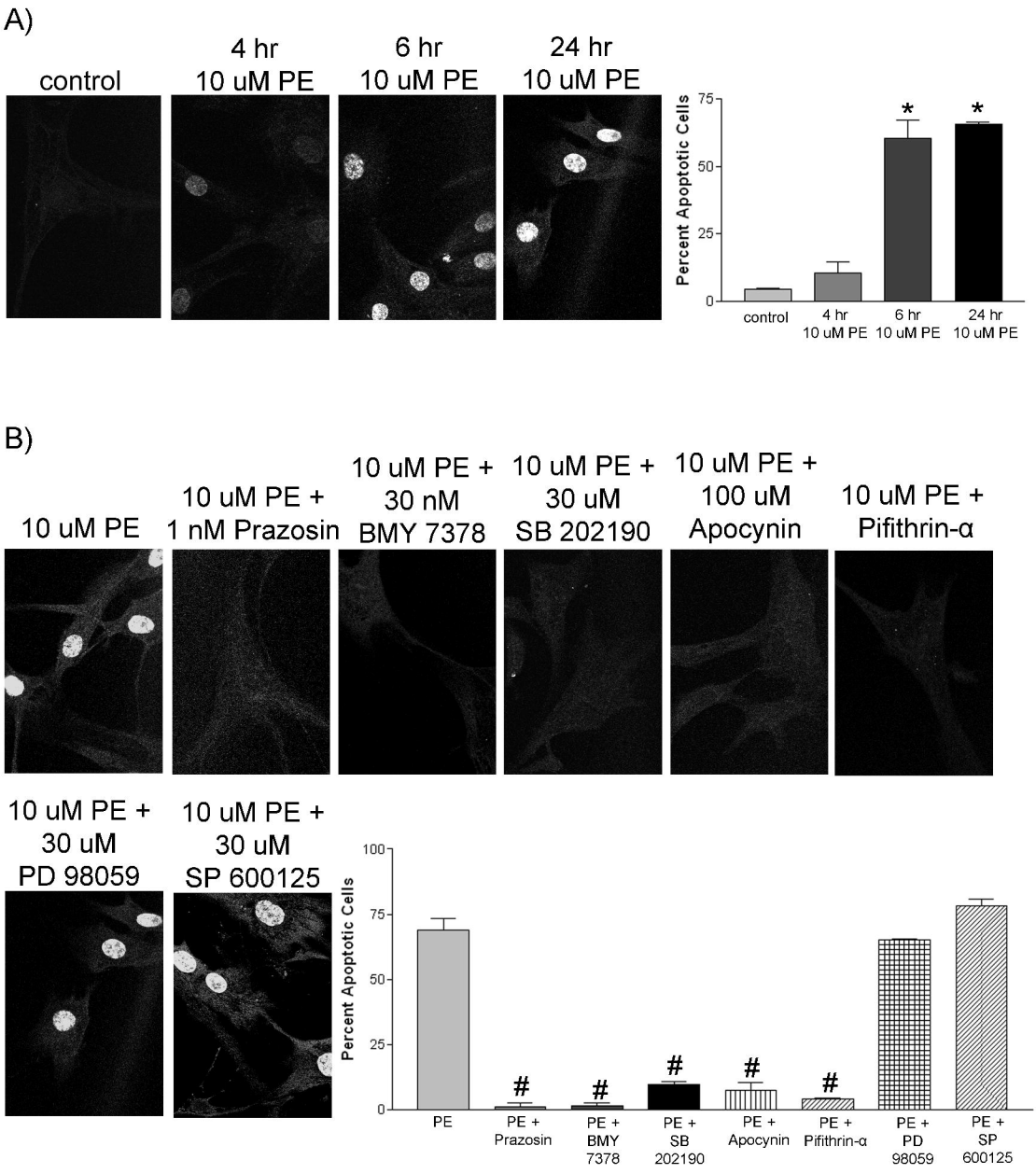

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Figure 5

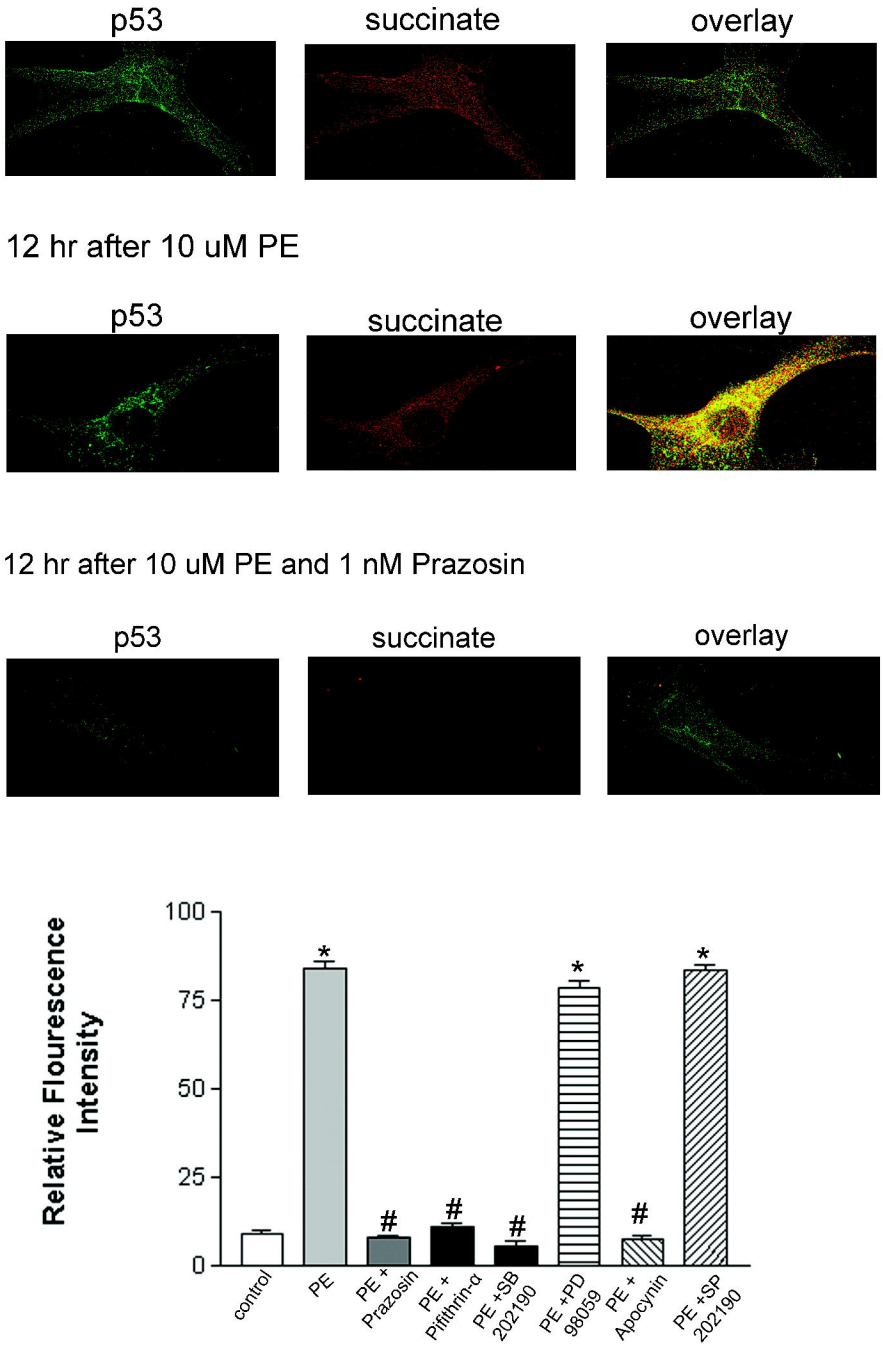

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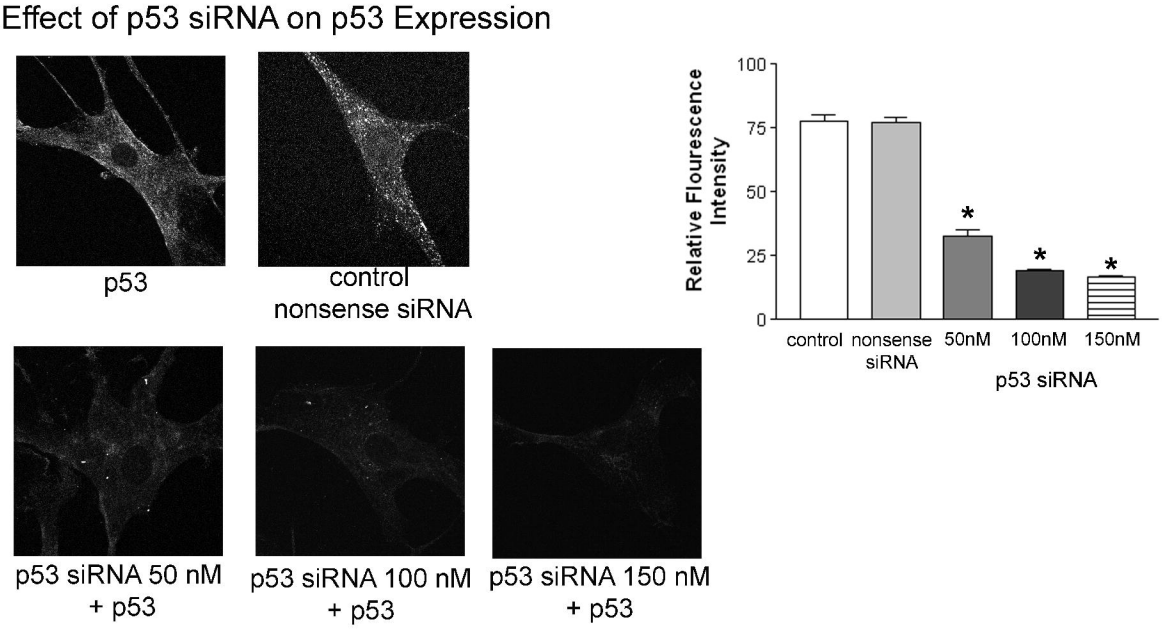
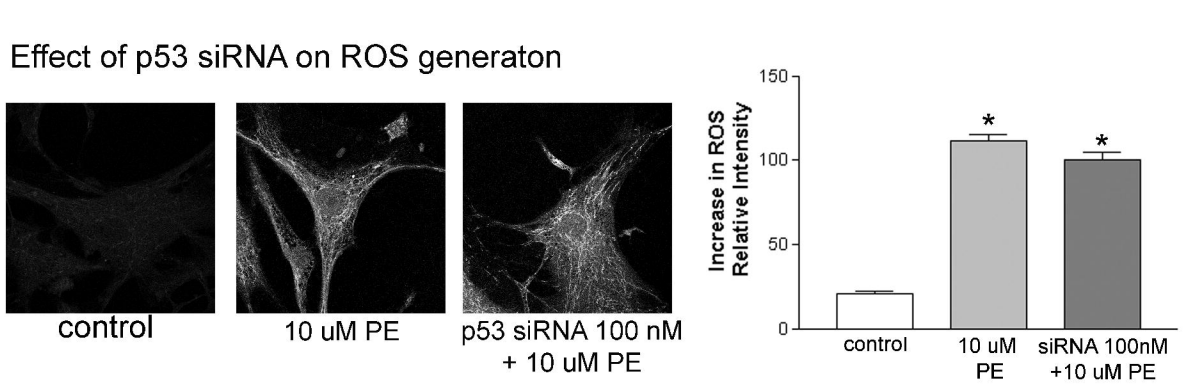
Figure 6

A)



B)



C)