Redox Regulation of Vascular Smooth Muscle Cell Differentiation

B. Su,* S. Mitra,* H. Gregg, S. Flavahan, M.A. Chotani, K.R. Clark,
P.J. Goldschmidt-Clermont, N.A. Flavahan

Abstract—Experiments were performed to determine the role of reactive oxygen species (ROS) in regulating vascular smooth muscle cell (VSMC) phenotype. After quiescence, cultured human VSMCs increased their expression of differentiation proteins (α-actin, calponin, and SM1 and SM2 myosin), but not β-actin. ROS activity, determined using the H₂O₂-sensitive probe dichlorodihydrofluorescein (DCF), remained high in quiescent cells and was inhibited by catalase (3000 U/mL) or by N-acetylcysteine (NAC, 2 to 20 mmol/L). A superoxide dismutase mimic (SOD; MnTMPyP, 25 μmol/L) or SOD plus low concentrations of NAC (SODNAC2, 2 mmol/L) increased DCF fluorescence, which was inhibited by catalase or by NAC (10 to 20 mmol/L). Inhibition of ROS activity (by catalase or NAC) decreased the baseline expression of differentiation proteins, whereas elevation of ROS (by SOD or SODNAC2) increased expression of the differentiation markers. The latter effect was blocked by catalase or by NAC (10 to 20 mmol/L). None of the treatments altered β-actin expression. SODNAC2-treated cells demonstrated contractions to endothelin that were absent in proliferating cells. p38 Mitogen-activated protein kinase (MAPK) activity was decreased when ROS activity was reduced (NAC, 10 mmol/L) and was augmented when ROS activity was increased (SODNAC2). Inhibition of p38 MAPK with pyridyl imidazole compound (SB202190, 2 to 10 μmol/L) reduced expression of differentiation proteins occurring under basal conditions and in response to SODNAC2. Transduction of VSMCs with an adenovirus encoding constitutively active MKK6, an activator of p38 MAPK, increased expression of differentiation proteins, whereas transduction with an adenovirus encoding dominant-negative p38 MAPK decreased expression of the differentiation proteins. These findings demonstrate that ROS can increase VSMC differentiation through a p38 MAPK–dependent pathway. (Circ Res. 2001;89:39-46.)

Key Words: reactive oxygen species ■ p38 MAPK ■ myosin ■ calponin

Vascular smooth muscle cells (VSMCs) exist in a diverse range of phenotypes. In normal mature blood vessels, the predominant phenotype is the contractile or differentiated VSMC, which has as its major function the regulation of blood vessel diameter and blood flow. During protective (ie, arteriogenesis) or pathogenic (ie, arteriosclerosis) vascular remodeling, VSMCs with a noncontractile or synthetic phenotype generate intimal vascular lesions. These VSMCs, termed dedifferentiated cells, have reduced expression of proteins required for normal regulation of contractile function (eg, smooth muscle–specific isoforms of myosin, actin, and calponin) and have increased capacity to generate extracellular matrix proteins. Dedifferentiated cells do not regulate contraction but instead control vascular construction.

The mechanisms regulating vascular smooth muscle differentiation and phenotypic modulation have not been fully defined. Indeed, the relationship between differentiated and dedifferentiated VSMCs is controversial. Although intimal dedifferentiated cells may derive from contractile cells, they also may result from a progenitor cell that becomes activated during vascular stress or injury. The widespread destruction of medial VSMCs that occurs during vascular remodeling is consistent with a terminal role for medial, contractile cells. During vascular remodeling, dedifferentiated VSMCs are capable of maturing into fully differentiated contractile VSMCs and this process can mimic the maturation process that occurs normally during vascular development.

Previous studies have demonstrated that reactive oxygen species (ROS) play an important permissive role in a number of responses involved in vascular remodeling, including proliferation, migration, and hypertrophy. The aims of the present study were to determine whether ROS have a modulatory role in regulating VSMC differentiation in vitro using cultured cells and to analyze the underlying signal transduction processes responsible for the modulation.

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From the Heart and Lung Institute (B.S., S.M., H.G., S.F., M.A.C., P.J.G.-C., N.A.F.) and the Department of Pediatrics, Ohio State University (K.R.C.), Columbus, Ohio.

*Both authors contributed equally to this work.

Correspondence to N.A. Flavahan, PhD, Heart and Lung Institute, Room 110E, 473 W 12th Ave, Columbus OH 43210. E-mail flavahan-1@medctr.osu.edu

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Materials and Methods

Cell Culture
Human aortic VSMCs (Clonetics Human Cell Systems, BioWhitaker, Inc, Walkersville, Md) were grown in Clonetics SMGM and studied from passage 7 through 11. For most experiments, cells were plated at 50 cells/mm², so that by the next day they had attained ~30% confluence. At that point, the cells were either harvested as proliferating cells or made quiescent by exchanging the growth media for quiescent media (50:50 mixture of DMEM and Ham’s F12 media with glutamine [200 µg/mL], penicillin/streptomycin [100 U/mL each], and ITS). When VSMCs are placed in culture, the expanding cell population represents immature, dedifferentiated cells, and this quiescent media is often used to induce a more differentiated phenotype.19–22 The influence of agents on VSMC phenotype was assessed after 3 days in the quiescent media, when the VSMCs had become quiescent. Preliminary experiments revealed that the optimal time for analyzing phenotypic modulation was after an additional 3-day treatment. Therefore, most experiments were performed after a 6-day quiescent period, with the first 3 days in quiescent media and the last 3 days in quiescent media with the test agent.

Western Blot Analysis of VSMC Phenotypic Modulation
Expression of VSMC differentiation proteins and β-actin was assessed by Western blot analysis of VSMC lysates, using mouse monoclonal antibodies that recognize the human proteins. Full details of the procedure are contained in an online data supplement available at http://www.circresaha.org.

p38 Mitogen-Activated Protein Kinase Assays
Aortic cells were rinsed with ice-cold PBS. The cells were lysed in buffer (New England BioLabs) containing antiproteases (as above), incubated for 5 minutes on ice, then scraped, sonicated on ice (4 times for 5 seconds each), and cleared by centrifugation (14 000 g) for 10 minutes at 4°C. Supernatants were used to assess p38 mitogen-activated protein kinase (MAPK) enzyme activity using the immunoprecipitation/ATF-2 phosphorylation assay kit from New England BioLabs, according to the manufacturer’s instructions.

Assessment of ROS Activity
Aortic cells were treated with the H₂O₂ -sensitive probe, 5-(and-6)-chloromethyl-2′, 7′-dichlorodihydrofluorescein diacetate (DCF, Molecular Probes), 5 µg/mL, for 30 minutes at 37°C in Krebs-Ringer bicarbonate solution (in mmol/L: 118.3 NaCl, 4.7 KCl, 1.2 MgSO₄ 1.2 KH₂PO₄, 2.5 CaCl₂, 25.0 NaHCO₃, and 11.1 glucose). When analyzing the effect of ROS inhibitors, the cells were incubated with the agents before DCF: 4 hours for catalase (to enable intracellular accumulation of the enzyme,17 2 hours for MnTMPyP (SOD), or 2.5 hours for N-acetylcycteine (NAC). After DCF incubation, in order to reduce stress-induced oxidant activation, the attached cells were cooled and harvested by trypsinization at 4°C. They were then collected by centrifugation (4°C, 500g), washed once in cold Krebs-Ringer solution, and analyzed by flow cytometry (FACSCalibur, Becton Dickinson).

Taqman/Realtime PCR
For quantitative measurement of mRNA, we used Realtime PCR with 18S ribosomal RNA as an internal control. For further detail, see the online data supplement.

Cell Cycle Analysis
Cells (5 × 10⁶) were trypsinized, pelleted, and resuspended in 0.5 mL cold PBS. While vortexing (at setting 4), 4.5 mL of cold ethanol was slowly added to the cells. Cell samples were stored at −20°C for ≥30 minutes before proceeding to DNA staining. The cells were pelleted by centrifugation (200g), washed twice with PBS, and resuspended in 1 mL PBS containing 50 µg/mL RNase A. After 20 minutes’ incubation at 37°C, propidium iodide was added to a final concentration of 5 µg/mL and incubated in darkness for ≥2 hours. Cell fluorescence was then determined using the FACSCalibur System. For each sample, 100 000 gated events were collected, and results were analyzed using ModFit LT (Verity Software House).

Silicon Gel Preparation and Cell Contraction
Preparation of the silicon gel substrate and analysis of cell contraction were performed as previously described23 and are detailed fully in the online data supplement.

Adenoviral Transduction
Cells were plated in a 6-well plate (50 cells/mm²) 1 day before transduction. The next day, growth medium (SMGM) was switched to quiescent medium, and cells were infected with replication-incompetent adenovirus at a multiplicity of infection of 250, optimized using Ad.β-gal and Ad.GFP viruses. After 16 hours, cells were recovered for 24 hours in SMGM followed by 72 hours in quiescent medium in the absence or presence of pyridyl imidazole compound (SB202190). Cells were then harvested for Western analysis. Dr J. Han (The Scripps Research Institute, La Jolla, Calif) kindly provided the adenoviruses Ad.MKK6 (encoding a constitutively active mutant of MKK6), Ad.P38DN (encoding a dominant negative mutant of p38 MAPK), and Ad.GFP (encoding green fluorescent protein).

Statistical Analysis
Statistical evaluation of the data was performed by Student’s t test for either paired or unpaired observations. When >2 means were compared, analysis of variance was used. If a significant F value was found, Scheffe’s test for multiple comparisons was used to identify differences among groups. Values were considered to be statistically different when P≤0.05.

An expanded Materials and Methods section can be found in the online data supplement available at http://www.circresaha.org.

Results

Modulation of ROS Activity in VSMCs
When human VSMCs were cultured in quiescent media, oxidant activity decreased by ~35% (Figure 1). Treatment with the antioxidant NAC (2 to 20 mmol/L) reduced ROS activity in a concentration-dependent manner in quiescent VSMCs (Figure 2), whereas a cell-permeable mimic of superoxide dismutase (SOD; MnTMPyP, 25 µmol/L) increased oxidant activity (Figure 2). Because DCF is sensitive...
Figure 2. Regulation of oxidant activity in VSMCs, determined by flow cytometry analysis of DCF fluorescence. A. Effect of the antioxidant NAC (2, 10, and 20 mmol/L), the superoxide dismutase mimic MnTMPyP (SOD, 25 μmol/L), or SOD (25 μmol/L) + NAC (2, 10, and 20 mmol/L) on the oxidant activity of quiescent VSMCs. Results are expressed as percentage of the control response and are presented as mean±SEM for 5 experiments. Inset shows FACS output for a single representative experiment. B. Effect of low concentrations of NAC (0.5, 1, 2 and 10 mmol/L) in the presence and absence of MnTMPyP (SOD, 25 μmol/L) on the oxidant activity of VSMCs. Results are expressed as percent of the control response and are presented as mean±SEM for 4 experiments.

Exogenous H₂O₂, at 10, 30, and 100 μmol/L increased DCF fluorescence to 166.8±15.7%, 200.6±15.8%, and 277.3±59.9% of control cells, respectively (n=4, P<0.05).

Redox Modulation of VSMC Phenotype
Consistent with their characterization as dedifferentiated VSMCs, proliferating cells expressed minimal levels of VSMC differentiation marker proteins (Figure 3). As previously reported,19–22 quiescence of VSMCs increased the differentiation characteristics of the cells, with increased expression of basic calponin, SM1 and SM2 myosins, and α-actin, but no change in the levels of β-actin (Figures 3A, 3B). When oxidant activity was decreased by NAC (2 to 10 mmol/L) or catalase (3000 U/mL), expression of the differentiation proteins was reduced, whereas expression of β-actin was unchanged (Figure 3). Furthermore, when oxidant activity was increased by SOD (25 μmol/L) or by NAC (2 mmol/L) plus SOD (25 μmol/L) (SODNAC2), there was a marked increase in expression of the differentiation proteins, but again the expression of β-actin remained unchanged (Figure 3). The effect of SODNAC2 to increase expression of the differentiation proteins was blocked by inhibiting oxidant activity with NAC (10 or 20 mmol/L) or catalase (Figure 3). In the presence of SOD (25 μmol/L), exogenous H₂O₂ (30 μmol/L) increased the expression of calponin (by 52.1±10.8%, n=4, P<0.05) and SM2 myosin (by 33.5±6.3%, n=4, P<0.05), but not β-actin (decreased by 9.2±2.6%, n=4, P<0.05).

The changes occurring in the expression of differentiation proteins was associated with altered levels of mRNA for the differentiation markers, determined by Realtime reverse transcriptase-PCR (Figure 4).

To determine the extent of the differentiation process, the contractile activity of cultured vascular smooth muscle cells was assessed using cells cultured on a silicone substrate. Cells cultured in this manner and treated with SODNAC2 contracted in response to endothelin (30 nmol/L), shortening by 33.6±6.7% (mean±SEM, n=29). Untreated quiescent cells also contracted in response to the agonist (shortening by 4.5±2.0%, n=14, P<0.05) but significantly less than did SODNAC2 cells. In proliferating cells, endothelin (30 nmol/L) did not evoke contraction but did stimulate calcium mobilization, assessed using fura-2–loaded cells (data not shown). By comparing lysates of cultured cells and of medial extracts of human aorta, expression of calponin and SM2 myosin in SODNAC2-treated cultured cells was estimated to be 18.7±2.1% and 17.9±2.3% (n=15), respectively, of expression in native smooth muscle cells.

Role of p38 MAPK in Redox Regulation of VSMC Differentiation
Experiments were performed to assess the downstream signaling mechanisms involved in the ROS-dependent increase in VSMC differentiation. The p38 and p42/44 MAPK signaling pathways in VSMCs are redox-sensitive, with p42/44 MAPK being highly sensitive to superoxide and p38 MAPK having increased sensitivity to H₂O₂.25,26 In quiescent VSMCs, the activity of p38 MAPK was decreased by NAC (10 mmol/L) and increased by SODNAC2 (Figure 5A). In
contrast, neither NAC (10 mmol/L) nor SODNAC2 had any effect on the activity of p42/44 MAPK (data not shown).

Inhibition of the p38 MAPK pathway by SB202190 (2 and 10 μmol/L) decreased the expression of differentiation proteins under normal quiescent conditions and the elevated levels after SODNAC2 treatment (Figure 5B) but did not affect expression of α-actin (data not shown). Inhibition of the p42/44 MAPK pathway by 2′-amino-3′methoxyflavone (PD98059, 3 to 30 μmol/L) did not affect expression of the differentiation proteins (data not shown).

Transduction of VSMCs with a replication-incompetent adenovirus encoding a constitutively active form of MKK6 (MKK6-CA), an activator of p38 MAPK, increased expression of calponin and SM2 myosin (Figure 6), an effect that was inhibited by SB202190 (10 μmol/L) (Figure 6). An adenovirus encoding a dominant-negative form of p38 MAPK (p38DN) decreased expression of the proteins, whereas transduction with control adenovirus had no effect (Figure 6). Expression of β-actin was not affected by MKK6-CA or p38DN (Figure 6).
ROS activity of VSMCs was not influenced by modulation of p38 MAPK activity. For example, inhibition of p38 MAPK by SB202190 (2 μmol/L) did not affect ROS activity under quiescent conditions (DCF fluorescence: control, 100%; SB202190, 91.4 ± 7.8%, n = 5, P = NS) or after SODNAC2 (percent of control: SODNAC2, 248.7 ± 54.7%; SODNAC2 + SB202190, 241.0 ± 64.4%, n = 5, P = NS). Similarly, activation of p38 MAPK after transduction of VSMCs with adenovirus encoding MKK6-CA did not alter ROS activity (control, 100%; MKK6-CA, 109.3 ± 5.26%, n = 3, P = NS).

Vascular Smooth Muscle Growth Responses

Oxidants have been implicated in growth and remodeling responses of VSMCs, including smooth muscle proliferation and cellular hypertrophy. Therefore, experiments were performed to determine whether the increased oxidant activity caused by SODNAC2 treatment was associated with smooth muscle growth. VSMCs became quiescent when cultured in the quiescent media and did not re-enter the cell cycle after SODNAC2 treatment (Figure 7A). VSMC hypertrophy refers to an increase in VSMC size and occurs during vascular development, hypertensive remodeling, or in cultured cells after exposure to hypertrophic stimuli. The size of VSMCs, assessed by forward-angle light scatter, did not increase after SODNAC2 treatment (Figure 7B).

Discussion

In the present study, proliferating human VSMCs displayed the characteristics of immature or dedifferentiated VSMCs. They had minimal expression of differentiation marker proteins and failed to contract to endothelin when cultured on a silicone substrate. Previous reports have demonstrated that proliferating cultured VSMCs can be converted to a more mature phenotype by placing the cells in quiescent media.
This was demonstrated in the present study by a selective increase in the expression of the differentiation proteins α-actin, SM1 myosin, SM2 myosin, and calponin, with no change in expression of β-actin. Although ROS are thought to play a key role in the reparative functions of dedifferentiated cells,16–18,33 VSMC quiescence was associated with only a small decrease in oxidant activity. When oxidant activity was reduced in these quiescent VSMCs, either by NAC or by catalase, the expression of the differentiation proteins decreased, whereas expression of nonmuscle β-actin was unaffected. These results indicate that increased expression of differentiation proteins after quiescence of VSMCs requires oxidant activity. Indeed, when oxidant activity was further increased by SOD or SODNAC2 there was a further selective increase in the expression of the differentiation proteins, with no change in the expression of β-actin. Again, this response was inhibited by catalase or by higher concentrations of NAC. Therefore, oxidant activity induced a more mature phenotype in VSMCs, which culminated in the resumption of contractile activity to endothelin-1.

The oxidant species responsible for increasing smooth muscle differentiation is likely to be H$_2$O$_2$. This is supported by the following observations: (1) Expression of the differentiation proteins closely mimicked the fluorescent activity of the H$_2$O$_2$-sensitive probe DCF; (2) SOD (or SODNAC2), which catalyzes the dismutation of superoxide to H$_2$O$_2$, increased H$_2$O$_2$ levels and increased expression of the differentiation proteins, but did not affect expression of β-actin; (3) catalase, which inactivates H$_2$O$_2$, inhibited expression of differentiation proteins occurring under basal conditions or after SODNAC2 treatment, but did not affect expression of β-actin; and (4) exogenous H$_2$O$_2$, increased expression of the differentiation markers. Therefore, basal production of H$_2$O$_2$ is important in the expression of a more differentiated phenotype after quiescence of VSMCs, and increased activity of endogenous H$_2$O$_2$ is associated with a further increase in the differentiated characteristics of the cells.

MAPK signaling pathways previously have been demonstrated to be oxidant-sensitive in VSMCs, with preferential activation of p38 MAPK by H$_2$O$_2$. Indeed, in human aortic VSMCs, the activity of the p38 MAPK, but not the p42/44 MAPK pathway, was decreased by NAC and increased by SODNAC2. Inhibition of the p38 MAPK signaling pathway by SB202190 decreased expression of the differentiation markers occurring under baseline conditions or in response to SODNAC2. This suggests that basal production of oxidants and subsequent activation of the p38 MAPK signaling pathway mediate the expression of differentiated characteristics in quiescent VSMCs. Further increases in oxidant activity (by SODNAC2) can further increase oxidant-mediated activation of p38 MAPK and the differentiated characteristics of VSMCs. Consistent with this proposal, expression of a dominant-negative mutant of p38 MAPK decreased expression of differentiation proteins, whereas activation of the p38 MAPK pathway using the constitutively active mutant of MKK6, an upstream stimulus for p38 MAPK, increased the expression of differentiation markers. Neither inhibition (with SB202190) nor activation (with MKK6-CA) of p38 MAPK...
altered oxidant activity in VSMCs. Therefore, these results indicate that activation of p38 MAPK, in either an oxidant-dependent or oxidant-independent fashion, increases the differentiation characteristics of VSMCs.

In VSMCs, CArG [CC(A/T)GG] or CArG-like motifs, located in the promoter regions of smooth muscle differentiation proteins, are thought to play a key role in regulating these genes during differentiation.5,15,34,35 A key step in transcriptional activation is the formation of a multiprotein complex containing serum response factor (SRF).35 Indeed, the CArG-binding activity of SRF is higher in differentiated compared with dedifferentiated cells, despite similar expression of SRF in the 2 cell types.34 Because p38 MAPK can increase SRF-mediated transcriptional activation,36,37 this may account for the ROS/p38 MAPK–dependent increase in smooth muscle differentiation observed in the present study.

Although oxidants and oxidant-mediated activation of p38 MAPK played an important role in determining VSMC phenotype, the influence of oxidants was not evident in proliferating cells. In those cells, oxidant activity was high relative to that in quiescent cells, but there was no stimulation of cellular differentiation. This may reflect the activity of serum factors, such as platelet-derived growth factor (PDGF), which are known to inhibit the expression of differentiation proteins.38–40 PDGF also increases oxidant activity,17 suggesting that its inhibitory effect on differentiation can occur despite elevated ROS levels. Indeed, the overall effect of oxidants in VSMCs likely will be determined by the activity of other signaling pathways. Therefore, although oxidants and p38 MAPK can increase the differentiation characteristics of VSMCs, activation of other signaling pathways may suppress or redirect these signaling pathways to alternate VSMC responses. Indeed, ROS have been implicated in regulating a diverse range of cellular responses, including migration, proliferation, apoptosis, senescence, and hypertrophy.16,18,27,28,33,41,42

VSMCs display 2 distinct growth responses: hyperplasia, characterized by increased DNA/protein synthesis and cell division, and hypertrophy, characterized by increased cell size and protein content without DNA synthesis or cell division. Increased oxidant activity is thought to play a key role in the proliferative response of VSMCs,38,39,41,42 and in the hypertrophic response to angiotensin II.26,31,42,43 However, SODNAC2 did not influence VSMC size and did not stimulate VSMC proliferation. Furthermore, unlike hypertrophy, which may result in large part from a generalized increase in protein synthesis,44,45 the effects of SODNAC2 in increasing expression of differentiation proteins was specific and was associated with increased transcript levels for the differentiation proteins. Therefore, the increased oxidant activity associated with SODNAC2 was not associated either with VSMC hypertrophy or hyperplasia. This observation appears to contrast with the proposed role of cellular H2O2 and p38 MAPK signaling in the hypertrophic effects of angiotensin II. Angiotensin II increased the size and protein synthesis of quiescent VSMCs, with the latter effect being reduced by antioxidants (NAC) or by inhibition of p38 MAPK signaling.26,31,42,43 This may reflect an interaction of these signaling systems with distinct signaling pathways activated by angiotensin II. Indeed, the p38 MAPK signaling pathway was identified as only one of multiple interacting components in the hypertrophic signal transduction pathway.26

Conclusions

The results of the present study demonstrate that endogenous ROS can increase VSMC maturation and differentiation through a p38 MAPK–dependent pathway. Previous studies have demonstrated independently that oxidant activity or p38 MAPK can induce differentiation of other cell types. Indeed, oxidant activity has been proposed as a generalized stimulus for cell differentiation during development.46,47 Therefore, this pathway may contribute to regulating smooth muscle maturation during development and vascular remodeling.

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