Important role for Rac1 in regulating reactive oxygen species generation and pulmonary arterial smooth muscle cell growth

Sandip Patil, Melisa Bunderson, Jason Wilham, and Stephen M. Black. Important role for Rac1 in regulating reactive oxygen species generation and pulmonary arterial smooth muscle cell growth. *Am J Physiol Lung Cell Mol Physiol* 287: L1314–L1322, 2004. First published September 17, 2004; doi:10.1152/ajplung.00383.2003.—Vascular NADPH oxidases have been shown to be a major source of reactive oxygen species (ROS). Recent studies have also implicated ROS in the proliferation of vascular smooth muscle cells. However, the components required for activation of the NADPH oxidase complex have not been clearly elucidated. Here we demonstrate that ROS generation in ovine pulmonary arterial smooth muscle cells (PASMCs) requires the activation of Rac1, implicating this protein as an important subunit of the NADPH oxidase complex. Our results, using a geranylgeranyl transferase inhibitor (GGTase-I-287), demonstrated a dose-dependent inhibition of Rac1 activity and ROS production. This was associated with an inhibition of PASMC proliferation with an arrest at G2/M. The inhibition of Rac1 by GGTI-287 led us to more specifically target Rac1 to investigate its role in the generation of ROS and cellular proliferation. To accomplish this, we utilized a dominant negative Rac1 (N17Rac1) and a constitutively active Rac1 (V12Rac1). These two forms of Rac1 were transiently expressed in PASMCs using adenovirus-mediated gene transfer. N17Rac1 expression resulted in decreased cellular Rac1 activity, whereas V12Rac1 infection showed increased activity. Compared with controls, the V12Rac1-expressing cells had higher levels of ROS production and increased proliferation, whereas the N17Rac1-expressing cells had decreased ROS generation and proliferation and cell cycle arrest at G2/M. However, the inhibition of cell growth produced by N17Rac1 overexpression could be overcome if cells were co-incubated with the Cu,Zn superoxide dismutase inhibitor DETC. These results indicate the importance of Rac1 in ROS generation and proliferation of vascular smooth muscle cells.

vascular reduced nicotinamide adenine dinucleotide phosphate oxidase

THE NADPH OXIDASE COMPLEX is a multisubunit enzyme that has been widely studied with respect to its role in the innate immune response by phagocytic cells (4). Activation of NADPH oxidase results in generation of reactive oxygen species (ROS), which are important in the host defense against infection (4). More recently, ROS have been demonstrated to be important modulators of vascular cell function. ROS have been shown to be generated in response to a number of ligands such as angiotensin II (10), epidermal growth factor (37), platelet-derived growth factor (21), basic fibroblast growth factor (16), phenylephrine (38), endothelin-1 (5, 34, 41), and serotonin (18, 19). In addition, the proliferation of smooth muscle cells (SMCs) has been strongly linked to the generation of ROS such as superoxide and H2O2 (5, 34, 41). It has been demonstrated that the major source of ROS in vascular cells is the NADPH oxidase complex (3, 23, 26, 28).

The Rac1 enzyme has been shown to play a critical role in the assembly and activation of NADPH oxidase in the neutrophil form of the enzyme complex (1, 13). However, the role of Rac1 in the vascular NADPH oxidase system remains unresolved. Rac1 is a 21-kDa GTP binding protein belonging to the Ras superfamily. Rac1 cycles between an inactive GDP bound and, upon stimulation, an active GTP bound form. This cycle is regulated by GDP exchange factors and GTPase activating factors. Although much of the work on the regulation of NADPH oxidase signaling has been in the phagocytic context (4), recent strides have been made in the understanding of its role in cardiac and vascular cells (11). However, key differences exist between the neutrophil and the cardiovascular NADPH oxidases such as the location and the rate of generation of ROS. Thus it will be important to evaluate the role of potential subunit components in the vascular NADPH oxidase complex and contrast and compare with the phagocytic form. To begin to examine the components required for formation of an active vascular NADPH oxidase complex we chose Rac1. Amongst the structural features involved in its activation, the Rac1 protein contains a cysteine residue in its COOH terminus to which a geranylgeranyl group is attached by a thioether linkage (36). Thus geranylgeranylation is a necessary post-translational modification that allows Rac1 to participate in the formation and activation of the NADPH oxidase complex. Proteins such as Rac1, Rho1, and Rap1A are geranylgeranylated by enzymes known as geranylgeranyl transferase I (GGTase-I). GGTase-I recognizes proteins that end with the motif CAAX, where X is leucine or isoleucine (6). Recently, CAAX peptidomimetics have been made that selectively inhibit the geranylgeranylation by GGTase-I (31).

Here we show that pharmacological inhibition of geranylgeranylation blocks the production of ROS and cell growth in vascular SMCs. By more specifically targeting Rac1 using dominant negative and constitutively active Rac1 proteins expressed from adenoviral constructs, we have shown that the modulation of ROS generation can alter SMC proliferation, indicating the critical requirement for Rac1 in regulating both NADPH oxidase activity and subsequently SMC growth. The effect of the overexpression of the dominant negative Rac1 mutation was the inhibition of SMC proliferation at the G2/M phase of the cell cycle. This inhibition could be overcome if...
ROS levels were increased in the SMCs. These data suggest that the targeting of Rac1 may have therapeutic potential for the treatment of cardiovascular disease in which increased oxidative stress is thought to play an important role.

MATERIALS AND METHODS

Cell culture and adenoviral infections. Pulmonary arterial smooth muscle cells (PASMCs) from 4-wk-old lambs were isolated by techniques previously described (41, 42). PASMC identity was confirmed as being positive for SMC actin, caldesmon, and calponin. Cells were maintained in Dulbecco’s modified Eagle’s medium (Mediatech) containing 10% fetal bovine serum (FBS), Hyclone) and antibiotics. Cells were maintained in a 5% CO₂ atmosphere at 37°C and split 1:4 at 80% confluence.

Adenoviral constructs for constitutively active (AdV12Rac1) and dominant negative (AdN17Rac1) Rac were a gift from Dr. Toren Finkel (National Institutes of Health). The viruses were amplified in dominant negative (AdN17Rac1) Rac were a gift from Dr. Toren Finkel (National Institutes of Health). The viruses were amplified in 6-well plates and treated as described above. Treatment was carried out for 72 h. Dihydroethidium (DHE, 20 μM; Molecular Probes) was added to the media 15 min before the end of the experiment. Cells were washed with PBS and imaged under a Nikon Eclipse TE-300 microscope. DHE-stained cells were observed after excitation at 518 nm and emission at 605 nm. Fluorescent images were captured with a CoolSnap digital camera, and the average fluorescent intensities (to correct for differences in cell number) were quantified using Metamorph imaging software (Fryer) as we have previously described (41). Statistical analysis was carried out as detailed below (see Statistical analysis).

Terminal deoxynucleotidyltransferase dUTP nick end labeling assays. Terminal deoxynucleotidyltransferase dUTP nick end labeling (TUNEL) assays were performed using the Dead End Fluorometric TUNEL System Kit (Promega) according to the manufacturer’s protocol. Briefly, cells grown in 96-well plates were fixed in 4% formaldehyde at 4°C for 24 h. The cells were washed, treated with 0.2% Triton X, and equilibrated in equilibration buffer followed by further incubation in assay buffer containing fluorescein-12 dUTP for 60 min at 37°C. The reaction was terminated with 2× saline-sodium citrate buffer incubated at room temperature with 4′,6-diamidino-2-phenylindole (DAPI, 5 μg/ml) stain in PBS for 15 min and viewed under a Nikon TE300 fluorescent microscope. DAPI is a blue fluorescent nuclear stain and was used to confirm the presence of cells that were negative for TUNEL staining. Again, fluorescent images were captured with a CoolSnap digital camera.

Flow cytometry. Cell cycle analysis was performed with propidium iodide-stained nuclei according to the manufacturer’s protocol (Becton-Dickinson). Briefly, cells were washed and reconstituted at 10⁶ cells/ml. Cells were stained with propidium iodide stain solution for 20 min at 37°C. To this an equal volume of 4 M NaCl salt solution was added. This was stored for 6 h before flow cytometry analysis. The distribution was determined using ModFit software.

Statistical analysis. All values are expressed means ± SE. Band intensities from Western blot analyses were determined densitometrically using Kodak 1D software version 3.5. Comparisons were made by ANOVA. A value of P < 0.05 was considered statistically significant.

RESULTS

Initially we used GGTL-287 (0–5 μmol/l) to examine its effects on Rac1 expression and activity in PASMCs. Results obtained indicated that GGTL-287 had no effect on Rac1 expression at any concentration used (Fig. 1, A and B). However, treatment with GGTL-287 produced a dose-dependent inhibition of Rac1 activity with activity being reduced by 62% at 25 μmol/l (P < 0.05 vs. untreated, Fig. 1, C and D). Next, using the oxidation of DHE to ethidium as an estimate of cellular ROS generation, we examined the dose-dependent effect of GGTL-287 on cellular ROS levels. The results obtained indicate that GGTL-287 dose dependently inhibited ethidium fluorescence, indicating a key role for Rac1 in the production of ROS in PASMCs (Fig. 2).

Because we have previously demonstrated the importance of ROS in regulating SMC growth (41), the inhibition of ROS...
generation led us to determine whether there were alterations in PASMC growth. As in the previous experiments, PASMCs were exposed to GGTI-287 (0–50 μmol/l), and the effect on proliferation to 10% FBS was determined at 72 h (Fig. 3). At concentrations of up to 5 μmol/l, GGTI-287 had no effect on the inhibition of cell growth compared with control. However, at 25 μmol/l, GGTI-287 inhibited the proliferation of PASMCs (Fig. 3A). Correlating with the effect of GGTI-287 on Rac1 activity (Fig. 1D), this result suggests that inhibition of PASMC growth requires that Rac1 activity be less than a threshold level (~40% of untreated cells, Fig. 1D). Using GGTI-287 at 25 μmol/l, we next performed cell cycle analysis using flow cytometry to compare the cellular distribution in various phases of the cell cycle (Fig. 3B). The results obtained indicated that there was a twofold increase in the number of cells in the G2/M phase compared with untreated cells (P < 0.05 vs. untreated, Fig. 3C).

Our previous studies have detected an increase in programmed cell death associated with a decrease in cellular ROS (41). Thus using the TUNEL assay, we determined whether the decrease in ROS induced by GGTI-287 exposure was associated with an increase in PASMC apoptosis. However, cells treated with GGTI-287 were TUNEL negative for all doses tested (Fig. 4), although the addition of 4-(2-aminoethyl)-benzenesulfonyl fluoride, which inhibits NADPH oxidase assembly, (8) induced SMC apoptosis as we have previously described (41).

GGTI-287 inhibits the activation of a number of small GTP binding proteins. Thus we next wanted to determine whether the effects we observed with this agent were specifically due to its ability to inhibit Rac1. To examine this, adenoviruses containing Rac1 mutants were used to infect PASMCs. The overexpression of the Rac1 mutants was initially confirmed by Western blot analysis (Fig. 5). Results obtained demonstrate that Rac1 levels were increased by twofold in cells infected with the dominant negative (N17Rac1) or constitutively active (V12Rac1) mutant proteins compared with uninfected cells or with cells infected with AdGFP (P < 0.05, Fig. 5, A and B). The lack of significant differences in Rac1 expression between the uninfected and AdGFP-infected cells indicates that adenoviral infection by itself did not alter Rac1 expression (Fig. 5, A and B). We next determined Rac activity. No significant differences were observed between the uninfected PASMCs and the GFP-infected cells, indicating that adenoviral infection by itself did not alter Rac activity. However, the results obtained indicate that Rac1 activity was altered by the presence of the rac1 regulatory protein.
or absence of serum. The levels of Rac1 activity in the uninfected PASMCs and the GFP-infected cells was significantly decreased in the absence of 10% FBS (Fig. 5, C and D). Cells infected with AdV12Rac1 (constitutively active) showed a twofold increase in Rac1 activity compared with controls in the absence of 10% FBS (Fig. 5, C and D).

Fig. 3. GGTI-287 inhibits the proliferation of PASMCs. A: PASMCs were plated in 96-well plates (2,000/well) and allowed to attach for 12 h. Cells were treated with increasing doses of GGTI-287 (0–25 μmol/l) in the presence of 10% FBS as a stimulus for proliferation. The number of viable cells was determined at 0 and 72 h. Cells were treated with GGTI-287 for 72 h in the presence of 10% FBS. Terminal deoxynucleotidyltransferase dUTP nick end labeling (TUNEL) assay was performed as described in MATERIALS AND METHODS. Cells were costained with 4',6-diamidino-2-phenylindole (DAPI) to show identify all cells in each field. GGTI-287 did not induce a marked increase in TUNEL-positive nuclei relative to untreated cells. However, the presence of 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF, 1 mmol/l) to prevent NADPH oxidase assembly did induce an increase in TUNEL-positive nuclei. This experiment was repeated 3 times with the same result.

Fig. 4. GGTI-287 does not induce apoptosis in PASMCs. Cells were plated in 96-well plates, allowed to attach, and then were treated or not with GGTI-287 (5 and 25 μmol/l) for 72 h in the presence of 10% FBS. Terminal deoxynucleotidyltransferase dUTP nick end labeling (TUNEL) assay was performed as described in MATERIALS AND METHODS. Cells were costained with 4',6-diamidino-2-phenylindole (DAPI) to show identify all cells in each field. GGTI-287 did not induce a marked increase in TUNEL-positive nuclei relative to untreated cells. However, the presence of 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF, 1 mmol/l) to prevent NADPH oxidase assembly did induce an increase in TUNEL-positive nuclei. This experiment was repeated 3 times with the same result.

The presence of 10% FBS, the AdV12Rac1-infected cells maintained the twofold increase in Rac1 activity compared with the controls in the presence of 10% FBS (P < 0.05, Fig. 5, C and D). PASMCs infected with the AdN17Rac1 (dominant negative) had similar levels of active Rac1 compared with controls in the absence of 10% FBS. However, Rac1 activity was not increased by the presence of 10% FBS (P < 0.05; Fig. 5, C and D).

To determine whether the modulation of Rac1 activity altered ROS generation in PASMCs, changes in ethidium fluorescence were determined in the presence and absence of 10% FBS. Confirming the importance of ROS in regulating SMC growth, the presence of 10% FBS significantly increased the levels of ROS generation in PASMCs infected with AdV12Rac1 and AdN17Rac1.
ethidium fluorescence in uninfected cells and cells infected with either AdGFP or AdV12Rac1, whereas no such increase was observed in cells infected with AdN17Rac1 (Fig. 6). Further comparisons of the ethidium fluorescence intensities demonstrate that the constitutively active Rac1-infected cells (AdV12Rac1) had significantly higher levels of ethidium fluorescence compared with either uninfected cells or cells infected with AdGFP ($P < 0.05$, Fig. 6). In addition, no significant differences were observed between uninfected cells or cells infected with AdGFP, indicating that adenoviral infection by itself did not alter cellular ROS levels (Fig. 6).

Next, we determined the effect of modulating Rac activity on PASMC proliferation. PASMCs were again infected with the Rac1 mutants or the adenovirus containing GFP, in the presence and absence of 10% FBS for 72 h. The results obtained indicated that 10% FBS increased the number of PASMCs by 75% over a 72-h time period (Fig. 7A). This growth was attenuated in serum-free conditions (Fig. 7A). Cells expressing constitutively active Rac1 (V12Rac1) showed a significantly greater increase in cell number compared with the GFP-infected cells, in both the presence and absence of 10% FBS ($P < 0.05$, Fig. 7A). Conversely, the PASMCs expressing the dominant negative Rac1 had significantly decreased proliferation in the presence of 10% FBS ($P < 0.05$, Fig. 7). Cell cycle analysis of these cells showed that the dominant negative N17Rac1 cells were accumulated in the G2/M phase of the cell cycle (Fig. 7, B and C). This is in contrast to GFP- and V12Rac1-infected cells that showed no such accumulation (Fig. 7, B and C). Again GFP infection did not alter the percentage of cells in G2/M, indicating that infection alone did not alter PASMC cell cycle (data not shown).

Finally, we determined whether the reduction in ROS levels associated with N17Rac1 overexpression was solely responsible for the reduction in SMC growth. To accomplish this we again infected SMCs with N17Rac1. After 24 h, the cells were exposed to increasing concentrations of the SOD1 inhibitor DETC (0–1 µM) for 4 h. The medium was then replaced with fresh DMEM containing 10% FBS. After a further 48 h, cell proliferation in the presence of 10% FBS (Fig. 7, C). Again GFP infection did not alter the percentage of cells in G2/M, indicating that infection alone did not alter PASMC cell cycle (data not shown).

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viability was determined. The results obtained indicate that N17Rac1 overexpression (Fig. 8A) reduced SMC growth by 48% (Fig. 8B). However, the presence of DETC was able to overcome the inhibitory effect of N17Rac1, indicating that increasing ROS levels (Fig. 8C) is sufficient to restore SMC cell growth.

DISCUSSION

Vascular SMC proliferation is a significant contributing factor in the onset and progression of pulmonary hypertension as well as other cardiovascular pathological states, such as atherosclerosis, restenosis, and stent placement (30). Recently ROS have been implicated as important mediators of SMC growth, and increased ROS generation has been implicated in conditions involving vascular remodeling such as hypercholesterolemia, hypertension, diabetes, and balloon injury (12, 25). Given the importance of ROS in vascular remodeling, a better understanding of how their generation is regulated will be important if better therapies to treat these pathologic conditions are to be developed. In this study, we have addressed the role of Rac1 in the generation of ROS and its role in regulating SMC proliferation. The results we have obtained indicate the critical importance of Rac1 in regulating SMC ROS generation and cell proliferation.
The exact mechanism by which Rac1 is involved in the activation of the vascular NADPH oxidase complex remains unclear. This is in contrast to phagocytic cells, where the role of Rac1 has been elucidated. In the phagocytic context it has been shown that one of the oxidase complex subunit p67phox is sequestered by p40phox. Rac1 binding to p67phox results in the disruption of p67phox/p40phox association allowing NADPH oxidase activation (29). However, whether this is also true of vascular oxidases has not been elucidated. Indeed, until this study, it has not been clear whether Rac1 is involved in regulating ROS generation in SMCs. To begin to elucidate the role of Rac1 in regulating the vascular NADPH oxidase complex, we have used various strategies to modulate the cellular activity of Rac1. The use of GGTIs, which block a broad range of small GTP binding proteins, inhibited Rac1 activity in PASMCs. This is in agreement with previous studies in which another GGTI, GGTI-298, was shown to inhibit Rac1 rat pulmonary aortic microvascular SMCs stimulated by FBS (31). However, this study found that GGTI treatment also resulted in a reduction in cell growth due to an increase in apoptosis. In our study, though, PASMC growth was inhibited and cells were not apoptotic, as determined by TUNEL analysis. However, in the previous study, only cells cultured in the absence of serum became apoptotic (31). As all our treatments were carried out in the presence of serum, this may explain why our studies did not induce PASMC apoptosis. However, a more likely explanation is that the GGTI-287 failed to reduce Rac activity and ROS levels below the threshold required to trigger apoptosis in our cells. We have previously shown, using SMCs isolated from the pulmonary vessels of fetal lambs, that ROS levels need to fall to <20% to induce apoptosis (39). This may also explain why GGTI-287 at 5 μmol/l does not inhibit PASMC proliferation. At this concentration GGTI-287 inhibits Rac activity and ROS levels by <50% (see Figs. 1D and 2), and we have previously shown, using SMCs isolated from the pulmonary vessels of fetal lambs, that ROS levels need to fall to <50% of control levels to produce an inhibition of cell growth (40).

Our next strategy involved the use of both constitutively active and dominant negative mutants of Rac1 to more specifically target this protein and better delineate its role in the regulation of ROS induction and SMC proliferation. We found that the dominant negative Rac1 phenotype inhibited the proliferation of SMCs. This inhibition resulted in a lower cell number compared with uninfected cells grown in the presence of 10% FBS. This correlated with N17Rac1-mediated inhibition of cellular ROS generation. In addition, increasing cellular ROS levels by inhibiting SOD1 was able to overcome the inhibitory effect of N17Rac1 overexpression, supporting our conclusion that ROS levels play an important role in regulating PASMC growth. We also found that the constitutively active V12Rac1 stimulated PASMC proliferation even in the absence of 10% FBS. This suggests that increasing ROS generation alone is sufficient to stimulate SMC growth. This is in agreement with our previous studies, in which the addition of H2O2 in the absence of serum stimulated SMC growth in cells isolated from late-gestation fetal lambs (41). V12Rac1-infected cells also exhibited higher levels of proliferation in the presence of 10% FBS than under serum-free conditions. However, our data also demonstrate that 10% FBS stimulates Rac1 activity and ROS generation. Thus the result that FBS increased proliferation in V12Rac1-infected cells may be explained by an increase in the activation of the NADPH oxidase complex by the growth factors present in FBS. Indeed several studies have indicated that growth factors can activate NADPH oxidase activity and ROS levels in SMCs (16, 21, 37).

Our studies have focused on the role of Rac1 in NADPH oxidase activity. However, a previous study showed that Rac2 is present in SMCs isolated from human aorta (27). This study found that Rac2 protein expression was induced by thrombin in a biphasic fashion with expression peaking at 1 h and again at 6 h after treatment (27). This study also found that in addition to changes in expression, thrombin also induced Rac2 translocation to the membrane fraction of human aortic smooth
muscle cells. Accumulation of ROS was also found to occur shortly after translocation of Rac2 to the membrane fraction (27). Together our data and this previous study suggest that either Rac1 or Rac2 can be utilized to form the active NADPH oxidase complex. The Rac isoforms share 92% homology (20), and Rac1 or Rac2 overexpression has been shown to increase ROS generation in cell culture models suggestive of overlapping function (32, 35). In addition, Rac2 appears to be the principal GTPase interacting with components of the NADPH oxidase, at least in some systems (9, 14, 15). However, our results utilizing the dominant negative Rac1 completely abrogated the proliferative response of our PASMCs to 10% FBS, suggesting that Rac1 may be the dominant Rac isoform, at least in PASMCs. It is not clear whether all SMCs express both Rac1 and Rac2 or only one or the other, and further studies will be needed to address the relative expression and activity of Rac2 compared with Rac1 in SMCs.

Our cell cycle analyses using the Rac1 inhibitor GGTI-287 and the N17Rac1 mutant showed a significant accumulation of cells in the G2/M phase. There was a twofold increase in the accumulation of cells in the G2/M phase upon GGTI-287 treatment and a fivefold greater number of cells in the G2/M phase compared with the G1/S phase. Our results are in agreement with other studies investigating the effect of Rac1 inhibition in cell proliferation. For example the overexpression of N17Rac1 in fibroblasts also inhibited proliferation and G2/M progression (24). Similarly, a novel Rac protein, racE, regulates cytokinesis in Dictyostelium (17). It should be noted that several studies of the inhibition of cell proliferation by GGTIs have suggested this inhibition occurs at the G1/S phase of the cell cycle (22, 31, 33). These were found to induce the hypophosphorylation of the retinoblastoma protein and also regulated the expression of cell cycle proteins such as cyclin A, p21, and the activation of cyclin-dependent kinase (CDK) 2 and CDK4 (2). However, these studies were carried out in tumor models and not in primary cells. As tumor cells usually demonstrate aberrant regulation of one or more cellular functions, this may account, at least in part, for the differences in the phase in which the cell cycle arrest occurred. Also, as mentioned above, GGTIs can alter proteins other than Rac1, and this may also produce effects on cell cycle progression. It should also be noted that a majority of our cells were still in the G1/S phase. It is unclear why the N17Rac1 results in a greater accumulation of cells in the G2/M phase than GGTI-287 treatment. However, this may be related to their respective reduction in cellular ROS levels. We have previously shown, using SMCs isolated from the pulmonary vessels of fetal lambs, that ROS levels need to fall to <50% of control levels to produce an inhibition of cell growth and to <10% to induce apoptosis (39, 40). As N17Rac reduces ROS levels by ~65% (Fig. 6) whereas GGTI-287 reduces ROS by ~50%, this may account for the relative increase in cells in the G2/M phase.

In summary, this study demonstrates an important role for Rac1 in the signal transduction pathway leading to the proliferation of SMCs via the generation of ROS. Inhibition of Rac1 results in a decrease in ROS generation and a reduction in cell growth. This inhibition of proliferation appears to be at the G2/M transition. Thus our data strongly suggest an important role for Rac1 in the activation of the NADPH oxidase complex and in the ROS generation required to stimulate the proliferation of SMCs. We speculate that the directed targeting of the Rac1 protein may have potential therapeutic efficacy in the treatment of cardiovascular disease where increased SMC proliferation is involved. However, further studies will need to be undertaken using animal models to better define this possibility.

REFERENCES


