N-Ethylmaleimide Inhibits Platelet-derived Growth Factor BB-stimulated Akt Phosphorylation via Activation of Protein Phosphatase 2A*

Chandrahasa R. Yellaturu†‡, Manjula Bhanoori†‡, Indira Neelī†, and Gadiarthi N. Rao†¶

From the †Department of Physiology and ‡Center for Vascular Biology, The University of Tennessee Health Science Center, Memphis, Tennessee 38163

The redox state plays an important role in gene regulation. Thiols maintain the intracellular redox homeostasis. To understand the role of thiols in redox signaling, we have studied the effect of thiol alkylation on platelet-derived growth factor-BB (PDGF-BB)-induced cell survival events in vascular smooth muscle cells. PDGF-BB stimulated Akt phosphorylation predominantly at Ser-473. N-Ethylmaleimide (NEM), a thiol alkylating agent, blocked PDGF-BB-induced Akt phosphorylation without affecting its upstream phosphatidylinositol 3-kinase (PI3K). On the other hand, LY294002 and wortmannin, specific inhibitors of PI3K, prevented PDGF-BB-induced phosphorylation of Akt and its downstream effector molecules, p70S6K, ribosomal protein S6, 4E-BP1, and eIF4E. NEM also abrogated the phosphorylation of p70S6K, ribosomal protein S6, 4E-BP1, and eIF4E induced by PDGF-BB, suggesting that thiol alkylation interferes with the PI3K/Akt pathway at the level of Akt. In addition, NEM blocked PDGF-BB-induced phosphorylation of BAD and forkhead transcription factor FKHR-L1, and these events correlated with increased apoptosis. NEM alone and in concert with PDGF-BB increased reactive oxygen species (ROS) production and protein phosphatase 2A (PP2A) activity in VSMC. The inhibition of PDGF-BB-induced Akt phosphorylation by NEM was completely reversed by PP2A inhibitors foscarnet and okadaic acid, ceramide synthase inhibitor fumonisin B1, and ROS scavenger N-acetylcysteine (NAC). NAC also attenuated the apoptosis induced by NEM, alone or in combination with PDGF-BB. Together, these findings demonstrate for the first time that PP2A mediates thiol alkylation-dependent redox regulation of Akt and cell survival.

The cellular redox state plays an important role in the regulation of gene expression in prokaryotes and eukaryotes (1–4). The following observations support this notion: 1) Oxidants regulate the activities of several transcription factors, including activator protein-1, nuclear factor kappa B, and p53 (5–7); 2) Oxidants are capable of activating several early response events, including stimulation of protein tyrosine phosphorylation, activation of mitogen-activated protein kinases and induction of expression of proto-oncogenes (8–11); 3) Oxidants are produced acutely in response to various agents, including growth factors and cytokines in several cell types (12, 13), and a requirement for their production in the mitogenic effects of receptor tyrosine kinase and G protein-coupled receptor agonists has been demonstrated (14, 15); and 4) In addition to producing oxidants, cells also possess enzymatic and non-enzymatic mechanisms for their removal (16–18), and this feature attests to the role of oxidants as second messenger molecules (19). Despite the growing body of information on the role of oxidants in the regulation of gene expression, the mechanisms by which these molecules transmit the extracellular signals from the plasma membrane to the nucleus are less clear. Thiols play a critical role in the reduction/oxidation reactions as well as in the structure and function of several enzymes, transcription factors, and transporters (1, 20, 21). Most interestingly, cells also possess several enzymatic mechanisms such as thiol-oxidases and glutaredoxins for regeneration of thiols from their oxidized state, features that orchestrate these molecules as primary targets for oxidant action (22, 23). Oxidation of cysteinyl thiols in the active site of protein tyrosine phosphatase 1B has been observed as a mechanism of its reversible inactivation in response to growth factors and oxidants facilitating tyrosine phosphorylation and activation of receptor tyrosine kinases (24, 25).

Oxidant stress has been implicated in the pathogenesis of a variety of diseases, including atherosclerosis and cancer (26, 27). Depletion of cellular thiols causes oxidant stress (26). In view of the above information, we hypothesize that the cellular thiol redox state plays a determinant role in agonist-induced cell survival/apoptotic signals from the plasma membrane to the nucleus leading to induction of expression of target genes enabling the cellular response. Assuming such an important role for cellular thiols in the signal transduction pathways, one would expect that blockade of these inorganic sulfur groups should affect the signal transduction events that are dependent on oxidation/reduction of these molecules either positively or negatively. The PI3K/Akt pathway plays an important role in cell survival and growth in response to a variety of agents, including cytokines, growth factors, and hormones (28–33). To understand the role of thiol-sensitive redox mechanisms in the regulation of cell survival and growth, we have studied the

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† Both authors contributed equally to this work.

‡ To whom correspondence should be addressed: Dept. of Physiology, University of Tennessee Health Science Center, 894 Union Ave., Memphis, TN 38163. Tel.: 901-448-7321; Fax: 901-448-7126; E-mail: grao@physiol1.utmem.edu.
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PI3K Assay—PI3K activity was measured as described previously (34). Briefly, after appropriate treatments, cells were lysed in 1% lysis buffer (20 mM HEPES, pH 7.4, 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM dithiothreitol, 1 mM Na3VO4, 1% Triton X-100, 10% glycerol, 2 μM leupeptin, 10 units/ml aprotinin, and 400 μM PMSF) for 20 min on ice. The cell lysates were centrifuged at 12,000 rpm for 15 min at 4 °C. The protein content of the supernatants was determined using a Micro BCA™ protein assay reagent kit (Pierce, Rockford, IL). Five-hundred micrograms of protein from control and each treatment was immunoprecipitated with 3 μg of anti-PI3K antibodies for 2 h at 4 °C, followed by incubation with 40 μl of 50% (w/v) protein A-Sepharose beads for an additional hour. The immunoprecipitates were washed three times with lysis buffer, three times with wash buffer, and then resuspended with TNE buffer (50 mM Tris-HCl, pH 7.4, 10 mM NaCl, 1 mM EDTA, and 10 μM Na3VO4). The kinase activity was measured by resuspending the immunoprecipitates in 30 μl of TNE buffer and incubating with 10 μl of 2 mg/ml phosphatidylinositol, 10 μl of 100 mM MgCl2, 2 μl of 100 mM ATP, and 20 μl of 1 μM γ[-32P]ATP for 10 min at 22 °C. The reaction was terminated by addition of 20 μl of 5 x HCl and 200 μl of chloroform:methanol (1:1) mix. The aqueous and organic phases were separated by centrifugation at 2000 rpm for 10 min. The organic phase containing the phosphoinositols was spotted onto a Silica Gel 60A TLC plate coated with 1% potassium oxalate and separated in a solvent system consisting of chloroform:methanol:water:ammonium hydroxide (90:70:14:6.5:4, v/v). The TLC plate was exposed to X-Omat AR x-ray film for 4–6 h at 80 °C and developed.

PP2A Assay—PP2A activity was measured using a kit following the supplier’s instructions (Upstate Biotechnology Inc.). After appropriate treatments, VSMC were lysed in 1 ml of lysis buffer consisting of 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 2 μM leupeptin, 100 units/ml aprotinin and 400 μM PMSF. Cell lysates consisting of 500 μg of protein from control and each treatment were immunoprecipitated with 3 μg of PP2A catalytic alpha subunit (PP2Ac) antibodies overnight at 4 °C, at which time 40 μl of 50% (w/v) protein G-Sepharose CL-4B beads was added and incubation continued for another 2 h. The immunoprecipitates were washed three times with lysis buffer and resuspended in 25 μl of assay buffer (50 mM Tris-HCl, pH 7.5, and 0.1 mM NaCl) incubated by the addition of 1 μl of 100 μM phosphopeptide substrate (200 μM) (KRPThr) and incubating at 37 °C for 10 min. The reaction was then terminated by the addition of 100 μl of Malachite Green solution. The reaction mixture was spun down, and the absorbance of the supernatant was measured at 620 nm in a Spectra Max 190 microtiter plate reader (Molecular Devices Inc., Sunnyvale, CA). Phosphatase activity was calculated using a phosphate standard curve.

Reactive Oxygen Species Detection—After appropriate treatments, cells were rinsed twice with DMEM and incubated for 10 min with 1 mg/ml DCFDA in DMEM. DCF fluorescence produced by ROS was measured on an arbitrary gray scale with a Nikon Eclipse TE300 fluorescence microscope following a previously published procedure (15).

Thiol Determination—After appropriate treatments, thiols in VSMC were determined according to the method of Ellman, using 5,5'-dithio-bis(2-nitrobenzoic acid) (35).

Western Blot Analysis—After appropriate treatments, VSMC were rinsed with cold phosphate-buffered saline and frozen immediately in liquid nitrogen. Cells were lysed by thawing in 250 μl of lysis buffer (phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml PMSF, 100 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM sodium orthovanadate) and scraped into 1.5-ml tubes containing equal amounts of protein were resolved by electrophoresis on 10% SDS and 10% polyacrylamide gels. The proteins were transferred electrothermally onto a nitrocellulose membrane (Hybond, Amersham Biosciences, Piscataway, NJ). After blocking in 10 mM Tris-HCl buffer, pH 8.0, containing 150 mM NaCl, 0.1% Tween 20, and 5% nonfat dry milk, the membranes were incubated with appropriate primary antibodies followed by incubation with horseradish peroxidase-conjugated secondary antibodies. The antigen-antibody complexes were detected using a chemiluminescence reagent kit (Amersham Biosciences).
RESULTS

The PI3K/Akt pathway plays an important role in cell survival and growth in response to a variety of agents, including cytokines, growth factors, and hormones (28–33). To understand the role of thiol in redox-signaling events related to cell survival/apoptosis, we have studied the effect of thiol alkyla-

tion on activation of Akt by PDGF-BB in VSMC. NEM has been used extensively as a specific thiol-alkylating agent (36, 37). To determine thiol alkyla-

tion by NEM, growth-arrested VSMC were treated with and without 20 μM NEM for 30 min, and free thiol levels were measured using 5,5'-dithiobis(2-nitrobenzoic acid) reagent (35). NEM (20 μM) alkylated 60% of the available thiol groups (control, 522 ± 11 nmol/mg of protein versus NEM treatment, 213 ± 4 nmol/mg of protein). At higher concentrations, NEM was found to be toxic to VSMC, and, therefore, it was used at 20 μM concentration throughout this study. Growth-arrested VSMC were treated with and without PDGF-BB (20 ng/ml) in the presence and absence of NEM (20 μM) for the indicated times, and cell extracts were prepared. Equal amounts of protein from control and each treatment were analyzed by Western blotting for Akt using its Ser-473 and Thr-308 phospho-specific antibodies. PDGF-BB stimulated Akt phosphorylation both on Ser-473 and Thr-308 residues in a time-dependent manner (Fig. 1A). Increases in PDGF-BB-stimulated Akt Ser-473 phosphorylation occurred at 5 min (5-fold) and peaked by 30 min (7-fold), and these levels sustained thereafter for at least 2 h. Maximal increases in PDGF-BB-stimulated Akt-308 phosphorylation were observed at 5 min (3-fold), and these levels decreased thereafter. Furthermore, PDGF-BB-induced phosphorylation of Akt was found to be several-fold higher on Ser-473 than Thr-308. NEM significantly (80%) inhibited PDGF-BB-stimulated Akt Ser-473 and Thr-308 phosphorylation. Because PDGF-BB-induced Akt phosphorylation on Ser-473 was several-fold higher than Thr-308, all the subsequent experiments were focused on Akt Ser-473 phosphorylation. To test whether the observed changes in Akt phosphorylation levels correlate with its activity, growth-arrested VSMC were treated with and without PDGF-BB (20 ng/ml) in the presence and absence of NEM (20 μM) for the indicated times, and cell extracts were prepared. Equal amounts of protein from control and each treatment were assayed for Akt activity using a kit (Upstate Biotechnology Inc.). As shown in Fig. 1B, PDGF-BB-induced Akt activity by 2- to 3-fold as compared with control, and it was significantly suppressed by NEM. PI3K is upstream to, and mediates Akt phosphorylation and activation in response to a variety of agonists, including growth factors and cytokines (31–33). Therefore, to understand the mechanism by which NEM inhibits PDGF-BB-stimulated Akt phosphorylation, we studied the effect of thiol alkylation on PI3K activity. PDGF-BB stimulated PI3K activity in a time-dependent manner with a 5-fold increase at 2 h (Fig. 2). Thiol alkylation by NEM alone caused an increase in PI3K activity, and it had an additive effect on PDGF-BB-induced activation of this kinase (Fig. 2).

Akt is downstream to and mediates several of the PI3K-dependent events, including phosphorylation of p70S6K, 4E-BP1, BAD, and FKHR family of transcriptional factors (31, 38–40), although other mechanisms that are independent of PI3K and Akt have also been reported, at least, in the phosphorylation of p70S6K and 4E-BP1 (41–43). Because thiol alkylation had no effect on PDGF-BB-stimulated PI3K activity, we were interested to learn the consequences of Akt inactivation by thiol alkylation on PDGF-BB-induced phosphorylation of p70S6K and 4E-BP1 and their downstream effector molecules ribosomal protein S6 and eIF4E. To gain information on this aspect, we first studied the role of PI3K on PDGF-BB-induced phosphorylation of Akt. LY294002 (25 μM) and wortmannin (1 μM), two structurally different and potent inhibitors of PI3K, blocked both basal and PDGF-BB-induced phosphorylation of Akt (Fig. 3, top panel). LY294002 also inhibited PDGF-BB-induced phosphorylation of p70S6K, ribosomal protein S6, 4E-BP1, and eIF4E (Fig. 3, lower panel). These results suggest that PDGF-BB-induced phosphorylation of Akt, p70S6K, ribosomal protein S6, 4E-BP1, and eIF4E is PI3K-dependent. We now tested the effect of thiol alkylation on PDGF-BB-induced phosphorylation of the above molecules. NEM completely inhibited PDGF-BB-induced phosphorylation of p70S6K, ribosomal protein S6, 4E-BP1, and eIF4E (Fig. 4).

Akt promotes cell survival via phosphorylating and inactivating pro-apoptotic molecules such as BAD and FKHR-L1 (31, 38–40). Upon phosphorylation BAD dissociates from Bcl-2, an anti-apoptotic protein, which in turn, prevents the release of cytochrome c from the mitochondria to the cytoplasm (44). The release of cytochrome c from the mitochondria to the cytoplasm is required for activation of caspase-9, an initial event in the execution of apoptosis (44–46). In the case of FKHR-L1, it is a
member of the forkhead family of transcriptional factors and plays a role in the regulation of cell cycle arrest and apoptosis via induction of expression of p27kip1 and retinoblastoma-like p130 protein (38–40). Because thiol alkylation prevented PDGF-BB-stimulated Akt phosphorylation, we also wanted to examine the effect of thiol alkylation on PDGF-BB-induced phosphorylation of its immediate substrate molecules BAD and FKHR-L1. Cell extracts of VSMC that were treated with and without PDGF-BB (20 ng/ml) in the presence and absence of NEM (20 μM) for the indicated times were analyzed by Western blotting for phosphorylation of BAD and FKHR-L1 using their phospho-specific antibodies. NEM, while alone causing a modest increase of 1.8-fold in the conversion of pro-caspase-3 into active form, in combination with PDGF-BB it increased active caspase-3 production by 3-fold (Fig. 6A). No active caspase-3 levels were detected in control or PDGF-BB-treated cells.

To find whether the increases in active caspase-3 levels result in increased VSMC apoptosis, growth-arrested VSMC were treated with and without PDGF-BB (20 ng/ml) in the presence and absence of NEM (20 μM) for 6 h, and apoptosis was measured by determining the cytoplasmic levels of phospho-specific antibodies. PDGF-BB stimulated BAD Ser-136 and FKHR-L1 Ser-253 phosphorylation in VSMC (Fig. 5). Maximal increases in PDGF-BB-stimulated phosphorylation of BAD and FKHR-L1 occurred at 30 min (2- to 3-fold), and these increases were sustained thereafter for at least 2 h. NEM completely inhibited PDGF-BB-stimulated phosphorylation of BAD and FKHR-L1. To test whether inhibition of phosphorylation of BAD and FKHR-L1 by thiol alkylation correlate with activation of caspase cascade, growth-arrested VSMC were treated with and without PDGF-BB (20 ng/ml) in the presence and absence of NEM (20 μM) for 2 h, and cell extracts were prepared and analyzed by Western blotting for caspase-3 using an antibody that recognizes both of its pro- and active forms. NEM, while alone causing a modest increase of 1.8-fold in the conversion of pro-caspase-3 into active form, in combination with PDGF-BB it increased active caspase-3 production by 3-fold (Fig. 6A). No active caspase-3 levels were detected in control or PDGF-BB-treated cells.

**Fig. 2.** Thiol alkylation does not inhibit PDGF-BB-induced PI3K activity. Growth-arrested VSMC were treated with and without PDGF-BB (20 ng/ml) in the presence and absence of NEM (20 μM) for the indicated times, and cell extracts were prepared. 500 μg of protein from control and each treatment was immunoprecipitated with 3 μg of anti-PI3K antibodies, and the kinase activity in the immunocomplexes was measured as described under “Materials and Methods.” Mean ± S.D. values of three independent experiments are shown in the bar diagram. *, p < 0.05 versus control; **, p < 0.01 versus control.

**Fig. 3.** PDGF-BB-stimulated phosphorylation of Akt, p70S6K, ribosomal protein S6, 4E-BP1, and eIF4E is PI3K-dependent. Growth-arrested VSMC were treated with and without PDGF-BB (20 ng/ml) in the presence and absence of indicated PI3K inhibitors LY294002 (25 μM) or wortmannin (1 μM) for 30 min, and cell extracts were prepared. 40 μg of protein from control and each treatment was analyzed by Western blotting for Akt, p70S6K, ribosomal protein S6, 4E-BP1, and eIF4E using their phospho-specific antibodies. pRPS6, phospho-ribosomal protein S6.

**Fig. 4.** Thiol alkylation inhibits PDGF-BB-stimulated phosphorylation of p70S6K, ribosomal protein S6, 4E-BP1, and eIF4E. Growth-arrested VSMC were treated with and without PDGF-BB (20 ng/ml) in the presence and absence of NEM (20 μM) for the indicated times, and cell extracts were prepared. 40 μg of protein from control and each treatment was analyzed by Western blotting for phosphorylation of BAD and FKHR-L1 using their phospho-specific antibodies. pRPS6, phospho-ribosomal protein S6.

**Fig. 5.** Thiol alkylation inhibits PDGF-BB-stimulated phosphorylation of BAD and FKHR-L1. Growth-arrested VSMC were treated with and without PDGF-BB (20 ng/ml) in the presence and absence of NEM (20 μM) for the indicated times, and cell extracts were prepared. 40 μg of protein from control and each treatment was analyzed by Western blotting for phosphorylation of BAD and FKHR-L1 using their phospho-specific antibodies. PDGF-BB stimulated BAD Ser-136 and FKHR-L1 Ser-253 phosphorylation in VSMC (Fig. 5). Maximal increases in PDGF-BB-stimulated phosphorylation of BAD and FKHR-L1 occurred at 30 min (2- to 3-fold), and these increases were sustained thereafter for at least 2 h. NEM completely inhibited PDGF-BB-stimulated phosphorylation of BAD and FKHR-L1. To test whether inhibition of phosphorylation of BAD and FKHR-L1 by thiol alkylation correlate with activation of caspase cascade, growth-arrested VSMC were treated with and without PDGF-BB (20 ng/ml) in the presence and absence of NEM (20 μM) for 2 h, and cell extracts were prepared and analyzed by Western blotting for caspase-3 using an antibody that recognizes both of its pro- and active forms. NEM, while alone causing a modest increase of 1.8-fold in the conversion of pro-caspase-3 into active form, in combination with PDGF-BB it increased active caspase-3 production by 3-fold (Fig. 6A). No active caspase-3 levels were detected in control or PDGF-BB-treated cells.

To find whether the increases in active caspase-3 levels result in increased VSMC apoptosis, growth-arrested VSMC were treated with and without PDGF-BB (20 ng/ml) in the presence and absence of NEM (20 μM) for 6 h, and apoptosis was measured by determining the cytoplasmic levels of hist-
tone-associated DNA fragments. As expected, growth-arrested VSMC exhibited a mild basal apoptotic activity, and this was reversed in response to treatment with PDGF-BB (Fig. 6 B).

NEM alone and in the presence of PDGF-BB induced VSMC apoptosis by 3- and 5-fold, respectively. Earlier studies have reported that PP2A plays a role in apoptosis via dephosphorylation and inactivation of Bcl-2 and CREB (47–49). In addition, ceramide-induced apoptosis was reported to be dependent on activation of PP2A (50, 51). To understand the molecular mechanism by which thiol alkylation induces apoptosis, the roles of PP2A and ceramide synthase were studied. Growth-arrested VSMC that were exposed or unexposed to NEM (20 μM) were treated with and without PDGF-BB (20 ng/ml) for 30 min, and cell extracts were prepared. 600 μg of protein from control and each treatment was immunoprecipitated with anti-PP2Ac antibodies, and PP2A activity was measured in the immunocomplexes using a phosphopeptide (KRpTIRR) as a substrate. *, p < 0.05 versus control.

PP2A and ceramide synthase were studied. Growth-arrested VSMC that were exposed or unexposed to NEM (20 μM) were treated with and without PDGF-BB (20 ng/ml) in the presence and absence of fumonisin B1 (25 μM), a potent inhibitor of ceramide synthase (54), for 30 min, and cell extracts were prepared. Equal amounts of protein from each condition were analyzed by Western blotting for Akt using its Ser-473 phospho-specific antibodies. Both PP2A and ceramide synthase inhibitors completely reversed the thiol alkylation-induced inhibition of PDGF-BB-stimulated Akt Ser-473 phosphorylation (Fig. 7). To find whether PP2A associates with Akt, co-immunoprecipitation experiments were performed. Equal amounts of protein from VSMC that were treated with PDGF-BB (20 ng/ml) in the presence and absence of NEM (20 μM) or left untreated were immunoprecipitated with anti-Akt or anti-PP2Ac antibodies, and the resulting immunocomplexes were subjected to Western blot analysis using the indicated antibodies. B, 600 μg of protein from control and each treatment was immunoprecipitated with anti-PP2Ac antibodies, and PP2A activity was measured in the immunocomplexes using a phosphopeptide (KRpTIRR) as a substrate. *, p < 0.05 versus control.

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versely, Western blot analysis of anti-Akt antibody immunocomplexes with anti-PP2A antibodies detected a protein with a molecular mass of 36 kDa (Fig. 8A). Western blot analysis of the immunoprecipitates of non-immune serum with anti-Akt or anti-PP2A antibodies did not detect either 70- or 36-kDa proteins (data not shown). These results suggest that PP2A exists as a complex with Akt. To obtain additional evidence for the role of PP2A in thiol alkylation-induced inhibition of PDGF-BB-stimulated Akt phosphorylation, the effect of NEM on PP2A activity was determined. Growth-arrested VSMC were treated with and without PDGF-BB (20 ng/ml) in the presence and absence of NEM (20 μM) for 30 min, and cell extracts prepared. Equal amounts of protein from each condition were immunoprecipitated with anti-PP2A antibodies, and PP2A activity was measured in the immunocomplexes using a phosphopeptide (KRPfTIRR) as a substrate. NEM increased PP2A activity 1.7-fold, alone and in concert with PDGF-BB (Fig. 8B).

Redox regulation of ceramide production by cytokines has been reported previously (54). To understand the mechanism by which NEM activates PP2A and thereby suppresses PDGF-BB-stimulated Akt phosphorylation, we tested its effect on ROS production. Growth-arrested VSMC were treated with and without PDGF-BB (20 ng/ml) in the presence and absence of NEM (20 μM) for 30 min, and ROS production was measured by DCF fluorescence (15). As shown in Fig. 9, NEM alone and in combination with PDGF-BB increased ROS production by 2- and 5-fold, respectively, as compared with control. PDGF-BB also increased ROS production at levels those are lower than the levels produced by NEM alone or in combination with PDGF-BB. In addition, although the effect of PDGF-BB on ROS production was found to be acute, the effects of NEM and NEM and PDGF-BB on ROS production were found sustained (data not shown). To determine the role of ROS in NEM-induced inhibition of PDGF-BB-stimulated Akt phosphorylation, we studied the effect of NAC, an ROS scavenger. Growth-arrested VSMC that were exposed or unexposed to NAC (20 mM) were treated with and without PDGF-BB (20 ng/ml) in the presence and absence of NEM (20 μM) for 30 min, and Akt phosphorylation was measured. NAC completely reversed NEM-induced inhibition of PDGF-BB-stimulated phosphorylation of Akt (Fig. 10A). NAC also completely suppressed the apoptosis induced by NEM alone or in combination with PDGF-BB (Fig. 10B). NAC alone had no effect either on Akt phosphorylation or apoptosis.

**DISCUSSION**

The important findings of the present study are as follows: 1) Thiol alkylation inhibited PDGF-BB-stimulated phosphorylation of Akt and its downstream effector molecules, p70S6K, 4E-BP1, BAD, and FKHR-L1; 2) Decreased p70S6K and 4E-BP1 phosphorylation also led to a decrease in the phosphorylation state of their effector molecules ribosomal protein S6 and eIF4E, respectively; 3) Decreased PDGF-BB-stimulated BAD and FKHR-L1 phosphorylation by thiol alkylation correlated with increased caspase-3 production and apoptosis; 4) The inhibition of PDGF-BB-stimulated Akt phosphorylation by thiol alkylation exhibited a requirement for activation of PP2A and ceramide synthase; 5) PP2A was found to be associated with Akt and thiol alkylation alone and in concert with PDGF-BB increased PP2A activity; and 6) Thiol alkylation increased ROS production by PDGF-BB, and NAC, an ROS scavenger, reversed NEM-induced inhibition of PDGF-BB-stimulated Akt phosphorylation. A large number of studies have demonstrated that PI3K-dependent Akt activation plays a critical role in cell survival (28, 29). One of the several
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mechanisms by which Akt enhances the cell survival activity is the phosphorylation of BAD, a pro-apoptotic protein, thereby causing it to dissociate from Bcl-2, an anti-apoptotic protein, which in turn, perhaps via inhibiting the activity of voltage-dependent anion channels present in the outer mitochondrial membranes, prevents the release of cytochrome c from the mitochondria to the cytoplasm (30, 44–46). In the cytoplasm, cytochrome c activates caspase-9, which, in turn, induces the conversion of pro-caspase-3 into active caspase-3 (44, 45). It has been reported that ceramides induce apoptosis as well as mediate cytokine-induced apoptosis (55, 56). Ceramides have also been shown to activate PP2A (50, 51), and a role for PP2A in apoptosis has been demonstrated (47). Importantly, redox regulation of ceramide production and voltage-dependent anion channel activity has also been reported (55, 57). The other mechanism by which Akt promotes cell survival is the phosphorylation and inactivation of forkhead family of transcriptional factors such as FKHR-L1 leading to down-regulation of apoptosis has been demonstrated (47). Importantly, redox regulation of cell cycle arrest molecules p27

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Addendum—While this report was being submitted for publication, a study from other laboratories reported that overexpression of calreticulin in smooth muscle cells activates caspase-9, which, in turn, induces the apoptosis (44, 45). It has been reported that ceramides induce apoptosis as well as mediate cytokine-induced apoptosis (55, 56). Ceramides have also been shown to activate PP2A (50, 51), and a role for PP2A in apoptosis has been demonstrated (47). Importantly, redox regulation of ceramide production and voltage-dependent anion channel activity has also been reported (55, 57). The other mechanism by which Akt promotes cell survival is the phosphorylation and inactivation of forkhead family of transcriptional factors such as FKHR-L1 leading to down-regulation of expression of cell cycle arrest molecules p27

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