JunB Forms the Majority of the AP-1 Complex and Is a Target for Redox Regulation by Receptor Tyrosine Kinase and G Protein-coupled Receptor Agonists in Smooth Muscle Cells*

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Gadiparthi N. Rao‡‡§§, Khurshed A. Katki‡, Nageswara R. Madamanchi‡, Yaxu Wu‡, and Michael J. Birrer¶

From the ‡Division of Cardiology, University of Texas Medical Branch, Galveston, Texas 77555 and the ¶Division of Cancer Prevention and Control, NCI, National Institutes of Health, Rockville, Maryland 20850

To understand the role of redox-sensitive mechanisms in vascular smooth muscle cell (VSMC) growth, we have studied the effect of N-acetylcysteine (NAC), a thiol antioxidant, and diphenyleneiodonium (DPI), a potent NADH/NADPH oxidase inhibitor, on serum-, platelet-derived growth factor BB-, and thrombin-induced ERK2, JNK1, and p38 mitogen-activated protein (MAP) kinase activation; c-Fos, c-Jun, and JunB expression; and DNA synthesis. Both NAC and DPI completely inhibited agonist-induced AP-1 activity and DNA synthesis in VSMC. On the contrary, these compounds had differential effects on agonist-induced ERK2, JNK1, and p38 MAP kinase activation and c-Fos, c-Jun, and JunB expression. NAC inhibited agonist-induced ERK2, JNK1, and p38 MAP kinase activation and c-Fos, c-Jun, and JunB expression except for platelet-derived growth factor BB-induced ERK2 activation. In contrast, DPI only inhibited agonist-induced p38 MAP kinase activation and c-Fos and JunB expression. Antibody supershift assays indicated the presence of c-Fos and JunB in the AP-1 complex formed in response to all three agonists. In addition, cotransfection of VSMC with expression plasmids for c-Fos and members of the Jun family along with the AP-1-dependent reporter gene revealed that AP-1 with c-Fos and JunB composition exhibited a higher transactivating activity than AP-1 with other compositions tested. All three agonists significantly stimulated reactive oxygen species production, and this effect was inhibited by both NAC and DPI. Together, these results strongly suggest a role for redox-sensitive mechanisms in agonist-induced ERK2, JNK1, and p38 MAP kinase activation; c-Fos, c-Jun, and JunB expression; AP-1 activity; and DNA synthesis in VSMC. These results also suggest a role for NADH/NADPH oxidase activity in some subset of early signaling events such as p38 MAP kinase activation and c-Fos and JunB induction, which appear to be important in agonist-induced AP-1 activity and DNA synthesis in VSMC.

Redox control plays an important role in gene regulation (1–6). Underlying the importance of redox mechanisms, oxidants have been implicated in the pathogenesis of cell proliferative diseases such as atherosclerosis and cancer and in aging (7–9). Although the exact mechanisms by which oxidant stress influences the pathogenesis of these diseases are not clear, several laboratories including ours have demonstrated that oxidants exhibit mitogenic activity in a variety of cell types including VSMC at nontoxic doses (10–15). In addition, oxidants mimic growth factors in several aspects: oxidants stimulate phosphorylation of growth factor receptor and nonreceptor tyrosine kinases (16–18), they activate extracellular signal-regulated kinases (ERKs) (19, 20), and they induce expression of several protooncogenes such as c-Fos and c-Jun (21–25). More importantly, a number of recent studies have demonstrated that growth factors stimulate production of H2O2 in a variety of cell types including VSMC (26–31). In addition, a requirement for H2O2 production in the mitogenic signaling events of growth factors has been reported (31).

NADH/NADPH oxidase is a flavin-containing plasma membrane-bound enzyme that generates superoxide anion upon activation (32). Superoxide anion can be dismutated enzymatically or nonenzymatically to H2O2, which is then scavenged by catalase or peroxidase (33). In an effort to identify the sources of oxidant generation, several investigators have reported that growth factors that induce either hyperplasia or hypertrophy stimulate NADH/NADPH oxidase activity in various cell types (34, 35). It was also reported that NADH/NADPH oxidase activity can be regulated in a growth-dependent manner (35). A role for NADH/NADPH oxidase activity in tumor necrosis factor-α- and fibroblast growth factor-induced c-Fos expression and tumor necrosis factor-α-induced Jun-NH2-terminal kinase-1 (JNK1) activation in chondrocytes has been reported (36, 37). In another study, inhibition of NADH/NADPH oxidase activity by antisense targeted depletion of its p22phox component blunt the hypertrophic effect of angiotensin II in VSMC (38). Because of these findings, a role for this enzyme in growth factor-induced signaling events has been suggested. However, the molecular events underlying the growth-regulating effects of NADH/NADPH oxidase are not clear. The purpose of the present investigation was to determine the role of redox-sensitive mechanisms in the modulation of agonist-induced early response events in VSMC. Here we report the following observations. 1) NAC, a thiol antioxidant, blocks agonist-induced ERK2, JNK1, and p38 MAPK activation, c-Fos, c-Jun, and JunB expression, AP-1 activity, and DNA synthesis in VSMC.

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‡ To whom correspondence should be addressed: 4.124B Old John Sealy Hospital, University of Texas Medical Branch, 301 University Blvd., Route 0567, Galveston, TX 77555-0567. Tel.: 409-747-1851; Fax: 409-772-1861; E-mail: grao@utmb.edu.

¶ The abbreviations used are: VSMC, vascular smooth muscle cell(s); ERKs, extracellular signal-regulated kinases; JNKs, Jun NH2-terminal kinases; MAPK, mitogen-activated protein kinase; NAC, N-acetylcysteine; AP-1, activator protein-1; PDGF-BB, platelet-derived growth factor-BB; GST, glutathione S-transferase; PBS, phosphate-buffered saline; Mops, 4-morpholino-2-propanesulfonic acid; CAT, chloramphenicol acetyltransferase; DPI, diphenyleneiodonium.
2) NADH/NADPH oxidase activity plays a role in some subset of agonist-induced early response events such as induction of p38 MAPK activation, c-Fos and JunB expression, AP-1 activity, and DNA synthesis. 3) JunB appeared to be the predominant member of the AP-1 complex formed in response to both receptor tyrosine kinase and G protein-co coupled receptor agonists. 4) AP-1 with c-Fos and JunB composition exhibited a higher transcriptional activity than AP-1 with other compositions tested. Together, these results strongly suggest that redox-sensitive mechanisms play an important role in the modulation of AP-1 activity and growth in VSMC.

**EXPERIMENTAL PROCEDURES**

**Materials**—Aprotinin, ATP, bis-N-methylacridinium nitrate (lucigenin), bovine myelin basic protein, EGTA, β-glycerophosphate, leupeptin, phenylmethylsulfonyl fluoride, sodium deoxycholate, sodium fluoride, sodium orthovanadate, sodium pyrophosphate, and thrombin were obtained from Sigma. PDGF-BB was from Genzyme (Cambridge, MA). Diphenylethionium was purchased from Toronto Research Chemicals, Inc. (Ontario, Canada). Anti-ERK1 (SC-93), ERK2 (SC-154), c-Fos (SC-052), JunB (SC-46), JNK1 (SC-474), and p38 MAPK (SC-762 and SC-535) rabbit polyclonal antibodies and GST-c-Jun (1–79) (SC-203) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-c-Jun rabbit polyclonal antibody (PC-06) was purchased from Oncogene Science (Uniondale, NY). AP-1 consensus double-stranded oligonucleotide and T4 polynucleotide kinase were from Promega (Madison, WI). Phospho-p38 MAPK antibodies were obtained from New England Biolabs, Inc. (Beverly, MA). 1(13)C]Chloramphenicol (52 mCi/mmol), [methy1-3H]thymidine (70 Ci/mmol), and [32P]ATP (8,000 Ci/mmol) were obtained from NEN Life Sciences, Inc. (Boston, MA).

**Cell Culture**—VSMC were isolated from the thoracic aortas of 200–300-g male Sprague-Dawley rats by enzymatic digestion as described earlier (13). Cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) heat-inactivated calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cultures were maintained at 37 °C in a humidified 95% air, 5% CO2 atmosphere.

**DNA Synthesis**—VSMC were plated onto 60-mm dishes, allowed to grow to 70–80% confluence, and then growth arrested by incubation in Dulbecco’s modified Eagle’s medium containing 1% (v/v) calf serum for 72 h. Growth-arrested VSMC were exposed to various agonists in the presence and absence of the indicated inhibitors for 24 h. Cells were pulse labeled with 1 μCi/μl [3H]thymidine for 2 h just before the end of the incubation period and harvested (20 μg/100 μl) using a Tris-phosphate-buffered citric acid (TPCA) solution containing 3% (w/v) trichloroacetic acid and vortexed vigorously to lyse the cells. The mixture was allowed to remain on ice for 20 min and was then passed through a GF/F glass microfiber filter. The filter was washed once with cold 5% trichloroacetic acid and once with cold 70% ethanol, dried, and placed in a liquid scintillation vial containing the mixture. The radioactivity was counted in a liquid scintillation counter using a chloroform:methanol mixture.

**ERK2 Assay**—ERK2 activity present in the immunoprecipitates was determined using in 20 μl of kinase buffer containing 5 μg of myelin basic protein, 20 μl ATP, and 1 μCi of [γ-32P]ATP per reaction and incubating at 30 °C for 20 min. For the JNK1 assay, incubation with the kinase buffer was the same as that for the ERK2 assay except that 1 μg of GST-c-Jun was used instead of myelin basic protein. The reactions were stopped by adding 20 μl of 4 × Laemmli sample buffer. The samples were heated at 95 °C for 5 min and analyzed by SDS-gel electrophoresis on 12% acrylamide gels. The dried gel was exposed to X-Omat AR x-ray film and developed. Activation of p38 MAPK was measured by Western blotting using phospho-p38 MAPK antibodies.

**Superoxide Anion Production Assay**—Growth-arrested VSMC were treated with or without inhibitors for the indicated time periods at 37 °C. Medium was aspirated and washed with PBS and pelleted by centrifugation at 1,000 rpm for 5 min at 4 °C. The cell pellet was resuspended in 1 ml of TEN buffer (40 mM Tris-HCl, pH 7.9, 7.5, 1 mM EDTA, pH 8.3, and 1 mM EDTA) double-stranded oligonucleotides (AP-1, 5′-CGCTGTAGTAGCTAGGCAA-3′) were labeled with [32P]ATP using a T4 polynucleotide kinase kit per the supplier’s protocol (Promega). Unincorporated nucleotides and, in some cases, final extracts were carried by chromatography in a G-25 spin column (Bio-Rad).

**Western Blot Analysis**—Growth-arrested VSMC were treated with and without the agonists in the presence and absence of appropriate inhibitors for the indicated time periods at 37 °C. Medium was aspirated, and cells were rinsed with cold phosphate-buffered saline (PBS) and frozen immediately in liquid nitrogen. 250 μl of lysis buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml phenylmethylsulfonyl fluoride, 100 μg/ml aprotinin, 1 μg/ml leupeptin, 20 μg β-glycerophosphate, 2 mM sodium fluoride, 2 mM sodium pyrophosphate, and 1 mM sodium orthovanadate (Na3VO4)) was added to the frozen monolayers, thawed on ice for 15 min, and scraped into 1.5-ml Eppendorf tubes. The cell lysates were cleared by centrifugation at 12,000 rpm for 30 min at 4 °C. The protein content of the supernatants was determined using Bradford reagent (Bio-Rad). Cell lysates containing equal amounts of protein were resolved by electrophoresis on a 0.1% SDS and 10% polyacrylamide gels. The protein was transferred electrophoretically to a nitrocellulose membrane (Hybond, Amersham Pharmacia Biotech). After blocking in 10 mM Tris-HCl buffer, pH 8.0, containing 150 mM sodium chloride, 0.1% Tween 20, and 5% (w/v) non-fat dry milk, the membrane was treated with appropriate primary antibodies followed by incubation with appropriate peroxidase-conjugated secondary antibodies. The antigen-antibody complexes were detected using chemiluminescence reagent kit (Amersham Pharmacia Biotech).

**ERK, JNK, and p38 MAPK Assays**—After appropriate treatments, cells were washed with cold PBS and solubilized on ice for 15 min in lysis buffer containing 20 mM Hepes, pH 7.4, 2 μg EGTA, 1 mM dithiothreitol, 50 mM β-glycerophosphate, 1% Triton X-100, 10 units/ml aprotinin, 2 μg/ml leupeptin, 1 mM Na3VO4, and 400 μM phenylmethylsulfonyl fluoride. The cell lysates were cleared by centrifugation at 13,000 rpm for 10 min at 4 °C. Cell lysates normalized for protein were immunoprecipitated by incubating with anti-ERK2 and anti-JNK1 rabbit IgG antibodies and JNK1 assay (2× 40 μl of 50% (w/v) protein A-Sepharose beads for an additional hour. The beads were washed three times with lysis buffer, three times with wash buffer (100 mM Tris-HCl, pH 7.6, 500 mM sodium chloride, 0.1% Triton X-100, and 1 mM dithiothreitol), and three times with kinase buffer (12.5 mM Mops, pH 7.5, 12.5 mM β-glycerophosphate, 7.5 mM MgCl2, 200 μM sodium fluoride, and 0.1 mM sodium orthovanadate). The ERK2 activity present in the immunoprecipitates was determined by resuspending in 30 μl of kinase buffer containing 5 μg of myelin basic protein, 20 μl ATP, and 1 μCi of [γ-32P]ATP per reaction and incubating at 30 °C for 20 min. For the JNK1 assay, incubation with the kinase buffer was the same as that for the ERK2 assay except that 1 μg of GST-c-Jun was used instead of myelin basic protein. The reactions were stopped by adding 20 μl of 4 × Laemmli sample buffer. The samples were heated at 95 °C for 5 min and analyzed by SDS-gel electrophoresis on 12% acrylamide gels. The dried gel was exposed to X-Omat AR x-ray film and developed. Activation of p38 MAPK was measured by Western blotting using phospho-p38 MAPK antibodies.

**Superoxide Anion Production Assay**—Growth-arrested VSMC were treated with or without inhibitors for the indicated time periods at 37 °C. The protein concentration of the supernatant was determined as described above. CAT activity was measured by the method of Gorman et al. (41). In brief, 50 μg of protein from each condition was incubated with 20 μl of 4 mM acetyl-CoA, 32.5 μl of 1× Tris-HCl buffer, pH 7.5, 4 μl of 50 μCi/ml [32P]Chloramphenicol in a total volume of 150 μl at 37 °C for 2–4 h. Controls without cell extract and/or with nontransfected cell extracts were incubated simultaneously. Acetylated and nonacetylated chloramphenicol was extracted with ethyl acetate and separated by thin layer chromatography on Silica Gel 1B plates using a chloroform:methanol mixture (19:1) as solvent, and the air-dried silica plates were subjected to autoradiography.
mm Hepes, pH 7.4. Superoxide anion production in VSMC in response to agonists was measured in the darkroom with an AutoLumat LB 953 luminometer. To start the assay, lucigenin was added to a final concentration of 250 μM to 1 × 10^6 cells followed by agonist in a total volume of 1 ml of assay buffer. Wherever appropriate, 20 mM NAC was added to cells 2 h before the assay, and DPI (12 μM) was added 30 min before the assay. Photoemission in terms of relative light units was measured every minute for 5 min.

All of the experiments were repeated at least three to four times with similar results. All samples were normalized for protein content before Western blot analysis, immunoprecipitation, electrophoretic mobility shift assay and CAT assay. Statistical analysis on [3H]thymidine uptake data was performed using Student’s t test.

RESULTS

To study the role of redox-sensitive mechanisms in agonist-induced cell growth, growth-arrested VSMC were treated with and without 10% serum, 10 ng/ml PDGF-BB, or 0.1 unit/ml thrombin in the presence and absence of 20 μM NAC or 12 μM DPI for 24 h, and DNA synthesis was measured by [3H]thymidine uptake. As shown in Fig. 1, A and B, all three agonists caused a significant increase in DNA synthesis in VSMC, and this effect was blocked by NAC and DPI.

To investigate the underlying mechanisms of NAC- and DPI-inhibited VSMC growth, we first determined the time course of agonist-induced expression of AP-1 components, c-Fos, c-Jun, JunB, and JunD. As shown in Fig. 2, A and B, serum, PDGF-BB, and thrombin induced c-Fos (12–20-fold), c-Jun (3–4-fold), and JunB (5–7-fold) expression in a time-dependent manner. Induction of c-Fos expression in response to serum, PDGF-BB, and thrombin, and thrombin was observed at 1 h, reached a near maximal level at 2 h, and declined thereafter. The maximal expression of c-Jun in response to these agonists was observed at 2 h, whereas JunB levels peaked 3 h poststimulation. Significant levels of JunD were present in growth-arrested VSMC which were increased by 2-fold upon treatment with serum. PDGF-BB and thrombin had no significant effect on JunD levels.

To test whether agonist-induced increases in the levels of c-Fos and members of the Jun family proteins are mediated via the generation of oxidants, we treated growth-arrested VSMC with serum, PDGF-BB, or thrombin in the presence and absence of NAC, a thiol antioxidant, and the protooncogene levels were determined. NAC significantly decreased c-Fos (70%), c-Jun (50%), and JunB (70%) expression in response to serum, PDGF-BB, and thrombin (Fig. 3). In contrast, NAC had no significant effect on JunD levels in either control or agonist-treated VSMC. NADH/NADPH oxidase has been implicated in cytokine- and growth factor-induced c-Fos expression and cytokine-induced JNK1 activation and c-Jun expression in chondrocytes (36, 37). To test the role of NADH/NADPH oxidase activity in agonist-induced expression of c-Fos and members of the Jun family proteins, growth-arrested VSMC were treated with and without serum, PDGF-BB, or thrombin in the presence and absence of DPI, and the protooncogene levels were determined. Expression of c-Fos in response to these agonists was almost completely (85%) blocked by DPI (Fig. 4). DPI also significantly (70%) inhibited JunB expression induced by these agonists. DPI alone induced c-Jun expression by 3-fold, and it exhibited an additive effect with agonist-induced expression of this protooncogene (Fig. 4). DPI had no significant effect on JunD levels in either control or agonist-treated cells.

The Fos and Jun family proteins constitute the transcription factor AP-1 (42). To investigate the relationship between the induction of expression of c-Fos and members of the Jun family proteins and AP-1-dependent transcription, we studied the expression of AP-1-responsive reporter gene in growth-arrested VSMC stimulated with serum, PDGF-BB, or thrombin in the presence and absence of NAC or DPI. VSMC were transiently transfected with a collagenase-CAT (coll-CAT) reporter plasmid in which CAT expression was driven by collagenase gene promoter containing a single AP-1 site, growth arrested, and then treated with and without the agonists in the presence and absence of NAC or DPI. All three agonists caused induction of the AP-1-dependent reporter gene expression, and this response was significantly blocked by both NAC and DPI (Fig. 5, A and B). Because NAC and DPI inhibited the AP-1 activity despite the differential effects of these compounds on serum-, PDGF-BB-, and thrombin-induced c-Jun expression, we next determined the composition of AP-1 formed in response to these agonists. Gel supershift analysis showed the presence of c-Fos and JunB in the AP-1 complex formed in response to
serum and thrombin (Fig. 6). Similar results were obtained with PDGF-BB (data not shown). To determine the functional activity of AP-1 with c-Fos and JunB composition, VSMC were cotransfected with expression plasmids for c-Fos and members of the Jun family in various combinations along with the AP-1 reporter plasmid coll-CAT, and CAT activity was measured 48 h after transfection. Consistent with the gel supershift analysis, AP-1 with c-Fos and JunB composition exhibited a higher transcriptional activity than AP-1 with other combinations tested (Fig. 7).

ERKs are a group of serine/threonine kinases that respond preferentially to various growth stimulants. These kinases are implicated in the expression of c-Fos in response to various agonists (43–46). To understand the possible mechanisms by which NAC and DPI inhibit c-Fos expression induced by these agonists, we investigated the effects of these compounds on serum-, PDGF-BB-, and thrombin-induced activation of ERK2 in VSMC. All three agonists caused significant increases (8–15-fold) in ERK2 activity as measured by an immunocomplex kinase assay using myelin basic protein as a substrate (Figs. 8 and 9). Although NAC completely inhibited the ERK2 activity induced by serum and thrombin, it had no significant effect on PDGF-BB-induced ERK2 activation (Fig. 8). Serum-, PDGF-BB-, and thrombin-induced ERK2 activity, however, was not affected by DPI (Fig. 9). NAC and DPI by themselves had no significant effect on ERK2 activity in growth-arrested VSMC.

JNKs are a group of MAPKs that are related to ERKs but respond preferentially to cellular stressors such as UV irradiation and agents that do not primarily cause growth (47–49). A role for JNKs has been suggested in the induction of expression of both c-Fos and c-Jun (43, 44, 50). To understand the possible mechanisms by which antioxidants affect the agonist-induced expression of c-Fos and members of the Jun family proteins, we studied the effects of NAC and DPI on serum-, PDGF-BB-, and thrombin-induced JNK1 activity. All three agonists activated JNK1 (10–25-fold) as determined by an immunocomplex kinase assay with GST-c-Jun (1–79) as a substrate (Figs. 8 and 9). NAC significantly inhibited (50–80%) the agonist-induced JNK1 activity (Fig. 8). On the contrary, DPI alone caused an increase (35% over control) in JNK1 activity, and it exhibited an additive effect with agonists on the activation of this enzyme (Fig. 9). It was reported that p38 MAPK cooperates with ERKs in the induction of expression of c-Fos in response to UV irradiation (51). To find the possible role of p38 MAPK in agonist-induced c-Fos expression, we tested the effect of serum, PDGF-BB, and thrombin on p38 MAPK activation. As determined by the phosphorylation of the enzyme, all three agonists activated p38 MAPK, and this effect was sensitive to inhibition by both NAC and DPI (Fig. 10).

To gain further evidence for a role of oxidants in receptor tyrosine kinase and G protein-coupled receptor agonist-induced growth signaling events, we tested the effect of serum, PDGF-BB, and thrombin on superoxide anion production in growth-arrested VSMC. As shown in Fig. 11, all three agonists signif-
Significantly stimulated superoxide anion production by 5 min, and this effect was blocked by both NAC and DPI.

**DISCUSSION**

The important finding of the present study is that JunB is the major constituent of the AP-1 complex formed in response to, and is a target for redox regulation by both receptor tyrosine kinase and G protein-coupled receptor agonists in VSMC. The present findings also show that antioxidant NAC inhibits serum-, PDGF-BB-, and thrombin-induced JNK1 and p38 MAPK activation, c-Fos, c-Jun, and JunB expression, and DNA synthesis in VSMC. Although NAC completely blocked the serum- and thrombin-induced ERK2 activity, it had no significant effect on PDGF-BB-induced activation of ERK2. These findings suggest a role for both redox-sensitive and redox-insensitive mechanisms in agonist-induced ERK2 activation in VSMC. ERKs are implicated in the induction of expression of c-Fos via phosphorylation and activation of the transcription factor Elk1 in response to growth factors (44, 52). Because NAC had no effect on PDGF-BB-induced ERK2 activity, and it inhibited completely the c-Fos expression and DNA synthesis induced by PDGF-BB, it is likely that other mechanisms that are independent of ERKs modulate the induction of expression of c-Fos by PDGF-BB. This also implies that activation of ERK2 alone may not be sufficient for growth induction by PDGF-BB. In fact, others have also reported that activation of ERKs alone is not sufficient for VSMC mitogenesis induced by PDGF-BB and angiotensin II (53, 54). It was reported that induction of c-Fos expression by UV requires a cross-talk between p38 MAPK and
Because all three agonists activated p38 MAPK, and this effect was inhibited by NAC, one likely mechanism by which NAC inhibits the PDGF-BB-induced c-Fos expression could be via down-regulating the p38 MAPK activity. A similar mechanism can be extrapolated to the inhibitory effect of DPI on agonist-induced c-Fos expression as DPI, although having no effect on agonist-induced ERKs activation, blocked p38 MAPK activation.

Reactive oxygen species production by NADH/NADPH oxidase has been implicated in cytokine- and growth factor-induced c-Fos expression and cytokine-induced JNK1 activation and c-Jun expression (36, 37). Because DPI, a potent inhibitor of NADH/NADPH oxidase, blocked only the c-Fos and JunB expression and p38 MAPK activation but not the c-Jun expression or ERK2 and JNK1 activation induced by serum, PDGF-BB, and thrombin, it is likely that NADH/NADPH oxidase activity is required only for some subset of agonist-stimulated early response events. Despite the differential effects of NAC and DPI on agonist-induced ERK2 and JNK1 activation and c-Jun expression, both caused a decrease in agonist-stimulated AP-1 activity and growth. Because c-Fos and JunB were present in the AP-1 complex formed in response to serum, PDGF-BB, and thrombin, and the expression of these protooncogenes was inhibited by both NAC and DPI, it is possible that these two protooncogenes account for the agonist-induced AP-1 activity and growth in VSMC. Indeed, cotransfection of VSMC with expression plasmids for c-Fos and members of the Jun family revealed that AP-1 with c-Fos and JunB composition possess more gene transactivating activity than AP-1 with the other compositions tested.

Superoxide anion and H2O2 are the frequently invoked reactive oxygen species implicated in growth factor-induced cellular signaling (10, 28, 31, 55, 56). In addition, growth factors have
been reported to activate NADH/NADPH oxidase, a major re- active oxygen species-generating system in a variety of cell types including VSMC (28, 34, 35). Our results show that both receptor tyrosine kinase and G protein-coupled receptor agonists stimulate the production of superoxide anion in VSMC via a mechanism involving NADH/NADPH oxidase activity. However, although serum-, PDGF-BB-, and thrombin-induced superoxide anion production was completely blocked by NAC and DPI, the early growth response events of these agonists exhibited differential sensitivity of inhibition to these compounds. Specifically, DPI inhibited agonist-induced p38 MAPK activation and c-Fos and JunB expression. On the other hand, NAC inhibited ERKs, JNKs, and p38 MAPK group of MAPK activation and c-Fos, c-Jun, and JunB expression. Considering these findings, it is clear that besides NADH/NADPH oxidase, other mechanisms are involved in the redox regulation of these early response signaling events of receptor tyrosine kinase and G protein-coupled receptor agonists. It has been reported that agents such as hemin and HgCl$_2$, which oxidize thiol groups, activate Ras and cause mitogenesis in lymphocytes (57, 58). NAC facilitates synthesis of glutathione, a major intracellular thiol donor, by providing cysteine (59). Glutathione, besides acting as a sulfhydryl donor, by providing cysteine (59). Glutathione, besides glutathione, a major intracellular scavenging reactive oxygen species, modulates the levels of several proteins including growth factor receptors, ion channels, and transcriptional factors (1–6, 60–63).

Another possible molecule that is involved in agonist-induced NADH-sensitive early response events could be arachi- donic acid. Both receptor tyrosine kinase and G protein-coupled receptor agonists stimulate the release of arachidonic acid in a variety of cell types including VSMC (64, 65). In addition, a requirement for arachidonic acid release and its subsequent metabolism via the lipoxigenase/cyclooxygenase P-450 monooxyge- nase pathways has been reported to be required for both receptor tyrosine kinase and G protein-coupled receptor agonist-induced growth in many cell types including VSMC (64, 66). Furthermore, activation of secretory type phospholipase A$_2$ (also known as group II phospholipase A$_2$), a group of rate-limiting enzymes for arachidonic acid release, requires disul- fide bond formation (67). It was also reported that expression of group II phospholipase A$_2$ in transgenic mice results in epider- mal hyperplasia (68). Although a role for arachidonic acid in agonist-induced thiol-sensitive mechanisms of oxidant generation can be extrapolated from these findings, future studies are required to test this hypothesis. In any case, the present study provides evidence for a role of several redox-sensitive mechanisms including NADH/NADPH oxidase activity in the early signaling events of both receptor tyrosine kinase and G protein-coupled receptor agonists in VSMC. In addition, the present study identifies JunB as the major component of the AP-1 complex formed in response to both receptor tyrosine kinase and G protein-coupled receptor agonists in VSMC and its possible role in redox-sensitive AP-1-mediated gene regulation.

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