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Reactive Oxygen Intermediates Mediate Angiotensin II-induced c-Jun·c-Fos Heterodimer DNA Binding Activity and Proliferative Hypertrophic Responses in Myogenic Cells*

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Angiotensin II (Ang-II) receptor engagement activates many immediate early response genes in both vascular smooth muscle cells and cardiomyocytes whether a hyperplastic or hypertrophic response is taking place. Although the signaling pathways stimulated by Ang-II in different cell lines have been widely characterized, the correlation between the generation of different second messengers and specific physiological responses remains relatively unexplored. In this study, we report how in both C2C12 quiescent myoblasts and differentiated myotubes Ang-II significantly stimulates AP1-driven transcription and c-Jun·c-Fos heterodimer DNA binding activity. Using a set of different protein kinase inhibitors, we could demonstrate that Ang-II-induced increase in AP1 binding is not mediated by the cAMP-dependent pathway and that both protein kinase C and tyrosine kinases are involved. The observation that in quiescent myoblasts Ang-II increase of AP1 binding and induction of DNA synthesis and, in differentiated myotubes, Ang-II stimulation of protein synthesis are abolished by the cysteine-derivative and glutathione precursor *N*-acetyl-L-cysteine strongly suggests a role for reactive oxygen intermediates in the intracellular transduction of Ang-II signals for immediate early gene induction, cell proliferation, and hypertrophic responses.

Octapeptide Ang-II,[1] a potent vasoconstrictor, is also a growth factor for vascular smooth muscle cells (VSMCs) (1–4). A number of *in vivo* and *in vitro* studies suggest that Ang-II may also be a critical factor in mediating cardiac hypertrophy (5–9). Hypertrophy is the fundamental adaptive process employed by postmitotic cardiac and skeletal muscle in response to mechanical load (10). Using a load-induced cardiac hypertrophy *in vitro* model, it has been recently demonstrated that mechanical stretch causes the release of Ang-II from cardiac myocytes and that locally produced Ang-II acts as the initial mediator of stretch-induced hypertrophic response (11).

In cardiac myocytes and nonmyocytes, Ang-II induces immediate early genes such as c-*fos*, c-*jun*, and *egr1* leading to hypertrophy and mitogenesis, respectively (12). In general, induction of immediate early genes is regulated by post-translational modification of pre-existing factors and is directly regulated by cellular second messenger systems (13). Many peptide growth factors, such as bombesin and endothelin-1, activate multiple second messenger systems, which act synergistically to induce complex mitogenic responses (14). In different cell types a variety of second messengers have been involved in the transduction of Ang-II signaling. In cardiac myocytes and VSMCs Ang-II activates phospholipase C through a G-protein-coupled receptor, liberates inositol triphosphate; induces calcium release from inositol triphosphate-sensitive calcium storage sites; activates protein kinase C, phospholipase A_2, phospholipase D, adenilate cyclase, and arachidonic acid metabolism; and stimulates the tyrosine kinases, c-Raf1, and mitogen-activated protein kinases cascade (12, 15–23). Interestingly, the activation of both phospholipase A_2 and phospholipase D stimulates the intracellular generation of ROIs through the formation of arachidonate and phosphatidic acid (PA), respectively. In turn, it has been suggested that they act as second messengers in many physiological and pathological responses (24), including early response gene activation and cell growth regulation (24–26). However, the interplay between all these transducers of Ang-II signaling and their relation with specific responses is still unclear.

All muscle cell types share several structural properties and the expression of most of the known specific genes of muscles. These basic features are faithfully reproduced in primary cultures of fetal myoblasts and newborn satellite muscle cells, as well as in continuous mammalian myogenic cell lines. We used mouse C2C12 skeletal myoblasts, because they reproduce myogenic differentiation in culture (27), to form long term differentiated and functional grafts in adult syngeneic ventricular myocardium (28) and to represent an attractive means of studying the effects of Ang-II in different conditions of proliferation and terminal differentiation. Systematic dissection of Ang-II transduction pathway in myogenic cells enabled us to show the involvement of ROIs in the intracellular transduction of Ang-II signals for immediate early genes induction, cell proliferation, and hypertrophic response.

EXPERIMENTAL PROCEDURES

Cell Cultures, Plasmids, and Transfections—Actively growing mouse myogenic C2C12 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (growth medium). Quiescent myoblasts were obtained by plating the C2C12 cells at low

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[1] The abbreviations used are: Ang-II, angiotensin II; H7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine; NAC, N-acetyl-L-cysteine; ROI, reactive oxygen intermediate; VSMC, vascular smooth muscle cell; PA, phosphatidic acid; CAT, chloramphenicol acetyltransferase; tk, thymidine kinase; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; TRE, TPA response element.

confluence in Dulbecco's modified Eagle's medium supplemented with 0.1% fetal bovine serum. To induce differentiation, subconfluent C2C12 myoblasts were cultured for 48–72 h in Dulbecco's modified Eagle's medium containing 1% fetal bovine serum (differentiation medium).

The pBL2CATdel plasmid, containing the chloramphenicol acetyltransferase (CAT) gene driven by the minimal herpesvirus thymidine kinase (tk) gene promoter (positions −109 to +55), is described elsewhere (29). The TRE-tk-CAT and the mtTRE-tk-CAT plasmids were derived from pBL2CATdel by inserting three copies of either a wild type or a mutated human collagenase TRE upstream from the tk promoter. To obtain the TRE-tk-CAT and the mtTRE-tk-CAT stable cell lines, C2C12 myoblasts were cotransfected by the calcium phosphate precipitation method with 5 μg of the CAT reporter plasmid and 250 ng of the neomycin resistance gene vector pAG60. After selection by G418 at 500 μg/ml for 14 days, individual clones were picked up and expanded. In unstimulated and Ang-II-stimulated cells, CAT activity was assayed as described (29).

N-(2-Guanidinoethyl)-5-isoquinolinesulfonamide, H7, staurosporine, genistein, tyrphostin 25, and tyrphostin 1 were dissolved in dimethyl sulfoxide and added to the cells 2 h before the Ang-II stimulation to final concentrations of 75 mM, 100 mM, 70 ng/ml, 100 mM, 20 mM, and 20 mM, respectively. NAC and pyrrolidinedithiocarbamate were dissolved in H_2O and added to the cells 1 h before the Ang-II stimulation to final concentrations of 20 mM and 60 mM, respectively.

Cell Extracts and Electrophoretic Mobility Shift Assay—Whole cell extracts from unstimulated and Ang-II-stimulated C2C12 cells were prepared as described (30). In the extracts, protein concentrations were determined using the method of Lowry *et al.* (31). 5 μg of cell extract were incubated with 1 μg of poly(dI·dC)·poly(dI·dC), and a large excess (2 fmol) of ^{32}P-5′-end-radiolabeled double strand oligonucleotide was then added. After 20 min at room temperature, samples were subjected to electrophoresis on a 4% polyacrylamide gel with 0.25 × TBE (1 × TBE = 0.089 M Tris borate, 0.089 M boric acid, and 0.002 M EDTA). After electrophoresis, the gel was fixed with 10% acetic acid, 10% ethanol, dried, and exposed to x-ray film at −70 °C.

Bromodeoxyuridine Incorporation—Cells were kept either in 0.1% serum for 36 h (myoblasts) or in 1% serum for 48 h (myotubes) and then stimulated with 10^{-5} M Ang-II or 200 μM H_2O_2 for 18 h. When indicated, cells were pretreated for 30 min with 500 nM [Sar^1,Ile^8]Ang-II or 20 mM NAC. Control cultures were not stimulated with Ang-II or H_2O_2. In both preparations, 10 mM bromodeoxyuridine was added for the last 18 h. Cells were fixed for 5 min at room temperature in 1% paraformaldehyde, rinsed in TBS (0.05 M Tris buffer/NaCl, pH 7.6), treated with 0.1% Triton X-100 in TBS, and incubated for 1 h at 37 °C in 2 N HCl. After neutralization in 0.1 M borate buffer, pH 8.5, cells were washed in TBS and processed with anti-bromodeoxyuridine monoclonal antibody (Dako) for the immunohistochemical staining according to the APAAP complex method (32).

[^{35}S]Methionine Labeling—Cells were kept either in 0.1% serum for 36 h (myoblasts) or in 1% serum for 48 h (myotubes); pretreated or not with either [Sar^1,Ile^8]Ang-II or NAC for 30 min; stimulated for 24 h with 10^{-5} M Ang-II or 200 uM H_2O_2 in the presence or absence of [Sar^1,Ile^8]Ang-II and NAC; and then labeled for 45 min with 30 mCi/ml [^{35}S]methionine (Trans-label, ICN). After washing, cells were lysed in RIPA buffer (1 mM Tris/HCl, 1% sodium deoxycholate, 1% Nonidet P-40, 150 mM NaCl, and 0.25 mM phenylmethylsulfonyl fluoride). Extracts were clarified by centrifugation at 13,000 rpm in a microfuge at 4 °C for 30 min. 100 μg of each extract were loaded on a 10% acrylamide-SDS gel and run at 35 mA. The gel was fixed in 10% acetic acid, 10% ethanol, dried, and exposed to x-ray film at −70 °C. Quantitative evaluation of [^{35}S]methionine incorporation was performed by analyzing the radioactive emission of each gel lane by means of a PhosphorImager (Molecular Dynamics).

RESULTS

Ang-II Activates AP1/TRE-directed Transcription in C2C12 Stable Transfectants—AP1 (for a recent review see Ref. 33) is a family of transcriptional factors whose major component is the heterodimeric complex made up by the products of c-*fos* and c-*jun* proto-oncogenes. The Jun-Fos complexes bind a cis-element termed TPA (12-*O*-tetradecanoylphorbol 13-acetate) response element (TRE). To test Ang-II ability to activate transcription regulated from a TRE site, we produced C2C12 cell lines stably transfected with either a wild type TRE-tk-CAT or a mutant mtTRE-tk-CAT plasmid. As shown in Fig. 1, Ang-II increases CAT expression severalfold from the TRE, whereas

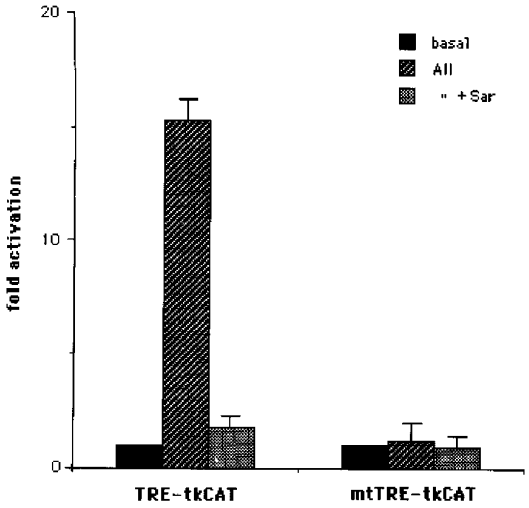

FIG. 1. **Effects of Ang-II on TRE-directed transcription in TRE-tk-CAT and mtTRE-tk-CAT stable C2C12 cell lines.** Cells from both stable cell lines were plated at 2×10^6/10-cm dish, cultured for 24 h, exposed for 24 h to Ang-II, and lysed by four cycles of freezing and thawing. 50 μg of total protein were assayed for CAT activity as described. The results are expressed as fold activation (*i.e.* the ratio of the percentage of conversion obtained in treated cells to the percentage of conversion obtained in untreated cells). Experiments were repeated three to five times with at least two different preparations of DNA. *AII*, Ang-II; " + *Sar*, [Sar^1,Ile^8]Ang-II.

no effect was observed on the mtTRE-tk-CAT cell line. This stimulation is specific, being inhibited by the selective angiotensin receptor antagonist [Sar^1,Ile^8]Ang-II (Fig. 1), and is dose-dependent, with a peak stimulation at 10^{-5} M (data not shown).

In Quiescent Undifferentiated C2C12 Myoblasts and Differentiated C2C12 Myotubes, Ang-II Increases c-Jun·c-Fos Heterodimer DNA Binding Activity—Modulation of TRE-directed transcription is an extremely complex phenomenon depending on the interplay existing between signals modulating either intrinsic transcriptional activity or DNA binding activity of the different TRE-binding proteins. Therefore, we first explored Ang-II eventual ability of modulating AP1 binding activity in the mouse skeletal muscle cell line C2C12. As shown in Fig. 2*A*, actively growing C2C12 myoblasts display a high AP1 binding activity, rapidly decreasing with serum starvation and completely abolished by the addition of specific cold TRE oligonucleotides although unaffected by the addition of unrelated cold NFκB oligonucleotides. During differentiation (Fig. 2*B*), AP1 activity drops at day 1 and subsequently increases slightly until reaching lower stable levels compared with those observed in cycling myoblasts. Also, a faster migrating band of unknown nature is observed in differentiated C2C12 myotubes (Figs. 2*B* and 3*C*) and in C2C12 myoblasts (Fig. 3*C*). Such a band can be seen in many cell types (25, 30) and cannot be competed by excess cold TRE oligonucleotide or affected by anti-c-Jun and anti-c-Fos antibodies (data not shown). Both in cycling myoblasts and differentiated myotubes, most of the TRE-bound complexes consist of c-Jun·c-Fos heterodimers, as demonstrated by the 90–95% reduction of the DNA binding activity when cell extracts were preincubated with anti-c-Fos and anti-c-Jun antibodies not cross-reacting with other members of the Fos and Jun families (data not shown).

Ang-II strongly increases AP1 binding activity in both quiescent myoblasts (Fig. 3*A*) and differentiated myotubes (Fig. 3*C*), and this phenomenon is utterly inhibited by [Sar^1, Ile^8]Ang-II (Fig. 3, *B* and *D*). Specific anti-c-Fos and anti-c-Jun antibodies were both able to almost totally eliminate the TRE binding activity in Ang-II-treated C2C12 myoblasts and myo-

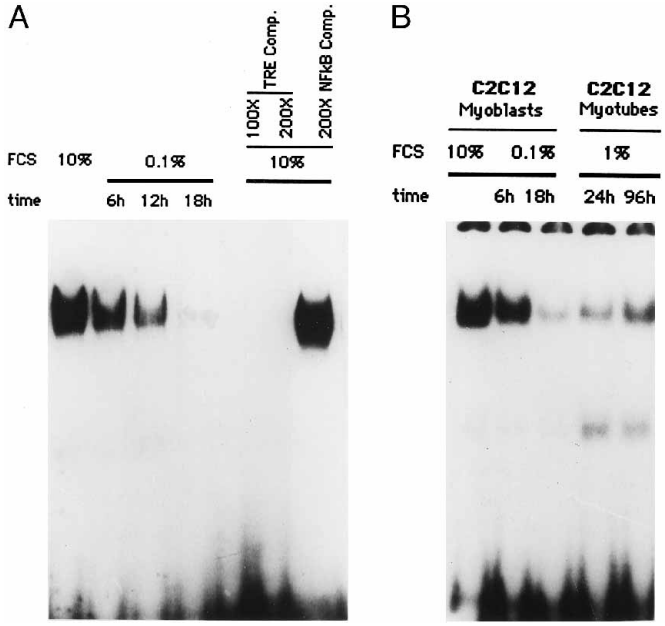

FIG. 2. **AP1 binding activity in C2C12 mouse muscle cells.** Cell extracts from actively growing or serum starved "quiescent" (*A*) and differentiated (*B*) C2C12 cells were prepared at the indicated time points as described elsewhere (28). 5 μg of each extract were tested in gel retardation assays with a ^{32}P-5′-end-radiolabeled oligonucleotide containing a canonical TRE site (5′-TCGAGTGTCTGACTCATGCTT-TCGA-3′). The specificity of the retarded complexes was assessed by preincubating the extracts with increasing amounts of cold specific TRE or unrelated NFκB probes. *FCS*, fetal calf serum.

tubes (Fig. 3, *B* and *D*), suggesting that after Ang-II stimulation, most of TRE-bound AP1 proteins consist of c-Fos and c-Jun. A variety of control antibodies (anti-p53, anti-E1A, anti-Myc) were unable to modify both basal and induced patterns (Fig. 3, *B* and *D*, and data not shown).

Ang-II-dependent Increase of AP1 Binding in C2C12 Cells Is Mediated by Multiple Intracellular Signaling Pathways—AP1 regulation involves both transcriptional and post-transcriptional events. Because the Ang-II-induced increase of AP1 binding occurs already after 10 min of stimulation and is unaffected by cycloheximide treatment at doses able to completely inhibit protein synthesis (Fig. 3, *B* and *D*), probably such an increase is ascribable to post-translational modifications of pre-existing AP1 proteins. Phosphorylation and dephosphorylation of c-Fos and c-Jun have been described as post-translational modifications having a critical role in AP1 function regulation (34–37). Therefore we tested the effect of a set of different protein kinase inhibitors on Ang-II-induced AP1 binding activity. Acting on its catalytic domain, staurosporine interferes with protein kinase C function but also exerts an inhibiting activity on both protein kinase A and pp60 v-Scr tyrosine kinase, as well as on other kinases. H7 and *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide are relatively selective inhibitors of protein kinase C and cAMP-dependent protein kinase A, respectively. Tyrphostin 25 and genistein are specific inhibitors of tyrosin kinases, and tyrphostin 1 is an inactive analogue of tyrphostin 25. As shown in Fig. 4*B*, quiescent C2C12 myoblast basal AP1 binding activity was not modified by any of these inhibitors. Staurosporine, H7, tyrphostin 25, and genistein abolish the Ang-II-induced increase of AP1 binding, whereas *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide has no effect (Fig. 4*A*). This result suggests that Ang-II signaling for AP1 binding increase involves the activation of both protein kinase C and tyrosine kinases, whereas protein kinase A does not seem to play a significant role.

ROIs Are Involved in Ang-II Induction of AP1 DNA Binding Activity—Additional regulatory mechanisms of AP1 activity involve reduction and oxidation events (25, 26). To evaluate the role of ROIs in Ang-II signaling in myogenic cells, we treated quiescent C2C12 myoblasts and C2C12 myotubes with H_2O_2 as a source of ROIs. We observed a clear dose-dependent induction of AP1 binding activity, specifically inhibited by the cysteine-derivative and glutathione precursor NAC (Fig. 4*B*) and the metal chelator and radical scavenger pyrrolidinedithiocarbamate (data not shown). Strikingly, NAC and pyrrolidinedithiocarbamate were also able to almost completely abolish the Ang-II-induced increase of AP1 binding (Fig. 4*B* and data not shown), suggesting a role for ROIs in the transduction of the Ang-II signal. To provide an insight on the site of action of ROIs in the transduction of the Ang-II signal in our cells, we tested the effects of NAC and H7 on TPA-induced AP1 binding. NAC treatment was unable to block the AP1 DNA binding induced by TPA, although H7 clearly did, thus suggesting that NAC exerts its inhibitory effect on targets upstream from protein kinase C or situated on an independent pathway (Fig. 5). The observation that H7 is able to block almost completely the induction of AP1 binding induced by H_2O_2 favors the hypothesis that in C2C12 cells, ROIs generation mediates the activation of protein kinase C by Ang-II (Fig. 5).

Ang-II-induced Generation of ROIs Influence DNA Synthesis in C2C12 Myoblasts and Protein Synthesis in C2C12 Myotubes—Many effects of ROIs may be involved in the induction of cell growth, and indeed oxidants do stimulate growth in various cell types (24). Because, as already mentioned, Ang-II is a growth factor of several muscle cell types, we examined the effects of Ang-II treatment on the rate of DNA synthesis in quiescent undifferentiated C2C12 cells and in C2C12 differentiated myoblasts. As expected, Ang-II has a clear mitogenic effect on undifferentiated myoblasts but has no effect on differentiated myotubes (Fig. 6). The induction of myoblasts proliferation by Ang-II is specifically inhibited by [Sar^1,Ile^8]Ang-II and is almost completely abolished by NAC (Fig. 6), thus suggesting that ROI generation plays an important role in the regulation of cell proliferation by Ang-II. Also H_2O_2 stimulates C2C12 myoblast proliferation, but Ang-II and H_2O_2 effects are not synergistic (Fig. 7). Finally, we evaluated the potential role of ROIs in mediating Ang-II hypertrophic effects. As detected by [^{35}S]methionine incorporation over 48 h (Fig. 7), fully differentiated myotubes responded to Ang-II with a significant increase in protein synthesis. The induction of protein synthesis by Ang-II was comparable with that induced by serum and was inhibited by [Sar^1,Ile^8]Ang-II and NAC. The ability of ROIs to influence protein synthesis in differentiated myotubes was confirmed when treating the cells with H_2O_2, and this effect was significantly inhibited by NAC (data not shown). These results strongly suggest that in differentiated C2C12 myotubes, the Ang-II hypertrophic effect (*i.e.* the increase of protein syntheis without DNA synthesis) is also mediated by the generation of ROIs.

DISCUSSION

Ang-II induces both proximal and distal signaling events ultimately leading to cell growth in a variety of myogenic and nonmyogenic cells. Ang-II binding to the Ang-II type 1 receptor initiates a cascade of early biochemical cellular events similar to those triggered by peptide growth factors. These include a rapid production of diacylglycerol and inositol triphosphate by phospholipase C-mediated hydrolysis of inositol phosholipids and activation of protein kinase C (12, 17), c-Raf1 serine threonine kinase (21), and mitogen-activating protein kinases (12, 38). Studies in rat liver epithelial cells, renal mesangial cells, and VSMCs have demonstrated that Ang-II stimulates tyrosine phosphorylation of several substrates, including phospho-

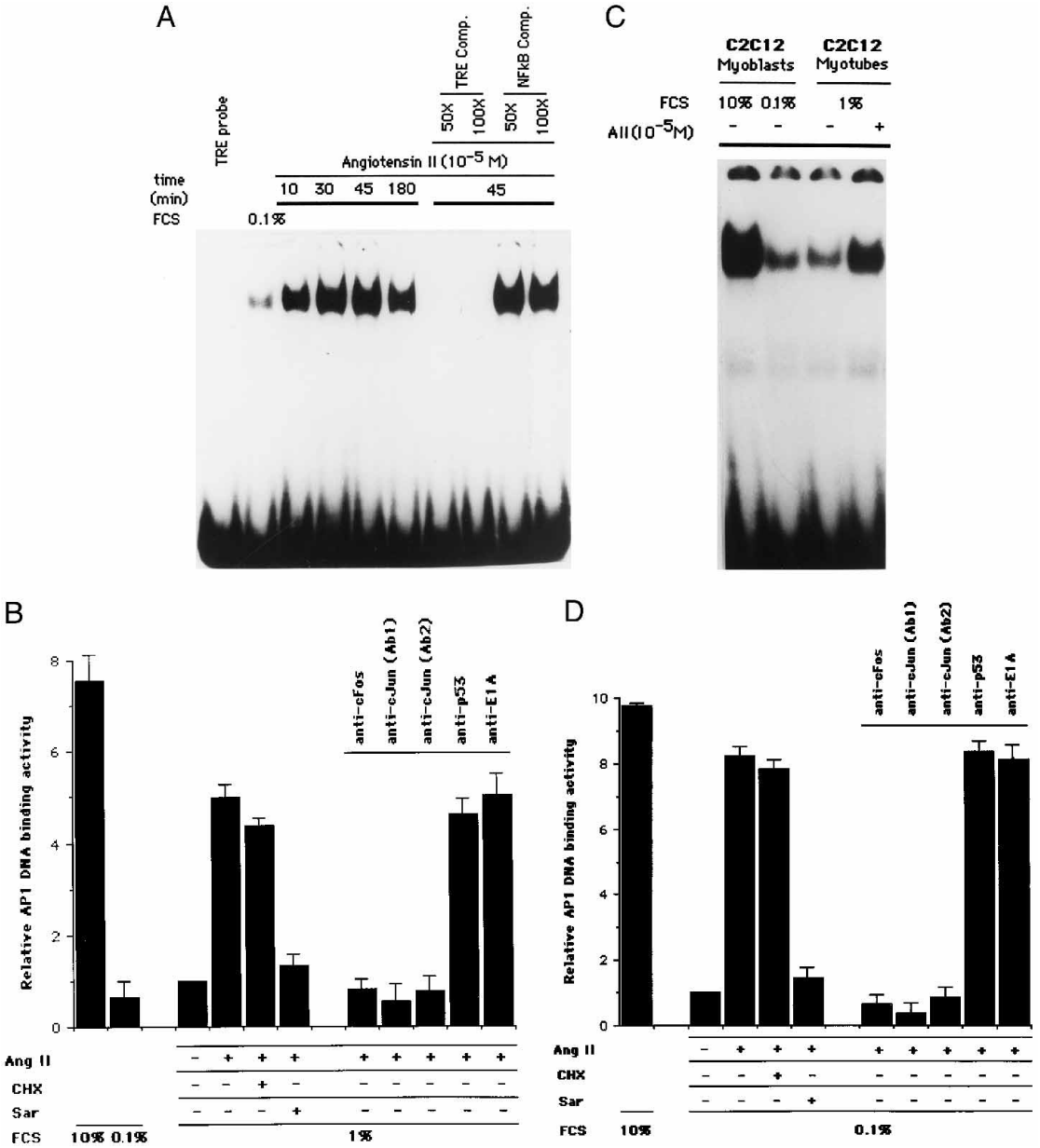

FIG. 3. **Induction by Ang-II of AP1 binding in quiescent C2C12 myoblasts (*A*) and differentiated C2C12 myotubes (*C*).** Cell extracts were prepared and tested as described in gel retardation assays. The specificity of the retarded complexes was assessed by preincubating the extracts with increasing amounts of cold specific TRE or unrelated NFκB probes. The relative AP1 DNA binding activity (mean ± S.E. from three to five experiments) was evaluated by laser densitometry analysis (GelScan, Pharmacia Biotech Inc.) and expressed as fold activation with respect to either unstimulated quiescent myoblasts (*B*) or unstimulated differentiated myotubes (*D*). The composition of TRE-bound complexes was evaluated by overnight preincubation of cell extracts from Ang-II-treated quiescent myoblasts (*B*) or differentiated myotubes (*D*) at 4 °C with 0.5 μg of a polyclonal antibody against the epitope 128–152 of c-Fos (Santa Cruz Biotechnology) and a polyclonal antibody directed against the N-terminal domain of c-Jun (Oncogene Science Ab-2) not cross-reacting with other Fos and Jun proteins. A cycloheximide concentration of 10 μg/ml was added to Ang-II-untreated and -treated cells (*C* and *D*). *CHX*, cycloheximide; *FCS*, fetal calf serum; *AII*, Ang-II.

lipase C-γ1 (19, 22, 38). In cardiac fibroblasts, it has been shown that Ang-II induces tyrosine phosphorylation of the p125 focal adhesion kinase ($p125^{Fak}$), a dominant tyrosine kinase substrate, after stimulation of many G-protein-coupled receptors and of $p46^{Shc}$ and $p56^{Shc}$. These play a crucial role in the activation of $p21^{Ras}$ and serve as a converging target for both growth factor and G-protein-coupled receptor-stimulated mitogenic responses (23). In VSMCs, Ang-II also causes a rapid and sustained activation of phospholipase D-mediated phosphatidylcholine hydrolysis, resulting in the formation of PA (39). Although a certain part of PA is converted to diacylglycerol, the PA that builds up could be involved in both calcium influx regulation and mitogenesis. Moreover, through PA generation, Ang-II stimulates NADH and NADPH oxidase activity in VSMCs, thus promoting superoxide generation (39). Finally, it has been shown that Ang-II has a strong inducing effect on the release of arachidonic acid from cultured cardiomyocytes and that arachidonic acid and inositol phosphate production occurs through distinct Ang-II type 1 and type 2 receptors and independent signal transduction pathways involving phospholipase C and phospholipase A_2, respectively (21).

Despite the increasing knowledge of the signaling pathways stimulated by Ang-II in different cell lines, the cross-talk between different second messengers and their correlation with specific physiological responses (*i.e.* vasoconstriction, hypertrophy, and hyperplasia) remains relatively unexplored. In this study we demonstrate that in myogenic cells, ROI generation plays a role in the intracellular transduction of Ang-II signals

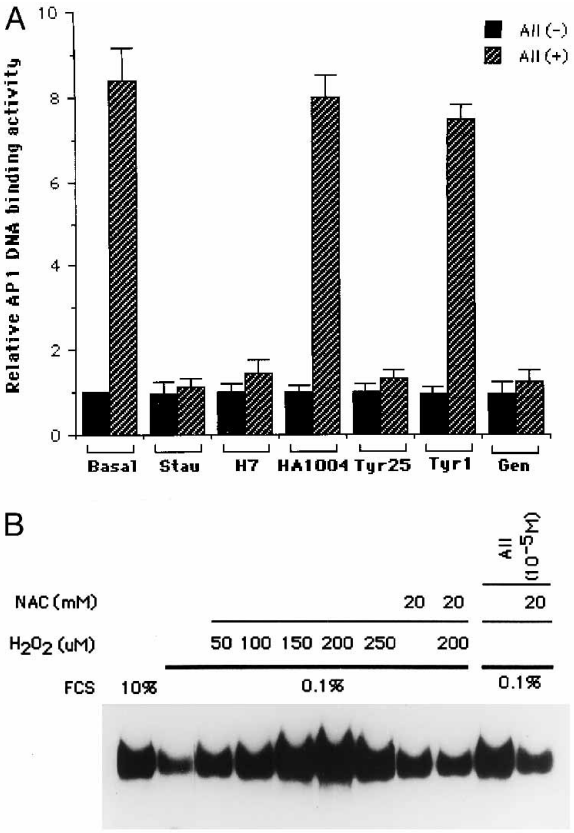

FIG. 4. **Effects of protein kinase inhibitors and anti-oxidants on Ang-II induction of TRE binding.** Cell extracts were prepared from unstimulated and Ang-II-stimulated C2C12 quiescent myoblasts treated with different protein kinases inhibitors (*A*) and from H_2O_2 stimulated cells treated with the cysteine-derivative and glutathione precursor NAC (*B*) (see "Experimental Procedures"). Gel retardation assays were performed, and the results are expressed as described in Fig. 3. *AII*, Ang-II; *HA1004*, *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide; *Tyr1*, tyrphostin 1; *Tyr25*, tyrphostin 25; *Gen*, genistein; *FCS*, fetal calf serum.

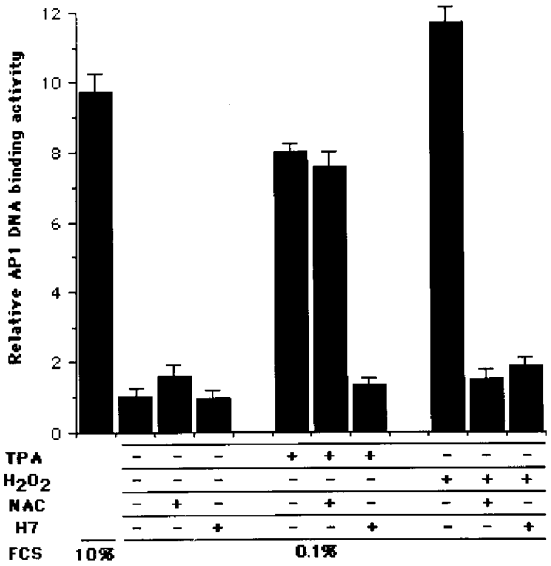

FIG. 5. **Effects of NAC and H7 treatment on TRE DNA binding induction by TPA (100 ng/ml) and H_2O_2 (200 μM) in C2C12 quiescent myoblasts.** NAC and H7 have been used as described under "Experimental Procedures." The relative AP1 DNA binding activity (mean ± S.E. from three to five experiments) was evaluated by laser densitometry analysis (GelScan) and expressed as fold activation with respect to either unstimulated quiescent myoblasts. *FCS*, fetal calf serum.

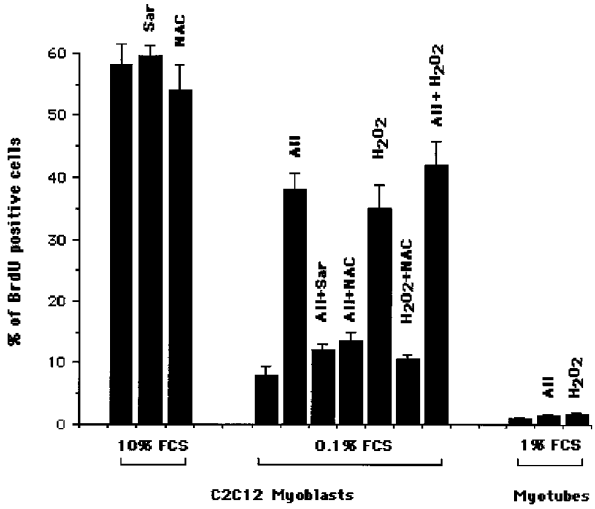

FIG. 6. **Ang-II induction of DNA synthesis in quiescent C2C12 myoblasts.** Cells were kept either in 0.1% serum for 36 h (myoblasts) or in 1% serum for 48 h (myotubes) and then stimulated for 24 h with Ang-II at 10^{-5} M or 200 uM H_2O_2 in the presence or absence of [Sar^1,Ile^8]Ang-II and NAC. Control cultures were prepared without stimulation with Ang-II or H_2O_2. In both preparations, for the last 18 h 10 uM bromodeoxyuridine was added. *BrdU*, bromodeoxyuridine; *FCS*, fetal calf serum; *AII*, Ang-II.

for immediate early c-*fos* and c-*jun* gene induction, cell proliferation, and hypertrophy. In both quiescent C2C12 myoblasts and differentiated C2C12 myotubes, Ang-II significantly stimulates AP1-driven transcription in C2C12 TRE-tk-CAT stable transfectants and AP1 binding. Using a set of different protein kinase inhibitors we show that the Ang-II-induced c-Jun/c-Fos heterodimer binding increase is not mediated by the cAMP-dependent pathway and that protein kinase C and tyrosine kinases are involved. Moreover, in quiescent C2C12 myoblasts Ang-II induction of both AP1 DNA binding activity and DNA synthesis is abolished by antioxidants. This strongly suggests a role for ROIs in the intracellular transduction of Ang-II signals for both immediate early gene induction and cell proliferation. Eukaryotic cells continuously produce the ROI H_2O_2, superoxide (O_2^-), and hydroxyl radical (OH^-) as by-products of electron transfer reactions (37). A condition of oxidative stress, characterized by above normal levels of ROIs, frequently occurs in cells exposed to UV light, gamma rays, or low concentrations of H_2O_2, but also upon cell stimulation with cytokines and natural ligands of other cell surface receptors (40). Although very high levels of ROIs, as produced by stimulated neutrophils, are strictly cytocidal and serve primarily to kill parasites in the organism, the increase of ROI levels observed in many conditions seems to induce many early growth signals, including a rise in intracellular pH (41), the expression of c-*fos*, c-*jun*, and c-*myc* proto-oncogenes, and the activation of transcription factors (40, 42, 43), protein kinases (44), protein phosphatases (45), and ion channels (46). A role for oxidative stress has been proposed in different pathological conditions, such as atherogenesis and carcinogenesis (47). Increased concentrations of active oxygen species have also been measured during the inflammatory stage of the restenosis process in response to angioplasty (48). Hyperplasia is an important aspect of these pathological conditions, and our results indicate that ROI generation mediate Ang-II mitogenic effects on quiescent C2C12 cells. Although in the induction of cell proliferation by growth factors, serum and TPA AP1-activity is required (49), in C2C12 myotubes Ang-II-dependent AP1 activation occurs independently from DNA synthesis stimulation. This suggests that in

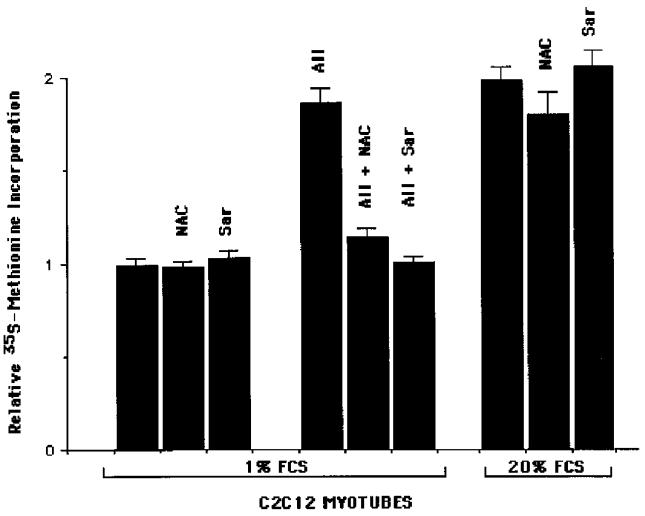

FIG. 7. **Ang-II induction of protein synthesis in differentiated C2C12 myotubes.** Cells were kept either in 0.1% serum for 36 h (myoblasts) or in 1% serum for 48 h (myotubes), stimulated for 24 h with Ang-II at 10^{-5} M or 200 uM H_2O_2 in the presence or absence of [Sar^1,Ile^8]Ang-II and NAC, and finally labeled with 200 mCi/ml [^{35}S]methionine (Trans-label). *FCS*, fetal calf serum; *AII*, Ang-II.

differentiated cells modulation of TRE-containing genes by AP1 might be important for other cellular responses. Our results support the hypothesis that in differentiated C2C12 myotubes Ang-II hypertrophic effects (*i.e.* increase of protein synthesis without DNA synthesis) are also mediated by the generation of ROIs. Thus, ROIs generation might represent a common second messenger involved in the induction of both early events (*i.e.* AP1 binding induction) and long term metabolic effects (hyperplasia or hypertrophy) in response to a single growth factor.

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