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 AP-1 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 erg1 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 trisphosphate; induces calcium release from inositol trisphosphate-sensitive calcium storage sites; activates protein kinase C, phospholipase A2, 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 A2 and phospholipase D stimulates the intracellular generation of ROS 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 ROS 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
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 chloramphenical acetyltransferase (CAT) gene driven by the minimal herpesvirus thymidine kinase 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-isoxazolinesulfonyamide, 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 H2O 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 of 32P-end-labeled 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 H2O2 for 18 h. When indicated, cells were pretreated for 30 min with 500 nM [Sar1,Ile8]Ang-II or 20 μM NAC. Control cultures were not stimulated with Ang-II or H2O2. 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 μCi/ml 35S-methionine (Trans-label, ICN). After washing, cells were lysed in RIPA buffer (1 mM Tris-HCl, 1% sodium deoxycholate, 0.1% Nonidet P-40, 150 mM NaCl, and 0.25 mM phenylmethylsulfonyl fluoride). Extracts were clarified by centrifugation at 13,000 rpm in a microtube 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 35S-methionine incorporation was performed by analyzing the radioactivity emission of each gel lane by means of a Phosphorimage (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 J un-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 no effect was observed on the mtTRE-tk-CAT cell line. This stimulation is specific, being inhibited by the selective angiotensin receptor antagonist [Sar1,Ile6]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 Differen-
tiated 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. 2A, 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 NF-kB oligonucleotides. During differentiation (Fig. 2B), 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. 2B and 3C) and in C2C12 myotubes (Fig. 3C). 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 un- 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 un-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 J un families (data not shown).

Ang-II strongly increases AP1 binding activity in both quiescent myoblasts (Fig. 3A) and differentiated myotubes (Fig. 3C), and this phenomenon is utterly inhibited by [Sar1,Ile6]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-
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$_2$O$_2$, 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. 4B) 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. 4B 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$_2$O$_2$ 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 myotubes. 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 ROIs generation plays an important role in the regulation of cell proliferation by Ang-II. Also H$_2$O$_2$ stimulates C2C12 myoblast proliferation, but Ang-II and H$_2$O$_2$ 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$_2$O$_2$, 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 synthesis 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 I 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 phospholipids 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--
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 A₂, 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.
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 ROS in the intracellular transduction of Ang-II signals for both immediate early gene induction and cell proliferation. Eukaryotic cells continuously produce the ROI H2O2, superoxide (O2•−), 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 H2O2 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 cytoidal 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 atherosclerosis 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
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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