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# Short Communication

# Modulation of Angiotensin II Signaling for GATA4 Activation by Homocysteine

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## ABSTRACT

**Homocysteine (Hcy) is a redox active thiol-containing compound with pro-oxidant and pathogenic properties in the cardiovascular system. Angiotensin II (Ang II) also plays important roles in age-associated cardiovascular disease. Recently, the GATA4 transcription factor was recognized as a mediator of heart failure. We investigated the interrelationship of these elements in NIH/3T3 fibroblasts and found that Ang II induces GATA4 activity and Hcy alters Ang II signaling. Electrophoretic mobility shift assays determined that treatment of cells with Ang II induced DNA binding activity to the GATA consensus sequence. This activation was transient with a peak occurring at 30 min. Supershift analysis revealed the GATA binding protein as GATA4. Ang II also induced NFAT activity with similar kinetics. Pretreatment of cells with Hcy (100 $\mu M$) delayed the peak of Ang II-induced NFAT and GATA activation to 60 min. Ang II-mediated activation of c-*fos* serum response factor (SRF) was similarly delayed by Hcy. These results suggest the pathogenic mechanism of Hcy action may be mediated in part via modulation of Ang II-signaling for gene transcription.** Antiox. Redox Signal. 1, 233–238, 1999.

## INTRODUCTION

HOMOCYSTEINE (Hcy) is a redox active thiol-containing biological compound generated during the metabolism of methionine. Epidemiological studies indicate that moderate hyperhomocysteinemia ( $\geq 14$ $\mu M$ Hcy) is an independent risk factor for cardiovascular disease (Clarke *et al.*, 1991; Selhub *et al.*, 1995). Hereditary hyperhomocysteinemia ( $> 100$ $\mu M$ Hcy) is associated with enzymatic defects in cystathionine synthase, Hcy methyltransferase, and methylenetetrahydrofolate reductase and results in premature arteriosclerosis, mental retardation, and other symptoms (Olszewski and McCully, 1993; McCully, 1996). It has been postulated that the production of reactive oxygen species (ROS) may be involved in the pathogenesis of Hcy-mediated diseases (Olszewski and McCully, 1993; Loscalzo, 1996).

Angiotensin II (Ang II), an essential component of the renin-angiotensin system, is also associated with hypertension, coronary heart disease, and heart failure (Dostal *et al.*, 1997). The action of Ang II to elicit cell growth signaling in vascular smooth muscle cells, cardiac myocytes, and fibroblasts may underlie, in part, its mechanism in the cardiovascular pathology associated with aging. Ang II also elicits early signal transduction events such as $Ca^{2+}$ release

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(Taubman *et al.*, 1989), $p21^{ras}$ activation (Schieffer *et al.*, 1996), tyrosine phosphorylation (Sadoshima *et al.*, 1995), p44/42 MAP kinase activation (Duff *et al.*, 1992; Sadoshima *et al.*, 1995), c-Jun amino$_2$-terminal kinase stimulation (Kudoh *et al.*, 1997), c-*fos* gene expression (Taubman *et al.*, 1989), and ROS generation (Griendling *et al.*, 1994).

The GATA4 transcription factor appears to be an important regulator of genes involved in cardiovascular disease (Herzig *et al.*, 1997; Molkentin and Olson, 1997). Molkentin *et al.*, (1998) reported that calcineurin, a $Ca^{2+}$-dependent protein phosphatase, elicited cardiac hypertrophy through activation of NFAT-GATA4 interactions. Because NFAT is activated by a low and sustained $Ca^{2+}$ influx (Dolmetsch *et al.*, 1997), activation of noncontractile pathways of $Ca^{2+}$ may turn on signals for cell growth. Because $Ca^{2+}$ signaling is redox sensitive (Suzuki and Ford, 1999), activities of $Ca^{2+}$-regulated transcription factors may be affected by redox-active agents.

We have investigated the influence of Hcy on NFAT and GATA4 activities elicited by Ang II.

## MATERIALS AND METHODS

NIH/3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 0.5 mg/ml gentamicin in an atmosphere of 5% $CO_2$ at 37°C in humidified air. Cells grown at 80–90% confluency were serum starved for 18 hr and treated with D,L-Hcy (Sigma Chemical Company, St. Louis, MO) and/or Ang II (Sigma Chemical) for various time periods.

DNA binding activities were monitored by electrophoretic mobility shift assays (EMSA) (Suzuki and Packer, 1995). To prepare nuclear extracts, cells were washed in phosphate-buffered saline (PBS) and incubated in a solution containing 10 m*M* HEPES, pH 7.8, 10 m*M* KCl, 2 m*M* $MgCl_2$, 0.1 m*M* EDTA, 0.1 m*M* phenylmethyl sulfonyl fluoride (PMSF), and 5 $\mu$g/ml leupeptin. IGEPAL CA-630 (Sigma Chemical) was then added at a final concentration of 0.6%, mixed vigorously, and centrifuged. Pelleted nuclei were resuspended in a solution containing 50 m*M* HEPES, pH 7.8, 50 m*M* KCl, 300 m*M* NaCl, 0.1 m*M* EDTA, 0.1 m*M* PMSF, and 10% (vol/vol) glycerol, then mixed and centrifuged. The supernatant was harvested and protein concentrations determined (Bradford, 1976). Binding-reaction mixtures contained 2 $\mu$g protein of nuclear extract, 1 $\mu$g poly(dI-dC)•poly(dI-dC), and $^{32}$P-labeled double-stranded oligonucleotide probe for GATA (5′-CAC TTG ATA ACA GAA AGT GAT AAC TCT-3′), NFAT (5′-CGC CCA AAG AGG AAA ATT TGT TTC ATA-3′) or SRF (5′-GGA TGT CCA TAT TAG GAC ATC T-3′) in 50 m*M* NaCl, 0.2 m*M* EDTA, 0.5 m*M* dithiothreitol (DTT), 10% (vol/vol) glycerol, and 10 m*M* Tris-HCl, pH 7.5. Electrophoresis of samples through a native 6% polyacrylamide gel was followed by autoradiography. Supershift experiments were performed with the rabbit polyclonal immunoglobulin G (IgG) for GATA4 (Santa Cruz Biotechnology, Santa Cruz, CA).

Reverse transcription-polymerase chain reaction (RT-PCR) analysis was used to monitor c-*fos* mRNA levels. Total RNA was extracted by phenol-chloroform extraction and ethanol

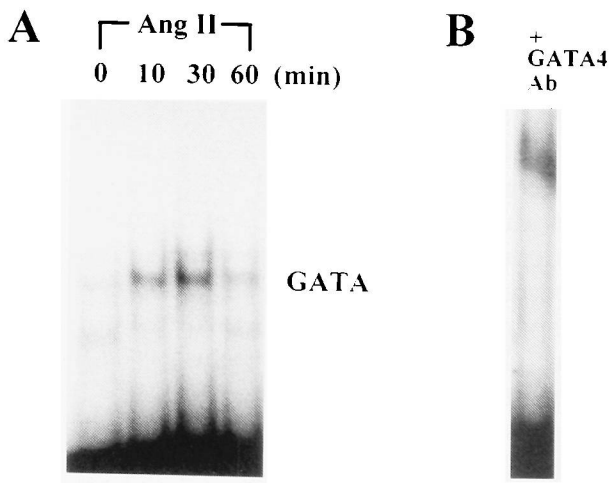


**FIG. 1. Ang II activates GATA4. A.** Serum-starved NIH/3T3 cells were treated with 1 $\mu$*M* Ang II for 0–60 min. Nuclear extracts were prepared and binding activity to the oligonucleotide containing GATA consensus sequence was monitored by EMSA. **B.** Supershift analysis of samples from cell treatment with Ang II for 30 min revealed the GATA binding protein is GATA4.

precipitation using TRIZOL Reagent (GIBCO BRL, Gaithersburg, MD), then reverse-transcribed by oligo(dT) priming and MMLV (Moloney marine leukemia virus) reverse transcriptase, and the cDNA was amplified by *Taq* DNA polymerase in a Perkin-Elmer Gene Amp PCR System 2400. PCR products were resolved on 1.5% agarose gels containing eithidium bromide. Sequences of PCR primers (Clonetech, Palo Alto, CA) are: c-*fos* [5′ Primer = 5′-GAG CTG ACA GAT ACA CTC CAA GCG-3′; 3′ Primer = 5′CAG TCT GCT GCA TAG AAG GAA CCG-3′ and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) [5′ Primer = 5′-ACC ACA GTC CAT GCC ATC AC-3′; 3′ Primer = 5′-TCC ACC ACC CTG TTG CTG TA-3′]. Denaturing, annealing, and polymerase reactions were performed 30 times at 94°C for 45 sec, 60°C for 45 sec, and 72°C for 2 min, respectively. Experiments were repeated at least three times to confirm the results.

## RESULTS

Nuclear extracts from serum-starved NIH/3T3 cells reveal very low levels of constitutive DNA binding activity to the GATA consensus sequence (Fig. 1A). Treatment of cells with a saturating concentration of Ang II (1 $\mu M$) increased GATA binding activity within 10 min. Kinetics of GATA activation was transient with a peak occurring at 30 min and stimulation absent by 60 min. Supershift analysis revealed the GATA binding protein induced by Ang II is GATA4 (Fig. 1B).

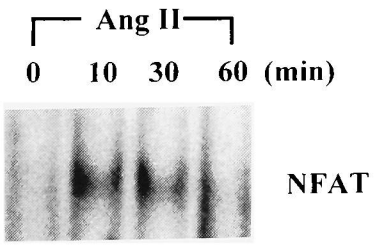


**FIG. 2. Ang II activates NFAT.** Serum-starved NIH/3T3 cells were treated with 1 $\mu M$ Ang II for 0–60 min. Nuclear extracts were prepared and binding activity to the oligonucleotide containing NFAT consensus sequence was monitored by EMSA.

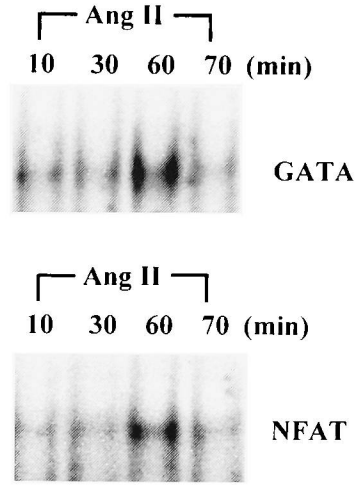


**FIG. 3. Hcy delays Ang II-induced GATA4 and NFAT activation.** Serum-starved cells were pretreated with 100 $\mu M$ Hcy for 1 hr and then treated with 1 $\mu M$ Ang II for durations indicated. Nuclear extracts were prepared and DNA binding activities to GATA and NFAT consensus sequences were monitored.

Calcineurin-activated NFAT interacts with GATA4 through protein–protein interactions, forming the NFAT-GATA4 pathway (Molkentin *et al.*, 1998). Treatment of cells with Ang II also induced NFAT DNA binding activity (Fig. 2) with transient kinetics similar to those of Ang II-mediated GATA activation.

Pretreatment of cells with 100 $\mu M$ Hcy altered the kinetics of Ang II-mediated GATA4 activation by delaying the maximal activation from 30 min (Fig. 1) to 60 min (Fig. 3). The kinetics of NFAT activation by Ang II were similarly altered as the peak of activation shifted to 60 min in Hcy pretreated cells.

Ang II induces c-*fos* gene expression (Taubman *et al.*, 1989). RT-PCR analysis revealed that Ang II elicited a transient induction of *c-fos* mRNA expression in serum-starved NIH/3T3 cells with a peak occurring at 30 min (Fig. 4). Pretreatment of cells with Hcy (100 $\mu M$) altered the kinetics of Ang II-mediated induction of c-*fos* gene expression by delaying the peak of induction to 60 min. No changes were observed in control G3PDH mRNA levels. EMSA revealed Ang II-mediated activation of DNA binding activity of serum response factor (SRF) to c-*fos* serum response element (SRE) was similarly altered with a delay of the peak from 30 to 60 min (Fig. 5).

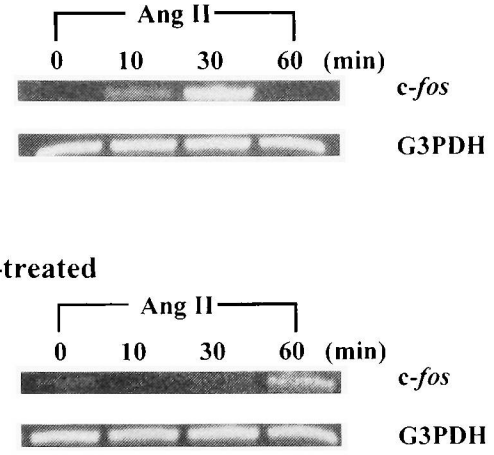


**FIG. 4. Hcy delays Ang II-induced c-*fos* gene expression.** Serum-starved cells with or without pretreatment with 100 $\mu M$ Hcy for 1 hr were treated with 1 $\mu M$ Ang II for durations indicated. Total RNAs were isolated and c-*fos* and G3PDH mRNAs were monitored by RT-PCR analysis.

## DISCUSSION

These results demonstrate Ang II induces NFAT and GATA4 transcription factors, regulators of genes involved in cardiovascular disease, such as the Ang II $type_{1a}$ receptor (Herzig *et al.*, 1997; Molkentin *et al.*, 1998). Further, clinically relevant concentrations of Hcy altered Ang II signal transduction patterns including kinetics of NFAT and GATA4 activation.

Aging does not significantly alter Ang II-signaling for vascular contraction in rats (Wakabayashi *et al.*, 1990; Lang *et al.*, 1995; Tschudi and Luscher, 1995) or in humans (McDonald *et al.*, 1995). Duggan *et al.* (1992) found no significant changes with age in basal plasma Ang II levels or Ang II receptor density and affinity in healthy normotensive people. Therefore, the increased risk for cardiovascular diseases with age, which may be associated with promotion of Ang II signaling, may not be due to increased levels of Ang II or its receptor. However, other age-associated factors such as increasing Hcy status (Nygard *et al.*, 1995) may modulate Ang II signal transduction patterns and elicit untoward outcomes.

We have found Ang II induces a transient activation of GATA4 and NFAT in NIH/3T3 fibroblasts, and that Hcy, at a concentration found in patients with hyperhomocysteinemia, significantly delays these events. Hcy also altered the kinetics of Ang II-mediated induction of c-*fos* gene expression and SRF activation. Because Ang II-signaling for c-*fos* SRF activation can also involve $Ca^{2+}$ signaling (Taubman *et al.*, 1989), Hcy may affect a low, sustained $Ca^{2+}$ release, which has been shown to elicit ERK- and NFAT-activated pathways (Dolmetsch *et al.*, 1997).

The pathogenic mechanism of Hcy is not well understood. Hcy is a thiol reductant, but its reductive property may elicit superoxide and hydrogen peroxide ($H_2O_2$) formation through one and two electron reductions of molecular oxygen, respectively. Thus, Hcy can act as a biological oxidant by generating ROS. Hcy could also reduce metal ions which together with $H_2O_2$ can generate hydroxyl radicals via a Fenton reaction. Starkebaum and Harlan (1986) observed copper-catalyzed $H_2O_2$ generation and injury to endothelial cells induced by Hcy. Further, Nishio and Watanabe (1997) reported that Hcy enhances actions of platelet-derived growth factor (PDGF) in vascular smooth muscle cells and elicits cell growth through the generation of $H_2O_2$.

Age-mediated changes in signal transduction patterns associated with oxidation or alterations of cellular redox balance may play an important role in the pathogenesis of cardiovascular disease. The present study demonstrates that normal signal transduction kinetics can be altered by redox-active compounds.

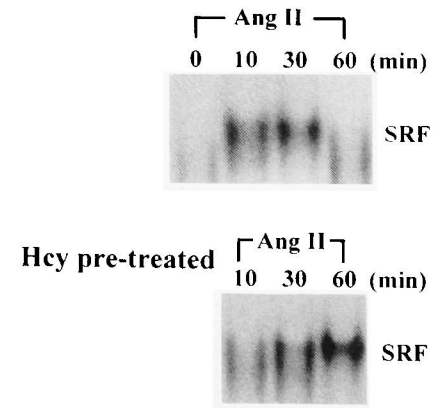


**FIG. 5. Hcy delays Ang II-induced SRF activation.** Serum-starved cells with or without pretreatment with 100 $\mu M$ Hcy for 1 hr were treated with 1 $\mu M$ Ang II for durations indicated. Nuclear extracts were prepared and binding activity to the oligonucleotide containing c-*fos* SRE consensus sequence was monitored by EMSA.

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## ABBREVIATIONS

Ang II, angiotensin II; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; $H_2O_2$, hydrogen peroxide; Hcy, homocysteine; IgG, immunoglobulin G; MMLV, Moloney murine leukemia virus; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; PMSF, phenylmethylsulfonyl fluoride; ROS, reactive oxygen species; RT-PCR, reverse transcription polymerase chain reaction; SRE, serum response element; SRF, serum response factor.

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