*Original Article*

# Globular Adiponectin Activates Nuclear Factor-κB and Activating Protein-1 and Enhances Angiotensin II–Induced Proliferation in Cardiac Fibroblasts

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**Adiponectin is present in the serum as a trimer, hexamer, or high–molecular weight form. A proteolytic cleavage product of adiponectin, known as globular adiponectin (gAd), also circulates in human plasma. The biological activities of these isoforms are not well characterized. Pressure overload in adiponectin-deficient mice results in enhanced concentric cardiac hypertrophy and increased mortality, suggesting that adiponectin inhibits hypertrophic signaling in the myocardium. Therefore, we examined whether gAd exerts the same effects on myocardium signaling. Nuclear factor-κB (NF-κB) and activating protein-1 (AP-1) activation were examined using cardiac fibroblasts prepared from the ventricles of 1- to 2-day-old Wistar rats and grown in culture. gAd activated NF-κB and enhanced tumor necrosis factor-α (TNF-α)-induced NF-κB activity. gAd also activated AP-1 and enhanced angiotensin II (Ang II)-induced AP-1 activity. gAd induced mRNA expression of c-fos and c-jun and activated extracellular signal–regulated kinase. Thus, gAd enhanced Ang II–induced DNA and collagen synthesis. Antibodies against adiponectin receptor (AdipoR)1 and AdipoR2 elicit activation of NF-κB or AP-1, two redox-sensitive transcription factors. Thus, rather than having an antihypertrophic effect, gAd might contribute to the activation of myocardium signaling, leading to myocardial hypertrophy. *Diabetes* 56:804–808, 2007**

Adiponectin is a circulating adipose-derived cytokine that is present in the serum as a trimer, hexamer, or high–molecular weight form. A proteolytic cleavage product of adiponectin, known as globular adiponectin (gAd), also circulates in human plasma (1). The biological activities of these isoforms are controversial, but it appears that high–molecular weight adiponectin has a mainly beneficial role in humans and rodents (2,3). Recombinant gAd is pharmacologically active and induces free fatty acid oxidation in incubated mouse muscle and cultured muscle cells (1). gAd is significantly more potent in reversing insulin resistance than uncleaved adiponectin (4). gAd is also a potent stimulator of nuclear factor-κB (NF-κB) activation, which in turn induces the expression of pro-inflammatory and adhesion molecule genes in vascular endothelial cells (5). Waki et al. (6) reported that adiponectin can be cleaved by leukocyte elastase secreted from activated monocytes and/or neutrophils and that this cleavage is a possible mechanism for the generation of the gAd fragment in the plasma. Although the pathophysiological importance of adiponectin cleavage by leukocyte elastase in vivo remains to be determined, adiponectin cleavage in inflammatory sites might facilitate the atherogenic process.

Pressure overload in adiponectin-deficient mice results in enhanced concentric cardiac hypertrophy and increased mortality, suggesting that adiponectin inhibits hypertrophic signaling in the myocardium (7). Therefore, we examined whether gAd exerts the same effects on myocardium signaling. Interstitial fibroblast proliferation and collagen accumulation are associated with compensatory remodeling of the hypertrophic myocardium (8,9), and structural remodeling leads to diastolic and systolic dysfunction (10). Angiotensin II (Ang II) is closely involved in cardiac remodeling by stimulating hyperplastic growth of cardiac fibroblasts (11) and synthesis of extracellular matrix protein (9). In the present study, to investigate the role of gAd in cardiac remodeling, we examined its effects on NF-κB and activating protein-1 (AP-1) activation in cultured rat cardiac fibroblasts. We further examined the extracellular signal–regulated kinase (ERK) 1/2 pathway and synthesis of DNA and collagen in Ang II–stimulated cardiac fibroblasts.

## RESEARCH DESIGN AND METHODS

**Cell culture.** Cardiac fibroblasts were prepared from ventricles of 1- to 2-day-old Wistar rats and grown as previously described (12,13). Subcultured fibroblasts from passages 4–6 were used in this experiment, and they were >99% positive for vimentin antibody immunostaining. In addition, they were negative for desmin (for myocytes), smooth muscle actin (for vascular smooth muscle cells), and a polyclonal antibody against von Willebrand factor (for endothelial cells) immunostaining. Subconfluent cells were serum-starved for 48 h and used for the experiments.

**NF-κB and AP-1 activation.** To study NF-κB activation, rat cardiac fibroblasts were stably transfected with a cis-reporter plasmid containing the luciferase reporter gene linked to five repeats of NF-κB binding sites (pNFκB-

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AdipoR, adiponectin receptor; Ang II, angiotensin II; AP-1, activating protein-1; C1qR, C1q receptor; ERK, extracellular signal–regulated kinase; gAd, globular adiponectin; IL, interleukin; NF-κB, nuclear factor-κB; siRNA, small interference RNA; TNF-α, tumor necrosis factor-α.

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Luc; Stratagene, La Jolla, CA) as previously described (14). Several clones were selected for the analysis of NF-κB activation. Luciferase activity was measured using a luciferase assay kit (Stratagene). Similarly, a cis-reporter plasmid containing the luciferase reporter gene joined to seven AP-1 sites (pAP1-Luc; Stratagene) was stably transfected into cardiac fibroblasts to study AP-1 activation (14).

**Analysis of DNA and collagen synthesis.** The effects of gAd on DNA and collagen synthesis in cardiac fibroblasts were evaluated by measuring the cell incorporation of [$^3$H]thymidine and [$^3$H]proline, respectively, as previously described (12,13). To examine collagen synthesis, 0.5 μCi [$^3$H]proline was added after treatment with Ang II (Sigma Chemical, St. Louis, MO), and the cells were incubated for 24 h. To examine DNA synthesis, 0.5 μCi [$^3$H]thymidine was added 12 h after treatment with Ang II, and the cells were incubated for 12 h. After labeling, the cells were rinsed twice with cold PBS and incubated with 10% trichloroacetic acid at 4°C for 30 min. The precipitates were then washed twice with cold 95% ethanol and solubilized in 1 mol/l NaOH. The radioactivity of aliquots of trichloroacetic acid–insoluble material was determined using a liquid scintillation counter.

**Immunoblot analysis.** Cardiac fibroblasts treated with Ang II in the presence or absence of gAd were lysed using cell lysis buffer (Cell Signaling, Beverly, MA) with 1 mmol/l pheylmethylsulphonyl fluoride. The protein concentration within each sample was measured using a Bio-Rad detergent–compatible protein assay. Subsequently, β-mercaptoethanol was added at a final concentration of 1%, after which each sample was denatured by boiling for 3 min. Samples containing 15 μg protein were resolved by electrophoresis on a 12% SDS-polyacrylamide gel, transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA), and incubated with phospho-ERK antibody and ERK antibody (1:1,000; Cell Signaling). The binding of each of these antibodies was detected using sheep anti-rabbit IgG horseradish peroxidase (1:5,000) and the ECL Plus system (Amersham, Buckinghamshire, U.K.).

**ERK activity.** To create reporter cardiac fibroblasts to evaluate ERK activation, we used a transreporting system using a GAL4 fusion transactivator as a pathway-specific sensor as previously described (15). The system uses a fusion transactivator plasmid containing the DNA binding domain of the yeast GAL4 (residues 1–147) protein, followed by the activation domain of Elk-1. We first transfected the pFR-Luc plasmid containing the luciferase gene controlled by a promoter that responds to GAL4 fusions (Stratagene) with a pSV40/Zeo2 plasmid containing a Zeocin expression cassette (Invitrogen, Carlsbad, CA) into cardiac fibroblasts. Through successive rounds of selection in antibiotic-containing medium (300 μg/ml Zeocin), Zeocin-resistant clones were isolated. Next, in separate experiments, we transfected the Zeocin-resistant cells with the fusion transactivator pFA2-Elk-1 plasmids (Stratagene). After performing successive rounds of selection with G418 (500 μg/ml), we chose the clones that gave the best response to ERK activation. These clones were then subjected to extensive analysis. The cells were grown to confluence in 24-well plates and then made quiescent by incubation with serum-free media for 24 h. Then the cells were incubated under experimental conditions for 6 h to analyze ERK activity.

**Real-time PCR.** For quantitative measurement of mRNA, 2 μg total RNA was treated with DNase I for 15 min and subsequently used for cDNA synthesis. The PCRs with the cDNA were performed in a LineGene system (BioFlux, Tokyo, Japan) under the following conditions: 95°C for 5 min, 40 cycles at 95°C for 15 s, 60°C for 15 s, and 72°C for 30 s.

**Small interference RNA transfection.** One day before transfection, plates were inoculated with an appropriate number of cardiac fibroblasts in serum-containing medium to ensure 50–70% confluence the following day. The small interference RNA (siRNA) (Santa Cruz Biotechnology, Santa Cruz, CA) for the C1q receptor (C1qR) mixed with siLentFect (Bio-Rad Laboratories) was added to the cells to a final concentration of 10 nmol/l. Twenty-four hours after transfection, the cells were serum-starved for another 24 h. Using those cells, NF-κB or AP-1 activation was examined in response to gAd.

**Materials.** gAd was obtained from PeproTech EC (London, U.K.). This protein is recombinant and derived from mouse globular domain ACRP30 cDNA expressed in *Escherichia coli*. This gAd was endotoxin free according to the limulus test (sensitivity, 0.06 units/ml; Sigma). Adiponectin receptor (AdipoR) antibodies were rabbit anti-mouse AdipoR1 and AdipoR2 IgG (Alpha Diagnostic International, San Antonio, TX). siRNA for C1qR was obtained from Santa Cruz Biotechnology. BAY 11-7082 was obtained from Biomol (Plymouth Meeting, PA).

**Statistical analysis.** The results are expressed as the means ± SD. ANOVA and Fisher's least significant difference test were used for multigroup comparisons, with a $P$ value of $<0.05$ considered significant.

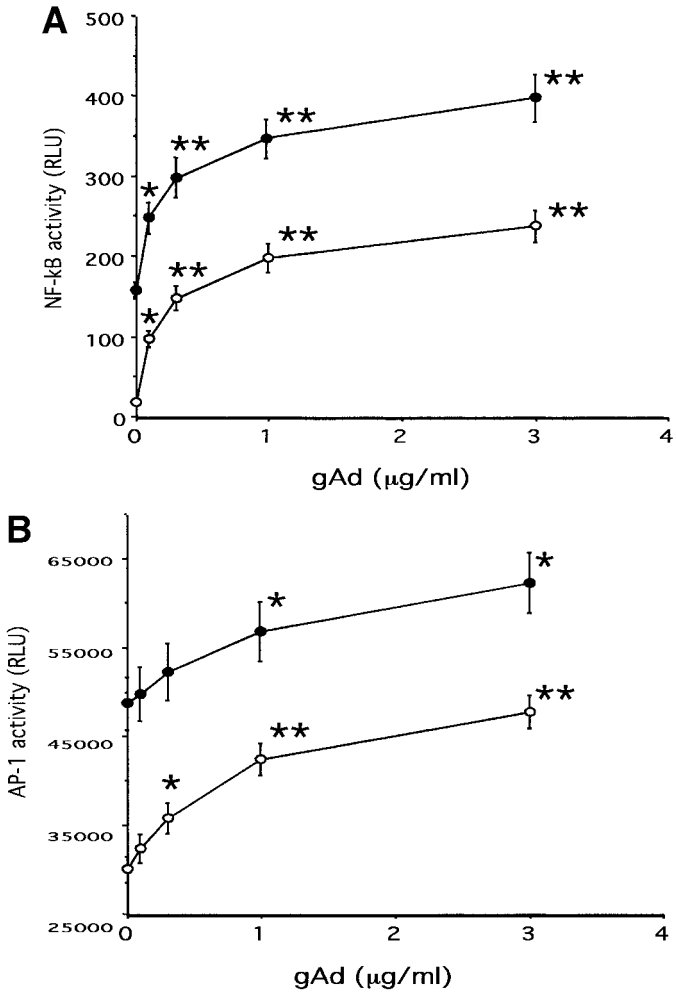


**FIG. 1. The effects of gAd on NF-κB–dependent (*A*) and AP-1–dependent (*B*) transcriptional activity. Quiescent cells (transfected with pNFκB-Luc or pAP1-Luc) were left untreated or were treated with various concentrations (0.1–3 μg/ml) of gAd (○). Cells were also treated with gAd concomitantly with TNF-α for NF-κB activation and with Ang II for AP-1 activation (●). After 2 h, cells were lysed, and luciferase activity was measured. Each point represents the mean ± SD ($n$ = 4). *$P$ < 0.05; **$P$ < 0.01 compared with those in the absence of gAd.**

## RESULTS

**gAd activates NF-κB and AP-1.** gAd dose dependently activated NF-κB in cardiac fibroblasts. It also enhanced TNF-α–induced NF-κB activation in a dose-dependent manner (Fig. 1*A*). Similarly, gAd dose dependently activated AP-1 in cardiac fibroblasts and enhanced Ang II–induced AP-1 activation (Fig. 1*B*).

**gAd enhances Ang II–induced ERK activity.** We examined the effect of gAd on Ang II–induced ERK phosphorylation. ERK levels were similar during the time course in the absence or presence of gAd. ERK phosphorylation levels, however, were significantly enhanced in the presence of gAd compared with those in the absence of gAd (Fig. 2*A*). We further examined gAd-induced ERK activity in cardiac fibroblasts. ERK activity was significantly increased by gAd and was substantially induced by Ang II. Cotreatment with Ang II and gAd further increased ERK activity (Fig. 2*B*).

**gAd enhances Ang II–induced c-fos and c-jun mRNA expression.** We examined the effect of gAd on Ang II–induced c-fos and c-fos mRNA expression. Very low

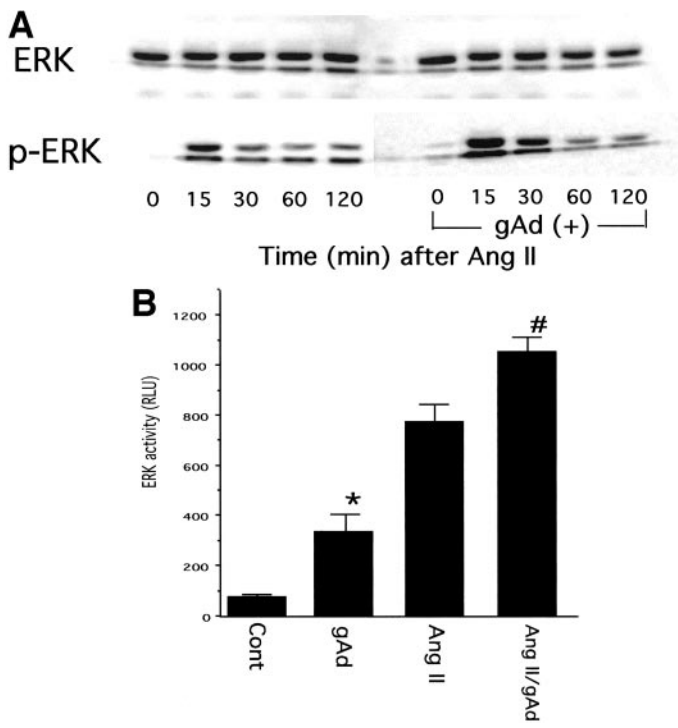


**FIG. 2. The effects of gAd on ERK activation in cardiac fibroblasts. *A*: gAd (3 μg/ml) increased Ang II ($10^{-6}$ mol/l)-induced ERK phosphorylation. *B*: The effects of gAd (3 μg/ml) on ERK activity induced by Ang II ($10^{-6}$ mol/l). Each bar represents the mean ± SD ($n$ = 4). *$P$ < 0.01 compared with control; #$P$ < 0.01 compared with Ang II.**

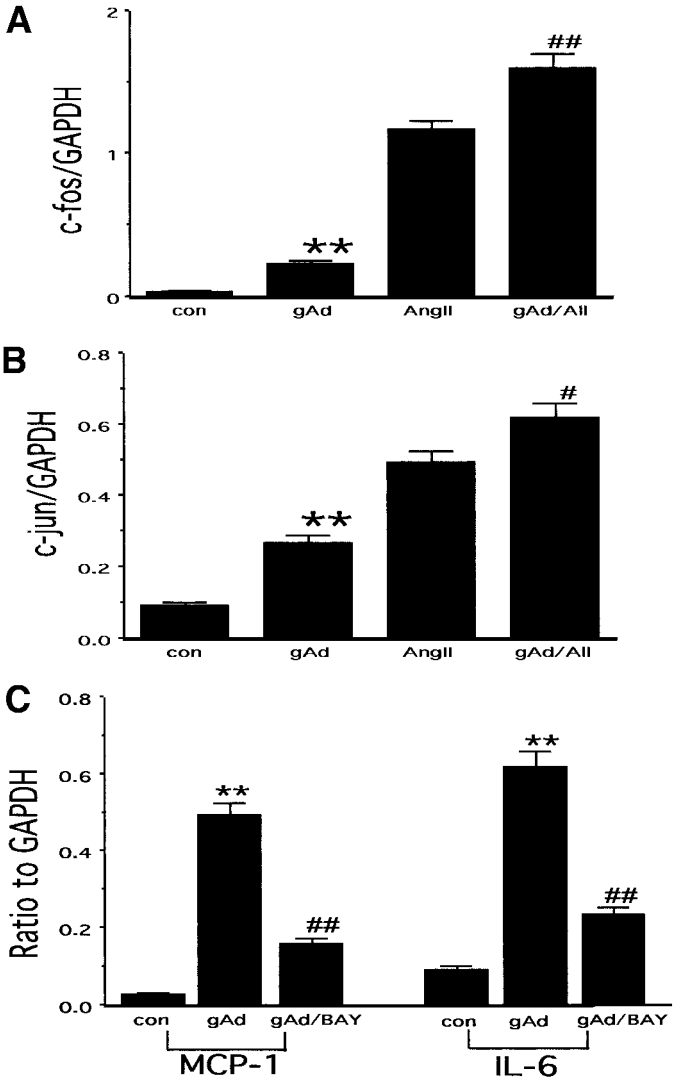


**FIG. 3. The effects of gAd (3 μg/ml) on Ang II ($10^{-6}$ mol/l)-induced c-fos and c-jun mRNA expression. c-fos (*A*) and c-jun (*B*) mRNA levels were examined by real-time PCR, which were shown as ratio to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). **$P$ < 0.01 compared with control; #$P$ < 0.05; ##$P$ < 0.01 compared with Ang II. *C*: gAd (3 μg/ml) enhances MCP-1 and IL-6 mRNA expressions, which were inhibited by the NF-κB inhibitor BAY11-7082 (20 μmol/l). Each bar represents the mean ± SD ($n$ = 3). **$P$ < 0.01 compared with control; ##$P$ < 0.01 compared with gAd.**

levels of c-fos mRNA levels were observed in untreated cells, but gAd treatment for 1 h significantly increased c-fos mRNA expression. Ang II treatment for 1 h increased c-fos mRNA levels, which were further enhanced by cotreatment with gAd (Fig. 3*A*). Similarly, c-jun mRNA levels were low in untreated cells, but gAd significantly increased c-jun mRNA expression 1 h after the addition of gAd. Ang II treatment for 1 h clearly increased the c-jun mRNA levels, which were further enhanced by the cotreatment with gAd (Fig. 3*B*). gAd treatment for 6 h enhanced MCP-1 and interleukin (IL)-6 mRNA expression, which was inhibited by the NF-κB inhibitor BAY11-7082 (Fig. 3*C*).

**gAd enhances Ang II–induced [$^3$H]thymidine and [$^3$H]proline incorporation in cardiac fibroblasts.** We next investigated whether gAd enhances Ang II–induced cell proliferation and collagen synthesis in cardiac fibroblasts. We measured the incorporation of [$^3$H]thymidine and [$^3$H]proline 24 h after administration of gAd in the absence and presence of $10^{-6}$ mol/l Ang II. gAd significantly increased [$^3$H]thymidine incorporation, but the increase in [$^3$H]proline incorporation in response to gAd alone was not significant (Fig. 4). Ang II stimulation increased [$^3$H]thymidine and [$^3$H]proline incorporation 3.1- and 1.6-fold, respectively. When cells were activated by cotreatment with gAd, there was a dose-dependent and significant enhancement of [$^3$H]thymidine and [$^3$H]proline incorporation (Fig. 4).

**AdipoR antibody stimulates NF-κB and AP-1 activation.** Antibodies against AdipoR1 markedly increased NF-κB activity, and antibodies against AdipoR2 also, to a much lesser extent, increased NF-κB activity. Activation by gAd was modest compared with the AdipoR1 antibody, but it significantly enhanced the antibody-stimulated NF-κB activity (Fig. 5*A*). Similarly, AdipoR1 antibodies significantly increased AP-1 activity, whereas antibodies against AdipoR2 increased AP-1 activity to a lesser extent (Fig. 5*A*).

**gAd stimulation of cardiac fibroblast is not mediated through C1qR.** C1qR siRNA was used to silence the C1qR in cardiac fibroblasts. We examined whether gAd-induced NF-κB activation differed between control cells and C1qR siRNA-pretreated cells. gAd-induced dose-dependent stimulation of NF-κB activation did not differ between control cells and siRNA-pretreated cells (Fig. 5*B*).

## DISCUSSION

The present study demonstrated that gAd potently stimulates NF-κB activation in cardiac fibroblasts. The transcription factor NF-κB is important for the regulation of a variety of genes involved in cellular inflammatory and proliferative responses. gAd also induced MCP-1 and IL-6 gene expression in cardiac fibroblasts, which was signifi-

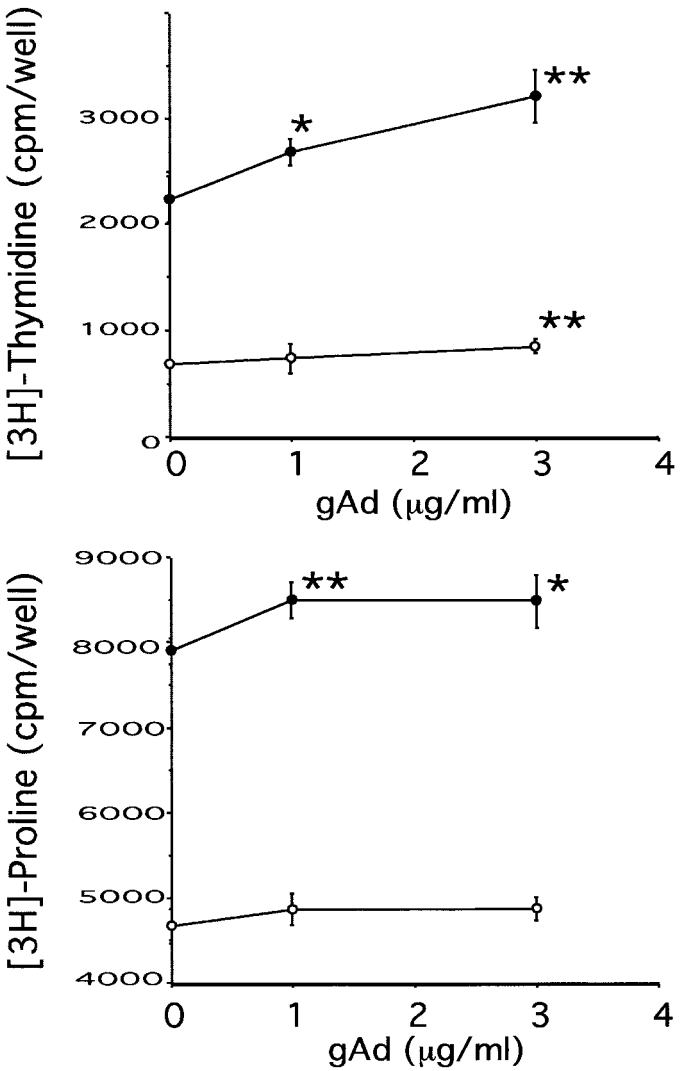


**FIG. 4. The effects of gAd on Ang II–induced DNA and collagen synthesis in cardiac fibroblasts. Effects of different concentrations of gAd (1 and 3 μg/ml) on [$^3$H]proline and [$^3$H]thymidine incorporation were evaluated in untreated cells (○) and in Ang II ($10^{-6}$ mol/l)-treated cells (●). Each point represents the mean ± SD ($n$ = 4). *$P$ < 0.05; **$P$ < 0.01 compared with incorporation in the absence of gAd.**

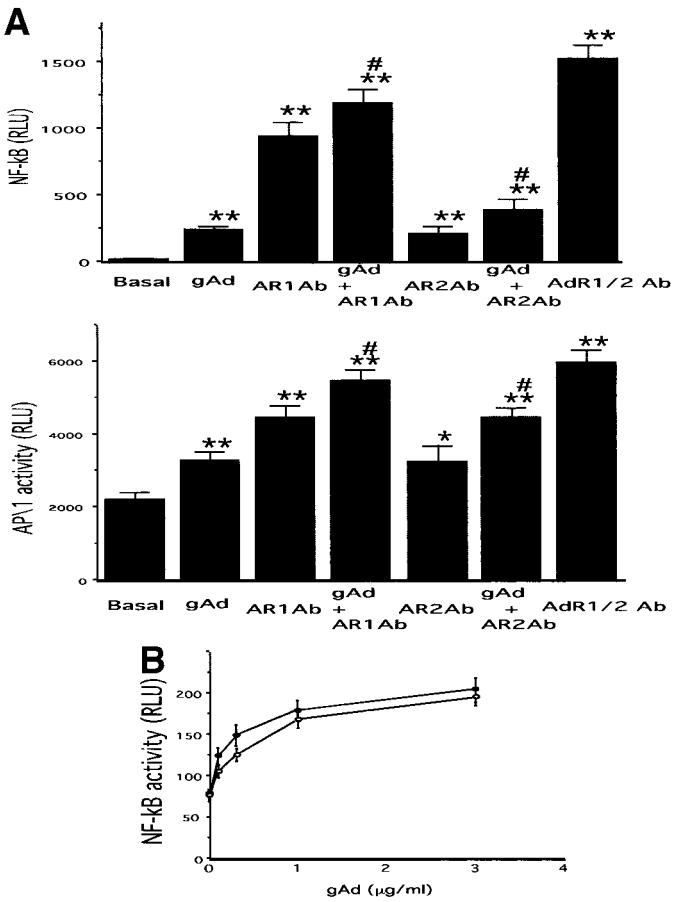


**FIG. 5. *A*: The effect of AdipoR antibodies on NF-κB and AP-1 activity. Antibody against AdipoR1 (10 μg/ml) or AdipoR2 (10 μg/ml) was added to the cells (transfected with pNFκB-Luc or pAP1-Luc) in the absence of gAd. After 2 h, cells were lysed, and luciferase activity was measured. Each bar represents the mean ± SD ($n$ = 4). *$P$ < 0.05; **$P$ < 0.01 compared with incorporation in the absence of gAd. #$P$ < 0.05 compared with AdipoR1 antibody or AdipoR2 antibody alone. *B*: Effects of C1qR siRNA transfection on gAd-induced NF-κB activation. Similar dose-dependent stimulation of NF-κB activation was observed with gAd (0.1–3 μg/ml) in control cells (○) and C1qR siRNA-pretreated cells (●). Each point represents the mean ± SD ($n$ = 4).**

cantly inhibited by the NF-κB inhibitor BAY11-7082, suggesting that the induction of MCP-1 and IL-6 gene expression by gAd is primarily mediated by NF-κB activation. Thus, one of the signaling pathways by which gAd activates cardiac fibroblasts appears to be via NF-κB activation. We also demonstrated that gAd stimulates AP-1 activation. In addition, gAd potently stimulates ERK activation, which might be functionally related to c-fos/c-jun gene expression and AP-1 activation in gAd-stimulated cardiac fibroblasts. Thus, another signaling pathway by which gAd activates cardiac fibroblasts appears to be the ERK→c-Fos/c-Jun→AP-1 pathway. gAd activates ERK and enhances Ang II–induced ERK phosphorylation and activation, which might lead to DNA and collagen synthesis in cardiac fibroblasts, resulting in cardiac hypertrophy.

In long-standing renovascular hypertension with marked remodeling of the heart, two-thirds of the myocardial cell population is composed of nonmyocyte cells, the majority of which are fibroblasts (9). Cardiac fibroblasts are responsible for the production and deposition of extracellular matrix proteins such as fibronectin and collagen types 1 and 3, which are induced in response to Ang II (9,16). Ang II receptors are localized predominantly on cardiac fibroblasts, and Ang II can indirectly stimulate hypertrophy of cardiac myocytes by stimulating cardiac fibroblasts to produce transferable factors (17). Therefore, the increased incorporation of thymidine and proline into cardiac fibroblasts induced by gAd might lead to further myocardial hypertrophy.

It has been shown that binding of insulin can also be modulated by anti-receptor antibodies. Depending on the epitope recognized, the interaction of antibodies with insulin receptor can be either stimulatory or inhibitory for equilibrium binding of insulin and can result in inhibition or acceleration of dissociation of previously bound ligand (18). In this study, we examined whether gAd activation of two redox-sensitive transcription factors is mediated through AdipoR1 and AdipoR2 (19) using antibodies against AdipoR1 and AdipoR2. The antibodies we used were raised with an 18–amino acid peptide from mouse AdR1 and a 14–amino acid peptide from mouse AdR2 as antigens, respectively, which are located on COOH terminus, extracellular domain of each receptor. It was observed that both antibodies were stimulatory by them-

selves without gAd and further increased gAd-stimulated NF-κB activity or AP-1 activity. Those results indicate that the antibodies against AdipoR1 or AdipoR2 are stimulatory at least for the activation of two redox-sensitive transcription factors and that gAd is likely to activate NF-κB or AP-1 through AdipoR1 and AdipoR2. AdipoR1 and AdipoR2 mRNA expression levels, as a ratio to glyceraldehyde-3-phosphate dehydrogenase, were 0.837 and 0.331, respectively, in vascular endothelial cells. Thus, if the mRNA levels of the receptor are parallel to the protein expression of AdipoRs, the activation of AdipoR1 appeared to be dominantly responsible for this signaling in response to gAd. Because adiponectin and C1q are similar in size and domain structure and gAd is similar to the globular domain of C1q (20), we examined whether the gAd-induced activation of NF-κB is mediated by C1qR by comparing cells not treated and cells pretreated with C1qR siRNA. There was no difference in the activation of NF-κB in response to gAd between those cells. Thus, C1qR appeared not to be involved in gAd-induced NF-κB activation.

Our data suggest that gAd signaling has a role in regulating the Ang II–induced proliferation in cardiac fibroblasts. Although experiments on cultured cells do not necessarily represent the in vivo state, the present results suggest that gAd might activate cardiac fibroblasts, possibly leading to cardiac hypertrophy. These results contribute to our understanding of the mechanisms involved in myocardial hypertrophy and provide a basis for possible strategies to prevent or reverse cardiac remodeling.

## ACKNOWLEDGMENTS

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