Angiotensin II modulates nitric oxide-induced cardiac fibroblast apoptosis by activation of AKT/PKB

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Tian, Bin, Jian Liu, Peter Bitterman, and Robert J. Bache. Angiotensin II modulates nitric oxide-induced cardiac fibroblast apoptosis by activation of AKT/PKB. Am J Physiol Heart Circ Physiol 285: H1105–H1112, 2003. First published May 22, 2003; 10.1152/ajpheart.01139.2002.—Previously we found that interleukin-1β (IL-1β)-activated inducible nitric oxide (NO) synthase (iNOS) expression and that NO production can trigger cardiac fibroblast (CFb) apoptosis. Here, we provide evidence that angiotensin II (ANG II) significantly attenuated IL-1β-induced iNOS expression and NO production in CFbs while simultaneously decreasing apoptotic frequency. The anti-apoptotic effect of ANG II was abolished when cells were pretreated with the specific ANG II type 1 receptor (AT1) antagonist losartan, but not by the AT2 antagonist DP-123319. Furthermore, ANG II also protected CFbs from apoptosis induced by the NO donor diethylenetriamine NONOate and this effect was associated with phosphorylation of Akt/protein kinase B at Ser473. The effects of ANG II on Akt phosphorylation and NO donor-induced CFb apoptosis were abrogated when cells were preincubated with the specific phosphatidylinositols 3-kinase inhibitors wortmannin or LY-294002. These data demonstrate that ANG II protection of CFbs from IL-1β-induced apoptosis is associated with downregulation of iNOS expression and requires an intact phosphatidylinositols 3-kinase-Akt survival signal pathway. The findings suggest that ANG II and NO may play a role in regulating the cell population size by their countervailing influences on cardiac fibroblast viability.

inducible nitric oxide synthase; donor; losartan

CARDIAC FIBROBLASTS (CFBs) are the major cell type involved in regulating the extracellular matrix (ECM) in the heart and play an important role in cardiac remodeling after myocardial infarction or in response to hemodynamic overload (2, 4, 24, 25, 29, 31, 37). In the normal heart, CFbs are quiescent and long lived but can proliferate under some circumstances such as ischemic heart disease, hypertension, and heart failure (17, 25, 36, 37). Proliferation of CFbs with deposition of collagen can decrease compliance of the ventricular chamber, leading to diastolic dysfunction and elevated filling pressure (17, 29, 37). Conversely, insufficient fibroblast proliferation and matrix deposition after myocardial infarction might result in an abnormally thin ventricular wall at risk of rupture. Therefore, the identifying factors that regulate the CFb population is important for understanding mechanisms that maintain normal physiological function and pathological remodeling of the left ventricle. ANG II and nitric oxide (NO) are two such factors that have the potential to influence the size of the CFb population by either stimulating proliferation or inducing apoptosis (30, 35).

ANG II has been shown to stimulate cell proliferation and ECM production by CFbs during the development of pathological ventricular hypertrophy caused by ischemic injury or hemodynamic overload (22, 24, 31, 36, 37). Current evidence indicates that ANG II can indirectly promote proliferation of CFbs by stimulating the synthesis of growth factors such as the platelet-derived growth factor (2, 5, 24, 33) because there is no evidence that ANG II can directly trigger cell division through activation of the cyclin-dependent pathway (2, 24). The size of a cell population is governed by the balance between cell proliferation and cell death, but little is known about the effect of ANG II on fibroblast viability in the heart.

We previously demonstrated that interleukin-1β (IL-1β)-induced inducible NO synthase (iNOS) expression and NO production can trigger CFb apoptosis (31), identifying NO as a candidate for regulation of CFb viability. It is unknown whether ANG II can modulate NO-induced CFb apoptosis, although ANG II has been shown to attenuate NO-induced vascular smooth muscle cell (SMC) apoptosis (30). Consequently, this study was performed to examine the impact of ANG II on endogenous and exogenous NO-induced CFb apoptosis. We found that ANG II significantly attenuated IL-1β and NO donor-induced CFb apoptosis through AT1 receptor-mediated mechanisms. This anti-apoptotic effect of ANG II is associated with downregulation of IL-1β-induced iNOS expression and requires a phosphatidylinositol 3-kinase (PI3-kinase)-Akt/protein kinase B (PKB)-dependent survival signal.

METHODS AND MATERIALS

Reagents. Mouse recombinant IL-1β was purchased from R&D System (Minneapolis, MN). The antibody against iNOS was from Santa Cruz Biotechnology (Santa Cruz, CA). The antibodies against phosphorylated Akt were from Cell Signaling (Beverly, MA). The NO donor diethylenetriamine (DETA) NONOate was purchased from Cayman Chemical.

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ANN II PREVENTS NO-INDUCED CFb APOPTOSIS

Primary culture of cardiac fibroblasts. All animal procedures were conducted in accordance with the National Institutes of Health Guide for the Care of Laboratory Animals, and were approved by the University of Minnesota Animal Care Committee. Cardiac fibroblasts were isolated from adult male Sprague-Dawley rats (180–280 g) as previously described with minor modification (35). Rats were anesthetized with pentobarbital sodium (25 mg/kg), and the hearts were removed. The left ventricles were minced and washed in Hanks’ balanced salt solution. Cells were released by digesting the tissue with a mixture of 0.1% trypsin (GIBCO-BRL Life Technologies; Grand Island, NY) and 100 U/ml of collagenase (Type IV) for 10 min per cycle at 37°C. Cells from the second to fifth digestion cycle were collected and cultured in flasks containing DMEM plus 20% FCS at 37°C, 10% CO2-90% O2 for 2 h. The attached cells (>95% are cardiac fibroblasts) were allowed to grow in DMEM containing 10% FCS at 37°C, 5% CO2-95% O2 until they were confluent. The cells were subcultured one or two more times before use. Fibroblasts were distinguished from other myocardioblast cell types by the presence of the fibroblast marker vimentin (catalog no. V-5255, Sigma), and absence of the endothelial cell marker von Willebrand factor (catalog no. F-3520, Sigma), or the muscle cell marker desmin (catalog no. D-1033, Sigma). The presence of these various markers was examined by immunofluorescent staining with appropriate secondary antibodies as previously described (35).

Induction of iNOS expression. Induction of iNOS expression was performed as previously described (35). Cardiac fibroblasts were subcultured in dishes containing 10% FCS (at 37°C, 5% CO2-95% O2) for 16 h. Cells were washed with prewarmed 1X PBS and switched to DMEM with low serum (0.1% FCS) and incubated for 24 h. All subsequent experiments were performed in low-serum DMEM. Cells were treated with IL-1β or the NO donor DETA NONOate in the presence or absence of the designated agents for the indicated time intervals. The concentrations of agents used in this study were chosen on the basis of our previous studies (35). The medium from the treated and nontreated cells was collected and stored at −70°C until use. Cells were lysed and lysate protein was subjected to immunoblot analysis for iNOS protein expression. Another parallel set of cells were fixed with precooled methanol and subjected to immunostaining for morphological analysis.

NO measurement. Assessment of NO production in the culture medium was performed with the use of the Griess reagent that measures NO2, the major NO metabolite in the cell culture system (35). One hundred microliters of sample or standard (sodium NO2 served as the standard) was added into each well of the 96-well plate and 100 μl of Griess reagent, a mixture of one part of Griess reagent A containing 0.1 g of N-[1-naphthyl]-ethylenediamine hydrochloride in 100 ml of water and one part of Griess reagent B containing 1 g of sulfanilamide in 100 ml of 3 N HCl was added. After incubation at room temperature for 15 min, the samples were read in a spectrophotometer at a 550-nm wavelength. The amount of NO2 was calculated with the use of a standard curve calibrated with NaN3O2 at concentrations of 0.1–80 μM.

Assessment of apoptosis. Apoptotic cells were identified by direct staining of the condensed nuclei or fragmented DNA in cells with bisbenzimide (Hoechst 33258) or TdT-mediated dUTP nick end labeling (TUNEL)-based staining. For Hoechst 33258 staining, the bisbenzimide stock solution was added directly into the culture medium (at a final concentration of 0.02%) and incubated with the cells for 20 min at 37°C. The cells were subjected to microscopic analysis with the use of an inverted phase-contrast fluorescence microscope (model MDIRB/E, Leica; Deerfield, IL). TUNEL-based staining was performed on methanol-fixed CFbs with the use of the TUNEL staining kit by following the manufacturer’s instructions (Roche; Indianapolis, IN). Image processing was performed with the use of NIH Image software, Adobe Photosh, and a Fuji Pixelaograph 3000 color printer. For quantitative assessment of apoptosis, TUNEL-positive cells were counted in 500 cells per sample and expressed as the percentage of apoptotic cells.

Western blot analysis. Western blot analysis for iNOS expression was performed as previously described (34, 35) with minor modification. Cells were harvested and lysed with lysis buffer (50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, and 0.5% Nonidet P-40) containing a protease inhibitor cocktail (Boehringer-Mannheim; Indianapolis, IN). The lysate was clarified by centrifugation at 16,000 g for 15 min at 4°C. Equal amounts of total protein were subjected to 8% SDS-PAGE and electrophoretically transferred to a High Bond nitrocellulose membrane (Amer sham Life Science; Arlington Heights, IL). After being blocked with 20 mM Tris-HCl, pH 7.6, 137 mM NaCl, and 0.05% Tween 20 (TTBS) containing 5% nonfat milk for 1 h, the membrane was incubated for 1 h at room temperature with appropriate primary antibodies (anti-iNOS antibody was diluted at 1:500 and anti-phospho-Akt antibody was diluted at 1:1,000) in blocking buffer (TTBS with 5% nonfat milk). After being washed three times for 10 min each in TTBS, the membrane was incubated with an appropriately diluted horseradish-peroxidase-labeled secondary antibody (1:2,000) in TTBS for 1 h at room temperature. The membrane was washed three times, reacted with enhanced chemiluminescence reagent (Amersham Life Science) and subjected to autoradiography. The strength of the signal was analyzed using densitometry and the results expressed as arbitrary units. Protein levels were standardized by comparison with anti-β-tubulin antibody.

Statistical analysis. Each experiment was repeated at least three times. Data are presented as means ± SE. Comparison between groups was performed by two-way ANOVA. Significance was considered as P < 0.05.

RESULTS

ANG II attenuates IL-1β-induced CFb apoptosis. To examine the effect of ANG II on IL-1β-induced apoptosis, CFbs were seeded in culture dishes or chamber slides and treated with IL-1β alone or combined with other reagents for 16 h, followed by assessment of apoptosis by immunostaining and TUNEL assay. In control CFbs, no apoptosis was detected (Fig. 1A,A). In accord with our earlier study (35), in IL-1β (5 ng/ml)-treated CFbs apoptosis was apparent as evidenced by nuclear condensation and cell shrinkage (Fig. 1A,B). The apoptotic frequency was markedly attenuated when ANG II was added immediately before the addition of IL-1β (10−6 M, Fig. 1A,C). The anti-apoptotic effect of ANG II was abolished by preincubating the cells for 3 h with the ANG type 1 (AT1)-selective receptor blocker losartan (3 × 10−5 M, Fig. 1A,D), but not by preincubating for 3 h with the selective AT2 receptor blocker P-123319 (3 × 10−5 M; Fig. 1A,E). CFb apoptosis was quantitated with the use of a TUNEL-
based assay (Fig. 1B). TUNEL-positive cells constituted <2% of the control group, whereas the frequency of TUNEL-positive cells was increased to 35% after exposure to IL-1β. The frequency of TUNEL-positive cells was decreased to 7.5% by treatment with ANG II. The antiapoptotic effect of ANG II was abolished when the cells were preincubated for 3 h with the selective AT1 receptor blocker losartan, whereas preincubation...
for 3 h with the selective AT2 receptor blocker had no effect on the antiapoptotic effect of ANG II. These data imply that ANG II is capable of rescuing CFbs from IL-1β-induced apoptosis through AT1 receptor-mediated mechanisms.

**ANG II decreases IL-1β-induced iNOS expression and NO production.** Because IL-1β-induced apoptosis in CFbs has been shown to be mediated, at least in part, by NO production, studies were performed to determine whether ANG II has an effect on IL-1β-induced iNOS expression. CFbs were treated with IL-1β alone or combined with other reagents for 16 h, followed by Western blot analysis for iNOS protein expression. iNOS protein expression was induced in CFbs treated with IL-1β (5 ng/ml), and this increase was significantly reduced when ANG II (1 × 10^{-6} M) was added immediately before IL-1β (Fig. 2A). The decrease in IL-1β-induced iNOS expression produced by ANG II was reversed by preincubation with losartan (3 × 10^{-5} M) for 3 h before the addition of ANG II, but not by preincubation with DP-123319 (3 × 10^{-5} M) for 3 h, implying that the ANG II receptor on iNOS expression is mediated via a type 1 ANG II receptor. ANG II alone had no effect on iNOS expression (Fig. 2A). Parallel changes were observed in the NO concentration in the medium collected from these CFbs (Fig. 2B). These data demonstrate that ANG II can rescue CFbs from endogenous NO-induced apoptosis, at least in part by downregulating iNOS expression induced by IL-1β.

**ANG II rescues cells from NO donor-induced Cfb apoptosis.** Although the anti-apoptotic effect of ANG II on IL-1β-induced Cfb apoptosis appeared to be at least in part due to downregulation of iNOS protein expression, we also examined whether ANG II is capable of protecting CFbs from apoptosis induced by exogenous NO. In these experiments, CFbs were exposed to the NO donor DETA NONOate (3 × 10^{-5} M) alone or combined with ANG II (10^{-6} M; ANG II was added 5 min before the addition of DETA NONOate) for 8 h, followed by analysis of nuclei by Hoechst 33258 staining. Compared with the control CFbs (Fig. 3A,A), apoptotic frequency in CFBs treated with DETA NONOate was significantly increased (Fig. 3A,B). Preincubation of the cells with ANG II for 5 min markedly attenuated DETA NONOate-induced apoptotic frequency (Fig. 3A,C). The antiapoptotic effect of ANG II was abolished when CFbs were preincubated with the selective AT1 inhibitor losartan (3 × 10^{-5} M) for 5 min before exposure to ANG II and DETA NONOate (Fig. 3A,D). Apoptosis quantified by TUNEL-based staining (Fig. 3B) demonstrated that ANG II inhibited CFB apoptosis induced by exogenous NO.

**ANG II stimulates Akt/PKB activity via PI3-kinase-dependent pathway.** Because ANG II was able to rescue CFbs from exogenous NO-induced apoptosis, we next addressed the question of mechanism. Because phosphorylation of Akt/PKB on Ser^{473} has been implicated as a universal survival mediator in many cell types, we examined the effect of ANG II on Akt/PKB phosphorylation. We found that ANG II rapidly phosphorylated Akt on Ser^{473} in a dose- and time-dependent fashion (Fig. 4, A and B), implying that phosphorylation of Akt by ANG II may activate an Akt-mediated survival signaling pathway. ANG II stimulated phosphorylation of Akt Ser^{473} was detected as early as 2.5 min, peaked between 5 and 10 min, and returned to the basal level within 30 min (Fig. 4A). The effect of ANG II on Akt phosphorylation at Ser^{473} was completely abolished by preincubation for 3 h with the PI3-kinase inhibitors wortmannin (20 nM) or LY-294002 (20 μM) (Fig. 4C). Furthermore, the antiapoptotic effect of ANG II on DETA NONOate-induced apoptosis was abolished when the cells were preincubated for 3 h with wortmannin or LY-294002 (Fig. 5). The apoptotic frequency in control CFbs was <2% and was increased to 45% after exposure to DETA NONOate. The apoptotic frequency was reduced to 6.5% in cells preincubated with ANG II for 5 min before exposure to DETA NONOate. The antiapoptotic effect of ANG II was abolished when the cells were preincubated for 3 h with either wort-
mannin (47%) or LY-294002 (46%) before treatment with DETA and ANG II. These data provide evidence that ANG II can rescue CFbs from NO-induced apoptosis by activation of a PI3-kinase-Akt/PKB-mediated survival-signaling pathway.

DISCUSSION

Cardiac fibroblasts are responsible for deposition of ECM in the heart in response to myocardial injury or hemodynamic overload (2, 4, 16, 24, 25, 29, 31, 37). The present study provides evidence that ANG II may participate in regulation of the fibroblast population size via AT1 receptor-mediated mechanisms that protect CFbs from apoptosis in response to endogenous or exogenous NO. The antiapoptotic effect of ANG II occurred via two distinct mechanisms. First, ANG II caused downregulation of iNOS protein expression and NO production induced by IL-1β. Second, when NO was supplied from an exogenous source, ANG II provided protection against apoptosis by activation of a PI3-kinase-Akt/PKB-mediated survival signaling pathway. Our data establish a role for both ANG II and NO in regulating the size of CFb population by their opposing influences on the viability of cardiac fibroblasts.

Accumulating evidence suggests that elevated levels of ANG II play an important role in regulation of cardiac remodeling after myocardial infarction or in response to hemodynamic overload (31, 36, 37). ANG II exerts several relevant biological effects on cardiac remodeling. Chronic infusion of low levels of ANG II can induce cardiac hypertrophy and myocardial fibrosis (14), and overexpression of AT1 receptors in transgenic mice induces cardiomyocyte hypertrophy and collagen deposition (28). In vitro studies have shown that ANG II potently stimulates the synthesis of a variety of ECM proteins by CFbs, including types I and III collagen (2, 22). In addition, ANG II causes proliferation of CFbs by stimulating autocrine production of growth factors such as platelet-derived growth factor (5, 33). In agreement with these findings, inhibition of the renin-angiotensin system with angiotensin-converting enzyme inhibitors or AT1 blockers has been shown to prevent or cause regression of excessive deposition of ECM.
collagen and CFb hyperplasia both in patients with hypertrophic cardiomyopathy and in experimental animal models of cardiac overload (32, 36, 37). Finally, the present study provides evidence that ANG II enhances CFb survival by activating a PI3-kinase Akt/PKB-mediated survival signal. Together, these data support the concept that ANG II can enhance CFb survival and function by its influence on proliferation and viability.

The influence of ANG II on viability is cell type and cell context specific. Several investigators (18, 30) have reported that ANG II can rescue SMC and cardiac myocytes from apoptosis. In contrast, ANG II has been reported to induce apoptosis in alveolar epithelial cells, endothelial cells, and vascular SMC (1, 10, 11, 20, 27). The mechanism(s) of ANG II-induced apoptosis in these cells is not well understood, although it has been shown that both AT1 and AT2 receptors were involved in ANG II-induced apoptosis (7, 9, 11, 13, 19, 27). The involvement of the AT2 receptor in ANG II-induced apoptosis is not unexpected because AT2 receptor overexpression has been reported to mediate ANG II-induced apoptosis, whereas deletion of the AT2 receptor antagonizes ANG II-induced apoptosis (15, 22, 38). However, involvement of the AT1 receptor in ANG II-induced apoptosis was unexpected because ANG II has been shown to promote proliferation or hypertrophy of several cell types (2, 9, 16, 33). In the present study, we found that ANG II prevented both endogenous and exogenous NO-induced CFb apoptosis through an AT1 receptor-mediated mechanism. It is worth noting that we did not observe detectable levels of AT2 receptor in cultured adult CFbs by immunostaining or by Western blot analysis (data not shown), indicating that lack of response to the AT2 blocker may have been due to extremely low expression of this receptor in adult rat CFbs. Nevertheless, our data support the hypothesis that ANG II can regulate CFb viability by AT1 receptor-mediated activation of PI3-kinase/Akt-dependent survival signaling.

The PI3-kinase/Akt/PKB signaling pathway has been demonstrated to elaborate important survival signals in many cell types (3, 6). ANG II can activate Akt/PKB in a PI3-kinase-dependent manner in several types of cells (12), but it had been unclear whether activation of the PI3-kinase-Akt/PKB pathway is involved in ANG II-mediated survival signaling in CFbs. We previously demonstrated that phosphorylation of Ser473 of Akt by antibody ligation of β1-integrin can prevent human lung fibroblast apoptosis induced by collagen contraction, suggesting that the PI3-kinase-Akt/PKB signal pathway can regulate lung fibroblast viability (34). In the present study, we have demonstrated that ANG II is capable of reversing NO-induced CFb apoptosis by providing a PI3-kinase-Akt/PKB-mediated survival signal. In an attempt to confirm the involvement of Akt phosphorylation in the ANG II activated survival signaling pathway, we transiently transfected CFbs with truncated Akt (lacking the catalytic domain) to determine whether this would interrupt the ability of ANG II to rescue the cells from NO-induced apoptosis. However, we were unable to perform this experiment because, on reducing or withdrawing serum in anticipation of adding the NO donor, we found that the majority of cells spontaneously underwent apoptosis (data not shown). Nevertheless, this indicates that Akt is important for CFb survival, because loss of Akt function itself induced CFb apoptosis during low-serum conditions. Because ANG II is in-

![Fig. 4. ANG II activates Akt/protein kinase B (PKB) in a phosphatidylinositol 3-kinase-dependent manner. CFbs were treated with 10^{-6} M of ANG II or preincubated with wortmannin or LY-294002, followed by Western blot analysis for phosphorylated Akt at Ser473. The phosphorylation of Akt was increased significantly within 2.5 min of ANG II exposure and returned to the basal level after 10 min (Fig. 4A). The phosphorylation of Akt at Ser473 was also increased in a dose-dependent fashion in response to ANG II stimulation (5 min) (Fig. 4B). The increase in Akt phosphorylation was abrogated when the cells were preincubated with the specific PI3-kinase inhibitors 20 nM wortmannin or 20 μM LY-294002 (Fig. 4C).](http://ajpheart.physiology.org/)

![Fig. 5. PI3-kinase inhibitors abolished protective effect of ANG II on NO donor-induced CFb apoptosis. Shown are the percentages of apoptotic cells quantitated morphologically with the use of Hoechst 33258 staining. *P < 0.05 vs. control or DETA + AII.](http://ajpheart.physiology.org/)
volved in multiple signaling pathways, such as activation of protein kinase C, ANG II may also activate other signaling pathways having antiapoptotic effects (8). Moreover, much evidence suggests that Akt/PKB mediates a universal survival pathway, which regulates the viability of many types of mammalian cells (6). >10 different downstream effectors of Akt have been identified, reflecting the complexity of this signaling pathway (3, 6). Additional studies will be needed to determine which downstream factors are involved in the ANG II-induced PI3-kinase-Akt/PKB survival-signaling pathway in CFbs.

NO has been reported to trigger apoptosis or protect cells from apoptotic stimuli, depending on the concentration of NO as well as the cell type (26). The relatively low physiological concentrations of NO generated by eNOS or nNOS can act as an antiapoptotic effector in several cell systems, including endothelial cells (26). In contrast, iNOS expression associated with pathological processes, such as inflammation, wound repair, or heart failure results in production of high NO levels that can trigger apoptosis in several cell types, including macrophages and SMCs (1, 23). Our previous study (35) demonstrated that both endogenous NO produced by iNOS or exogenous NO can induce CFb apoptosis in vitro, suggesting that NO may participate in regulation of the population size of cardiac fibroblasts.

In summary, the present data provide evidence that ANG II can modulate NO-induced CFb apoptosis by downregulating iNOS expression induced by IL-1β and by activating a PI3-kinase-Akt/PKB-mediated survival signal that opposes NO-stimulated apoptosis. Our data support the hypothesis that ANG II and NO may play a role in balancing the population size of cardiac fibroblasts by their countervailing influences on cell viability.

DISCLOSURES

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