Bradykinin-Induced Reductions in Collagen Gene Expression Involve Prostacyclin

Ann Marie Gallagher, Hisahiro Yu, Morton P. Printz

Abstract—Cardiac fibrosis after myocardial infarction and in chronic hypertension involves an increase in the synthesis and deposition of collagen within the myocardium. Angiotensin-converting enzyme (ACE) inhibitors limit hypertrophy and fibrosis; their mechanism of action remains controversial, although kinins have been implicated to play a role. Because both bradykinin and prostaglandins (PG) have been shown to reduce collagen gene expression in cardiac fibroblasts, the goal of this study was to determine whether the bradykinin effect was mediated through enhanced prostaglandin formation by cardiac fibroblasts. Bradykinin increased [3H]arachidonic acid metabolite release 2.3-fold over control and stimulated a dose-dependent increase in 6-keto PGF$_{1\alpha}$ (the stable metabolite of PGI$_2$) release from these cells, in which 1 nmol/L bradykinin produced a 4-fold increase in 6-keto PGF$_{1\alpha}$ release. Beraprost (a PGI$_2$ analogue) reduced steady-state proα1(I) and proα1(III) collagen mRNA levels by 35.6±6.6% and 34.2±10.0%, respectively. Bradykinin-induced reductions in collagen type I and III gene expression were reversed by pretreatment with indomethacin. Our results indicate that one mechanism by which bradykinin modulates collagen biosynthesis via the rabbit cardiac fibroblast involves formation of arachidonic acid metabolites, particularly PGI$_2$. The results of the present study argue that stabilization of endogenous kinins (as by ACE inhibitors) would enhance prostacyclin production and result in the attenuation of collagen gene expression, with potential implications for collagen synthesis and deposition within the myocardium. (Hypertension. 1998;32:84-88.)

Key Words: bradykinin ■ collagen ■ prostaglandins ■ fibroblasts ■ rabbits

After myocardial infarction and in chronic hypertension, the collagen content of the LV is increased. ACE inhibitors have been shown to be efficacious in the treatment of patients with these pathologies and to improve survival.1–3 Furthermore, in animal studies, one consequence of the use of ACE inhibitors after coronary artery ligation or aortic banding is a reduction in LV hypertrophy4–6 and collagen content of the myocardium.6–9 This attenuation in collagen deposition and LV mass is blunted by coadministration of a BK receptor antagonist,7,10,11 implying a role for the kinin system in regulating LV remodeling. Because ventricular remodeling involves both hypertrophic growth of myocytes and increases in interstitial fibrosis, ACE inhibitors potentially may alter either or both components. In recent studies from our laboratory and others, BK has been reported to reduce collagen gene expression via cardiac fibroblasts,12,13 but the signaling pathways involved in BK-induced modulation of collagen expression have not been fully explored.

Kinins stimulate the release of AA metabolites, including prostaglandins and prostanooids from a variety of cell types14–18 including cardiac fibroblasts,19,20 but data are conflicting on whether this effect is via modulation of the cyclooxygenase pathway.18,21 BK receptors have been shown to interact with phospholipase A$_2$,14,16 phospholipase C,14,16 and phospholipase D$^2$ and thereby enhance the release of AA. Both PGE$_2$ and PGI$_2$ have been shown to reduce collagen synthesis,20,23–25 and we recently found that the stable PGI$_2$ analogue beraprost also reduced collagen synthesis by rat cardiac fibroblasts.20 However, BK-induced stimulation of prostaglandin formation has not been fully examined as a primary mechanism by which BK attenuates collagen expression. Therefore, the goal of the present study was to assess whether the ability of BK to modulate collagen gene expression involves enhanced prostaglandin formation, specifically PGI$_2$, by the cardiac fibroblast.

Methods

All experiments conformed to American Association for the Accreditation of Laboratory Animal Care guidelines for use of animals in research, and the experimental protocols were approved by the University of California at San Diego Animal Subjects Committee.

Cardiac Fibroblast Isolation

New Zealand White rabbits were killed with sodium pentobarbital (100 mg/kg) via a medial ear vein. One heart was used for each cell preparation for a total of 6 hearts. The hearts were excised, and atrial tissue was removed. Ventricles were minced, and the cells were dispersed in a collagenase (Boehringer Mannheim)-pancreatin (Gibco BRL) digestion solution. Cell suspensions from 5 separate sequential digestions were combined, centrifuged, and resuspended in DMEM with 10% fetal bovine serum (FBS). The cells were plated onto 150-mm cell culture dishes for 45 minutes to permit attachment.

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of cells, after which time the medium containing unattached cells was removed and replaced with fresh medium. This differential plating permitted preferential attachment of fibroblasts to the cell culture dish. The cells that were isolated with this procedure were characterized immunocytochemically and were immunopositive for α-smooth muscle actin, vimentin, and fibronectin but not for desmin or α-sarcomeric actin. Fibroblast cultures exhibited no intracellular uptake of acetylated LDL labeled with 1,1,3,3-tetramethylindocarbocyanine perchlorate (Di-I-Ac-LDL, Biomedical Technologies Inc), which is a marker for endothelial cells.

### AA Metabolite Release

Cardiac fibroblasts were grown to confluence in 100-mm cell culture plates in DMEM supplemented with 10% FBS. Once confluence was reached, the growth medium was removed and replaced with DMEM containing 0.5% FBS and [3H]AA (3 μCi per plate, New England Nuclear). Cultures were incubated in radioisotope-containing medium for 18 hours, at the conclusion of which the medium was removed and the cells were gently rinsed in Ca2+/Mg2+-free PBS (pH 7.4). Rinsed monolayers were then equilibrated in HEPES-buffered medium (20 mmol/L HEPES) at 37°C for 30 minutes, after which 10% of the medium was used in liquid scintillation counting as an estimate of basal metabolite release. The 10% volume was replaced with incubation medium containing sufficient BK to yield a final concentration of 1 μmol/L peptide. After 30 minutes of stimulation, the radioactivity in 10% of the medium was counted to estimate the total metabolites released. The balance of the medium (90%) was removed and the cells were gently rinsed in ethyl acetate as previously described.1,2 The extracted sample was dried under nitrogen, redissolved, and subjected to HPLC analysis. Protein content in parallel sets of plates was determined by Lowry assay.

### Measurement of Endogenous Production of PGI2

The release of endogenous PGI2 was measured by assay for the stable metabolite 6-keto PGF1α. The experimental design was similar to that described above but without the addition of exogenous AA. Cell supernatants from 35-mm cell culture plates were collected and stored at −80°C before assay. After centrifugation to remove cellular debris, 6-keto PGF1α synthesis from endogenous AA was measured by enzyme-linked immunoassay (Cayman Chemical) according to the manufacturer’s instructions. Results are expressed as nanograms of 6-keto PGF1α per milligram of protein.

### RNA Isolation and Northern Blot Analysis

Cardiac fibroblasts were grown in 150-mm dishes to confluence and serum-deprived for 24 hours. Cultures were then treated with vehicle (DMEM with 15 μmol/L captopril (Squibb)), 250 μmol/L BK (Bachem), 10 μmol/L beraprost [sodium (+)-1R*, 2R*, 3aS*, 8bS*]-2,3,3a,8b tetrahydro-2-hydroxy-1-[(E)-(3S*)-3-hydroxy-4-methyl-1-oxen-6-ynyl]-1H-cyclopent[b]benzofuran-5- butyrate; kindly provided by Yamanouchi Pharmaceutical Co, Ltd, Tokyo, Japan], 10 μmol/L indomethacin (Sigma Chemical Co), or 10 μmol/L indomethacin followed 30 minutes later with 250 μmol/L BK for 24 hours. Total cellular RNA was extracted using the RNeasy kit (Qiagen), and RNA was quantified by absorbance at 260 nm. Total RNA (5 μg per lane) was separated by gel electrophoresis on a 1% formaldehyde-containing agarose gel, transferred to nylon membrane (Micron Separations Inc), and immobilized on the membrane by UV cross-linking. cDNA probes for collagen type I and III were labeled with [α-32P]dCTP by random primer labeling (NEBlot kit, New England Biolabs Inc) and hybridized to the RNA on the membranes at 65°C for 16 hours. The membranes were washed 3 times in wash buffer (1 mmol/L EDTA/40 mmol/L Na2HPO4, pH 7.2, 1% SDS) and exposed to film (Fuji Photo Film Co, Ltd) for 8 to 72 hours at −80°C. To correct for loading differences, nylon membranes were dehybridized and rehybridized with a 29-bp DNA oligomer to 28S ribosomal RNA.2 All data are expressed as the ratio of collagen mRNA/28S signals obtained by densitometry. The collagen I (670-bp cDNA for human proα1(I) collagen mRNA) and collagen III (705-bp cDNA for human proα1(III) collagen mRNA) probes were kindly provided by David Amiel (Department of Orthopedics, University of California at San Diego).

### Statistics

A t test was used to assess the difference between vehicle and BK treatment for [3H]AA. ANOVA was used to assess the effect of BK concentration on 6-keto PGF1α release. ANOVA with unpaired t tests for post hoc comparisons was used to assess the effects of BK and beraprost on collagen gene expression. Variances in mRNA levels differed between indomethacin-treated and vehicle control groups; therefore, Kruskal-Wallis tests were used to compare collagen mRNA levels for vehicle, indomethacin, BK, and BK with indomethacin pretreatment groups. Mann-Whitney post hoc analyses were used to assess differences in collagen mRNA levels for vehicle and indomethacin groups and for BK with and without indomethacin pretreatment. All P values presented are Bonferroni-corrected values.

### Results

BK enhanced release of total radioactive AA metabolites from prelabeled rabbit cardiac fibroblasts by 2.3-fold compared with vehicle-treated cells (Figure 1). HPLC analysis of the metabolites released from prelabeled rabbit cardiac fibroblasts after BK stimulation indicated that the major AA metabolite released from these cells comigrated with the standard for 6-keto PGF1α, the stable metabolite of PGI2 (data not shown). In contrast, the peak corresponding to PGE2 was only minimally increased by the addition of BK. After BK stimulation, the amount of radioactivity in the peak corresponding to 6-keto PGF1α increased 39-fold relative to vehicle, whereas the peak corresponding to PGE2 increased only 2.5-fold. To determine whether cultured rabbit cardiac fibroblasts produce PGI2 from endogenous stores of AA, an enzyme-linked immunoassay was used to detect 6-keto PGF1α. Under basal conditions, rabbit cardiac fibroblasts produced 36.0±14.8 ng 6-keto PGF1α per milligram of cellular protein, and BK stimulation a dose-dependent increase.
in release of 6-keto PGF₁α with an EC₅₀ of 0.15 nmol/L (Figure 2).

BK and beraprost, an exogenous PGI₂ analogue, were examined to assess and compare their effects on steady-state collagen mRNA expression. The addition of BK (250 nmol/L) reduced basal proα₁(I) and proα₁(III) collagen mRNA levels by 38.6±9.0% and 46.0±10.2%, respectively, whereas beraprost (10⁻⁵ mol/L) reduced collagen type I and III mRNA levels by 35.6±6.6% and 34.2±10.0%, respectively (Figure 3).

Because BK stimulated the release of PGI₂ and both BK and PGI₂ reduced collagen gene expression, we tested whether the effect of BK on collagen mRNA was through PGI₂. The production of PGI₂ requires obligatory cyclooxygenase activity on released AA to form the substrate for PGI₂ synthase. Therefore, blockade of cyclooxygenase should prevent the formation of PGI₂, and this was confirmed by HPLC (data not shown). If the BK effect on collagen gene expression involves release of PGI₂, then a cyclooxygenase inhibitor should prevent BK-induced reductions in collagen mRNA expression. Pretreatment with indomethacin fully prevented the BK-induced attenuation in collagen type I gene expression but only partially reversed the BK effect on collagen type III (Figure 4). Treatment with indomethacin alone did not significantly change collagen gene expression relative to vehicle control, although blockade of basal prostaglandin formation tended to increase the gene expression for both collagen subtypes (Figure 4).

**Discussion**

The results of the present study indicate that the ability of BK to reduce collagen gene expression is mediated by enhanced
formation of metabolites of the cyclooxygenase pathway, particularly PGI₂.

The mechanism by which BK stimulates prostaglandin synthesis has been studied using various cell types. BK receptors are coupled to GTP-binding proteins that activate phospholipases A₂ and C,

and in some cell types, phospholipase D, resulting in enhanced release of AA. The AA liberated from cardiac fibroblast membrane phospholipids then becomes a substrate for cyclooxygenase and potentially lipoxygenase, resulting in the generation of prostaglandins, prostanoids, hydroxyeicosatetraenoic acids (HETEs), and other potential products. In the present study, BK was shown to stimulate AA release and concomitantly stimulate PGI₂ release from rabbit cardiac fibroblasts.

In the present study, as in our previous findings with rat cardiac fibroblasts, a PGI₂ analogue significantly inhibited collagen gene expression and lowered proα(I) and proα(III) collagen mRNA levels. Indomethacin pretreatment prevented the BK-induced attenuation in collagen type I gene expression and partially but significantly reversed collagen type III inhibition. We would therefore conclude that the effect of BK on collagen mRNA is likely mediated in part through cyclooxygenase and an enhancement in prostaglandin (specifically PGI₂) formation. This conclusion is similar to early findings of Goldstein and Polgar in studies of human lung fibroblasts. Inhibition of cyclooxygenase activity by indomethacin would block formation of endoperoxides and thereby all resulting prostaglandins. Therefore, although we have documented that PGI₂ inhibits collagen synthesis and that cyclooxygenase inhibition reverses this effect, we cannot exclude the possibility that additional prostaglandin/prostanoid products of BK action on the cardiac fibroblast are also important in modulating collagen gene expression. Because PGE₂ formation by the rabbit cardiac fibroblast was minimally altered by BK, our data suggest that endoperoxides generated by BK are predominantly converted to PGI₂, which acts as a negative regulator of collagen gene expression. Our results also provide indirect evidence that basal expression of collagen may be partly influenced by basal prostaglandin formation, since blockade of cyclooxygenase, and local prostaglandin production, exhibited a tendency to increase gene expression for both collagen subtypes. Previous reports in noncardiac fibroblasts have shown a similar tendency toward increased basal collagen type I biosynthesis with blockade of prostaglandin synthesis.

The steady-state mRNA levels for both collagen genes were reduced by BK and PGI₂ to a similar extent; however, blockade of prostaglandin synthesis revealed potential differences in the regulation of collagen subtypes by the cardiac fibroblast. Blockade of cyclooxygenase completely reversed the BK-stimulated attenuation of proα(I) collagen mRNA but only partially reversed the BK-induced reduction of proα(III) collagen mRNA. Differential regulation of procollagen type I and III mRNA has been reported previously by Carver et al., who found that cardiac fibroblasts subjected to mechanical load increased procollagen type III but not type I mRNA levels. From in vivo studies, changes in the ratio of collagen type I to type III proteins within the heart during development and in disease states suggest that differential regulation of the two collagen subtypes may occur via transcription, translation, or in posttranslational modifications. Further study is needed to elucidate differences in the regulation of the collagen subtypes at the level of both mRNA and protein and the significance of this apparent differential regulation by BK.

The mechanism by which ACE inhibitors modulate and reduce cardiac fibrosis and limit LV remodeling in the intact animal and in humans remains controversial. Recent studies have implicated kinins in important roles in this cardioprotective effect of the ACE inhibitors. Initial studies found that both ACE inhibitors and angiotensin receptor antagonists reduced collagen deposition associated with myocardial infarction and LV hypertrophy. However, McDonald et al. found that treatment with an ACE inhibitor, but not an angiotensin receptor antagonist, prevented the increase in LV mass induced by myocardial necrosis in dogs. In addition, BK antagonists prevented the attenuation in collagen deposition and LV mass associated with ACE inhibition, suggesting that the maintenance of BK levels is at least as important as blockade of angiotensin II production in the ability of ACE inhibitors to limit ventricular remodeling. Furthermore, Wollert et al. found that BK receptor blockade alone enhanced interstitial collagen deposition after myocardial infarction in the rat, further supporting a role of endogenous kinins as modulators of collagen biosynthesis. The results of the present study are consistent with the hypothesis that BK, or other kinins, whose stability is enhanced by ACE inhibition, limits fibrosis through stimulated AA metabolite formation (particularly PGI₂) by the cardiac fibroblast.

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References


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