Journal of Cellular Biochemistry 97:1104–1120 (2006)

Microarray and Phosphokinase Screenings Leading to Studies on ERK and JNK Regulation of Connective Tissue Growth Factor Expression by Angiotensin II 1a and Bradykinin B2 Receptors in Rat1 Fibroblasts

B. Liu,[1] J. Yu,[1] L. Taylor,[1] X. Zhou,[2] and P. Polgar[1]*

[1]Department of Biochemistry, Boston University School of Medicine, Boston, Massachusetts 02118
[2]Dental Research Institute, School of Dentistry, University of California at Los Angeles, Los Angeles, California 90095

Abstract Rat1 fibroblasts stably transfected with the rat angiotensin II (AngII) AT1a and bradykinin (BK) B2 receptor cDNAs gained the ability to bind Ang II and BK. Wild-type Rat1 cells bound neither ligand. Exposure to either effector led to characteristic Gαi and Gαq signal cascades, the release of arachidonic acid (ARA), and the intracellular accumulation of inositol phosphates (IP). Microarray analyses in response to BK or AngII showed that both receptors markedly induce the CCN family genes, CTGF (CCN2) and Cyr61 (CCN1), as well as the vasculature-related genes, Cnn1 and Egr1. Real time PCR confirmed the increased expression of connective tissue growth factor (CTGF) mRNA. Combined sequence-based analysis of gene promoter regions with statistical prevalence analyses identified CREB, SRF, and ATF-1, downstream targets of ERK, and JNK, as prominent products of genes that are regulated by ligand binding to the BK or AngII receptors. The binding of AngII or BK markedly stimulated the phosphorylation and thus the activation of ERK2, JNK, and p38MAPK. A BKB2R and an AT1aR chimera which displayed only negligible G-protein-related signaling were constructed. Both mutant receptors continued to activate these kinases and stimulate CTGF expression. Inhibitors of ERK1/2 and JNK but not p38MAPK inhibited the BK- and AngII-stimulated expression of CTGF in cells expressing either the WT or mutant receptors, illustrating that ERK and JNK participate in the control of CTGF expression in a manner that appears to be independent of G-protein. Conversely, addition of BK or AngII to the cell line expressing WT AT1aR and BKB2R downregulated the expression of collagen α1(I) (COL1A1) mRNA. However, these effectors did not have this effect in cells expressing the mutant receptors. Thus, a robust G-protein related response is necessary for BK or AngII to affect COL1A1 expression. J. Cell. Biochem. 97: 1104–1120, 2006.

Key words: bradykinin; angiotensin II; AT1 receptor; bradykinin B2 receptor; connective tissue growth factor; MAP kinases; collagen 1A1; JNK; ERK; p38MAPK; microarray; phospho-specific kinase screening

The rat angiotensin type 1a (AT1a) and bradykinin B2 (BKB2) receptors are G-protein coupled receptors (GPCRs), are of similar molecular size and share moderate sequence homology [Yu et al., 2002]. Both receptors mediate signal transduction responses through Gαq and Gαi. Nevertheless, these receptors are often antagonistic in their physiological and pathological functions. Both are active vasoregulators and participate in fibrogenic events. Whereas AT1R is vasoconstrictive, the BKB2R is a vasodilator [de Gasparo et al., 2000; Prado et al., 2002]. The mechanisms leading to the physiologic actions of these two receptors are not well understood. Evidence suggests that at least some of the downstream responses to these ligands do not involve traditional G-protein targets [Thomas et al., 2004].

The two receptors are rarely both sufficiently expressed in the same cell type to directly compare their signal transduction responses [Chretien et al., 1998; Garcia-Sainz and Avendano-Vazquez, 1999]. Toward that goal,

Grant sponsor: NIH; Grant numbers: NHLBI HL25776, NIA AG00115.

*Correspondence to: P. Polgar, Department of Biochemistry, Boston University School of Medicine, 80 East Concord Street, Boston, MA 02118.
E-mail: polgar@biochem.bumc.bu.edu

Received 2 July 2005; Accepted 5 October 2005
DOI 10.1002/jcb.20709

we co-expressed the two receptors in Rat1 fibroblasts which otherwise do not express either receptor. DNA microarray and phospho-specific protein kinase screening were performed to survey the signaling cascades of the two receptors. Results of the microarray revealed a number of similar gene inductions but also differential gene inductions and pointed to the marked activation of the *CTGF* and *CYR61* genes within the CCN (Cysteine-rich 61, connective tissue growth factor (CTGF) and nephroblastoma-overexpressed) signal peptide family. These immediate-early genes coordinate complex biologic processes during differentiation and tissue repair [Bleau et al., 2005]. Accumulating evidence has linked CTGF overexpression with various fibrotic and inflammatory diseases, and therefore has been of particular interest [Gupta et al., 2000; Leask et al., 2002b; Rachfal and Brigstock, 2003]. We reported previously that BKB2R positive Rat1 cells upregulate CTGF mRNA in response to BK and that cells expressing BKB2R mutant receptors lacking meaningful Gαi and Gαq signaling continued to induce CTGF expression [Yu et al., 2004].

In this report we focus on the possible mechanisms by which both the AT1a and BKB2 receptors upregulate CTGF mRNA expression in the Rat1 cells. We continue to explore the G-protein dependency of this process by using BKB2R and AT1aR chimeric exchanges, which have essentially lost their classic G-protein-linked signaling. These mutant receptors continued to increase CTGF mRNA levels but lost their ability to downregulate COL1A1 mRNA expression.

MATERIALS AND METHODS

Materials

[^{3}H]Ang II (52.5 Ci/mmol), [^{3}H] BK (78 Ci/mmol), [myo-[1, 2-^{3}H] inositol (45–80 Ci/mmol), and [^{3}H] arachidonate (60–100 Ci/mmol) were obtained from NEN Life Science Products. Analytical grade Dowex-X8 (AG-1-X8, 100–200 mesh) was from Bio-Rad. Anti-phospho-ERK, anti-ERK, anti-phospho-JNK, anti-JNK, anti-phospho-P38MAPK, anti-P38MAPK antibodies, and restriction endonucleases were purchased from *New England Biolabs*. Y27632, PD98059, SB23058, and SP600125 were obtained from Calbiochem. Protease inhibitor cocktail was from Roche Diagnostics Co. QuikChange mutagenesis kit was obtained from Stratagene Corporation (La Jolla, CA). Oligonucleotides were synthesized by Invitrogen, Inc. All other reagents were from Sigma unless stated otherwise.

Cell Culture and Transfection

Rat1 cells were obtained from Dr. Robert Weinberg (Whitehead Institute, Massachusetts Institute of Technology) as a generous gift. Rat1 cells were grown in Dulbecco's modified Eagle's medium (DMEM), containing 5% fetal bovine serum supplemented with 50 U/ml penicillin and 50 μg/ml streptomycin at 37°C in a humidified incubator with 5% CO_2. Transfection was carried out using LipofectAMINE 2000 (Invitorgen, Inc.) according to the manufacturer's protocol. Transfection medium was replaced with complete medium 16 h later. Transfected Rat1 cells were then trypsinized and detached the next day, and seeded in a 60-cm^2 dish containing complete medium and 500 μg/ml of Geneticin (G418, Invitrogen, Inc.). G418-resistant cells were isolated for 2 weeks and then tested for their capacity to bind [^{3}H]BK or [^{3}H]AngII. To establish Rat1 cells expressing both AT1a and BKB2 receptors, Rat1 cells expressing the AT1a receptor were transfected with pCMIN(Zeo)-BKB2R and selected with 150 μg/ml zeocin (Invitrogen). The zeocin-resistant cell culture was then expanded and tested for specific binding to [^{3}H] AngII and [^{3}H] BK.

Construction of cDNA Hybrid AT1a and BKB2 Receptors

To construct a chimeric BKAT(Tail) mutant in which the entire C-terminus of BKB2 was replaced with that of AT1a receptor, PCR was performed to amplify the C-terminus of AT1a using the rat AT1a receptor cDNA as a template. The PCR product was digested with AfeI and BamHI, and ligated into the pBluescriptII SK(+)-rBKB2R vector digested with the same enzymes. For construction of ATBK(IC2), two silent restriction sites AgeI (at position 126) and BstEII (at position 147) were created by site-directed mutagenesis on AT1a cDNA in PCDNA 3.1. The corresponding sense and anti-sense oligonucleotides for the IC2 region of BKB2 receptor (5′-CCGGTACCTG GCCATCGTCA AGACCATGTC CATGGGCCGG ATGCGCGGGG-3′ and 5′-GTGACCTTGG CCACCAGTAC CCC-GCGCATC CGGCCCATGG ACATGGTCTT-3′)

were then annealed at equal molar ratio and ligated into the AgeI/BstEII digested vector cassette. The XhoI-XbaI fragments containing the chimera receptors were subcloned into the bicistronic mammalian expression vector, pCMIN(+) [Zhou et al., 2000] for transfection. pCMIN(Zeo) was constructed from pCMIN(+) by replacing the neo-resistance gene with a zeocin-resistance gene. All chimeric constructs were sequenced by an in-house facility using an automatic DNA sequencer (Applied Biosystem, Inc., model 370A). Plasmid DNA for transfection into mammalian cells was isolated with the Qiagen® Plasmid Midi kit.

Receptor Binding Assays

Receptor binding studies of the AT1 and BK B2R receptors in intact Rat1 cells were carried out as described previously [Prado et al., 1997]. Briefly, 80%–100% confluent cell monolayers in 24-well plates (Costar, Cambridge, MA) were incubated in binding buffer containing various concentrations of [^{3}H]AngII and [^{3}H]BK in the absence (total binding) or presence (nonspecific binding) of 5 μM AngII and BK for 2 h at 4°C. Cells were washed three times with ice cold buffer and then solubilized with 0.2% sodium dodecyl sulfate. Radioactivity was determined in a scintillation counter. The K_d and B_{max} were then determined using SigmaPlot 8.

Phosphatidylinositol Turnover

Rat1 cells stably transfected with AT1a and BKB2 receptors were incubated with 1 μCi/ml *myo*-[^{3}H]inositol in 1 ml of growth medium for 16–24 h, and the levels of inositol phosphates (IPs) were determined 24 h later as described previously [Prado et al., 1997]. Briefly, cells were exposed to DMEM containing 20 mM $LiCl_2$ and 20 mM HEPES, pH 7.4, 10 min prior to ligand stimulation. Cells were then exposed to 100 nM BK or AngII for 30 min at 37°C, and the incubations were terminated by removal of the media and addition of 0.5 ml of 10 mM ice-cold formic acid. Cells were scraped off and the formic acid-soluble material isolated by centrifugation and neutralized by adding 10 ml of 5 mM sodium tetraborate. Total [^{3}H]IP was isolated by anion exchange chromatography using a Dowex AG 1-X8 formate resin (Bio-Rad) from which this product was eluted with 2 M ammonium formate, pH 5.0. Radioactivity was determined in a Packard liquid scintillation counter.

Arachidonic Acid Release

Rat1 cells stably transfected with AT1a and BK B2 receptors were labeled with [^{3}H]arachidonate (0.2 μCi/well) for 16 h, as described previously [Prado et al., 1997]. Briefly, cells were washed and incubated with 500 μl of DMEM containing 2 mg/ml bovine serum albumin and incubated with 100 nM BK or AngII for 20 min at 37°C. Medium was removed and centrifuged at 800*g*. The control group was treated identically except for the addition of BK or AngII. Radioactivity in the supernatant was determined in a scintillation counter after addition of 2 ml of Ecolite scintillation fluid.

Real-Time PCR Analysis for CTGF and Collagen Expression

Wild-type and stably transfected Rat1 cells were incubated with 100 nM AngII or BK, and 5 ng/ml TGFβ in the presence or absence of previously established optimal concentrations of ERK1/2. JNK and p38 MAPK inhibitors. RNAs were isolated using RNeasy kit (Qiagen) and cDNAs were made using Superscript II (Invitrogen). Gene expression of CTGF and collagen were analyzed from the cDNAs using an ABI 7700 system (Applied Biosystems, Inc.). The results were normalized with GAPDH. The primers for real time PCR for CTGF were: 5′-TGTGTGATGAGCCCAAGGA-3′ and 5′-TCAGGGCCAAATGTGTCTTC-3′; for COL1A1 were: 5′-GTCGAGGGCCAAGACGAA-3′ and 5′-CACGTCTCGGTCATGGTACCT-3′. To account for possible variation of the cycling amplification of this procedure and of the RNA isolation, the results of real time PCR are more appropriately compared within a given run than from one run to the other. For this reason, we included the appropriate controls (such as wild-type receptor action) with each PCR analysis. As seen by the error bars in the Results section, the data of triplicate incubations are closely reproducible within each separate PCR analysis. However, some disparity exists between separate real-time PCR runs, as seen by the extent of CTGF expression in WT cells in response to stimulation by BK and AngII.

Western Blot Analysis

Rat1 cells stably transfected with AT1 and BKB2 receptors were incubated with or without inhibitors, PD98059, SP600125, or SB202190 for 30 min at 37°C, and then stimulated with

100 nM AngII or BK for 10 min. The cells were then washed twice with ice-cold PBS. Cell lysates were prepared by addition of ice-cold lysis buffer (20 mM HEPES (pH 7.2), 30 mM NaF, 1 mM Na_3VO_4, 1× Complete protease inhibitor cocktail (Roche Applied Science), and 1% Triton X-100) and sedimented at 12,000 rpm in a microcentrifuge at 4°C for 20 min. The proteins were fractionated on 10% SDS–PAGE gels and Western blots were carried out using antibodies against the phosphorylated or unphosphorylated ERK1/2, JNK, or p38MAPK (Cell Signaling Technology).

Microarray Gene Chips

Rat1-AT1/BKB2 cells were incubated in the presence or absence of 100 nM AngII or BK at 37°C for 2 h. Total RNA was isolated using RNeasy kit (Qiagen) and microarray analysis was performed by the Microarray Resource (Boston University School of Medicine). Briefly, double-stranded cDNA was synthesized from 10 μg of RNA using SuperScript double-stranded cDNA synthesis kit (Invitrogen) and purified using a Phase-Lock Gel (PLG Heavy Brinkmann Instruments, Westbury, NY). Biotin-labeled cRNA was then generated using RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, NY) and purified using RNeasy affinity columns (Qiagen). After treatment at 94°C for 35 min in 40 mM Tris-acetate, pH 8.1, 100 mM KOAc, 30 mM MgOAc, 15 μg of fragmented cRNA was hybridized to the Affymetrix GeneChip Rat Expression Set 230A array at 45°C for 16 h and 60 rpm using controls supplied by the manufacturer (Affymetrix). Arrays were then washed and stained according to the standard Antibody Amplification for Eukaryotic Targets protocol (Affymetrix). The stained Gene Chip arrays were scanned at 488 nm using an Affymetrix Gene Chip Scanner 3000 (Affymetrix). The scanned images were then quantified and scaled using Microarray Suite 5.0 software (Affymetrix). The signal intensities from the 22 probes for each gene were used to determine an overall expression level for the gene as well as a detection of confidence score. The gene expression levels were linearly scaled to an average of 500 U on each chip.

Phospho Specific Protein Kinase Screening

Rat1 cells stably transfected with AT1a and BKB2 receptors were incubated in the presence or absence of AngII or BK at 37°C for 10 min. Cells were harvested and homogenized on ice in lysis buffer containing 20 mM HEPES (pH7.2), 2 mM EGTA, 5 mM EDTA, 30 mM NaF, 1 mM Na_3VO4, and 0.5% Nonidet P-40. Cell lysate was prepared by centrifugation of the homogenate at 100,000*g* for 30 min following which 750 μg of the supernatant was boiled in SDS–PAGE sample buffer (Invitrogen, Inc.) for 5 min. The protein concentration of the cell extract was determined using a Bradford Assay kit (Bio-Rad) with bovine serum albumin as protein standard. Samples were then submitted to Kinexus Bioinformatics Co. (Vancouver, Canada). Kinexus resolved the kinases on a single-lane SDS–PAGE gel followed by electrophoretic transfer to a nitrocellulose membrane. The membranes were then probed with antibody mixtures. Radiolabeled antibodies that bound to their target antigens on the membrane were detected and quantitated by Kinexus using a proprietary Kinetworks profiling technology.

Data Analysis

The K_d and B_{max} of ligand binding were calculated using the Pharmacology Module of SPSS, Inc. SigmaPlot® 8 Program. Data are expressed as mean ± SE. Significance was established by using the Student's *t*-test. Differences were considered significant at a value of $P < 0.05$.

RESULTS

Microarray Determination of RNA Induction by BK and AngII

Angiotensin II and bradykinin (BK) effect cellular functions by binding to and activating their cognate receptors. Rat1, a rat fibroblast cell line, does not exhibit meaningful binding of either effector. The AT1R and BKB2R cDNAs were stably transfected into Rat1 cells. The resulting Rat1-AT1/BKB2 cells expressed both receptors with K_d and B_{max} for AT1aR at 1.6 nM and 1.76×10^5 receptors/cell, and for BKB2R at 5.5 nM and 4.51×10^5 receptors/cell, respectively. This cell line provided an opportunity to directly determine similarities and differences of the signaling cascade profiles between these two receptors.

Microarray-based expression analysis was carried out on the Rat1-AT1/BKB2 cells incubated in the presence or absence of 100 nM

AngII or BK for 2 h. We were principally interested in the induction of immediate-early genes, particularly the CTGF family of genes. These genes are activated rapidly at the transcription level in response to cellular stimuli before any new proteins are synthesized. Two hours incubation time is a common practice to detect the mRNA levels for these genes. Interestingly, these two effectors exerted concordant effects on a large group of genes but a differential effect on a smaller group of genes. Results of simultaneous gene induction or suppression greater than 1.5-fold of basal by AT1a and BKB2 receptors are shown in Table I. Specific genes underwent extreme induction. For example, the nuclear receptor (*NR4a3*) gene was induced approximately 800- and 400-fold by BK and AngII, respectively. The immediate early gene transcription factor Nr4a1 was induced 50–60 folds by both receptors. Calponin 1, a smooth muscle marker [Szymanski, 2004], was induced 28-fold by BK. Cysteine-rich 61 (CYR61) [Brigstock, 2003] was induced 10-fold by both effectors. The serine protease inhibitor 1(Serpine1) was induced sevenfold by AngII. Generally, the profile of effects of BK and of AngII were similar and in the same direction. However, differential effects also occurred. For example, the expression of Ras-like protein Tc10 mRNA was doubled by BK but unchanged by AngII. The synaptic vesicle glycoprotein 2b (Sv2b) and the OB-receptor gene related protein (Obrgrp) were increased by AngII but unchanged by BK. Zinc finger protein 386 (Znf386) was inhibited by approximately 50% by BK, but unchanged by AngII. Expression of genes associated with the regulation of connective tissue production was also affected by both receptors. These included the *COL1A1*, *CTGF*, *PTGS2*, and *LOX* genes. Both BK and AngII increased CTGF mRNA expression by approximately twofold compared to basal levels. On the other hand, COL1A1 gene expression was decreased, particularly by BK.

CTGF Expression

The upregulation of CTGF was of particular interest to us. AngII is a known hypertensive and fibrogenic agent. It has been described to increase *CTGF* gene expression [Gonzalez et al., 2002; Iwanciw et al., 2003; Ruperez et al., 2003]. This suggests a role for CTGF in AngII-mediated fibrosis in the heart, lung, and renal systems [Takehara, 2003]. Little has been reported as to the role of BK in CTGF expression. The microarray results suggested an increase in CTGF mRNA level by both AngII and BK. However, microarrays generally provide only semi-quantitative data. Real time PCR provides considerably more accurate numbers [Park et al., 2004]. We determined this response by using real-time PCR. CTGF mRNA expression was increased in the presence of TGFβ (5 ng/ml) by more than threefold over basal in the untransfected Rat1 cell. Neither BK nor AngII had any effect on CTGF mRNA levels (Fig. 1). This agreed with the binding data showing that WT Rat1 cells do not exhibit AT1a or BKB2 receptors. In Rat1-AT1/BKB2 cells, real-time PCR results showed CTGF expression increased approximately five- and ninefold over basal upon stimulation with 100 nM BK or AngII, respectively (Fig. 1).

Expression of CTGF in Cells Containing Mutant Receptors

Mutant receptors were generated and expressed which displayed minimal G protein-dependent phosphotidylinositol (PI) turnover and arachidonic acid (ARA) release to assess the role of G-protein signaling in the expression of CTGF mRNA. The sequences of the two mutant receptors are depicted in Table IIA. The entire (residues 311–366) C-terminus of BKB2R exchanged with that of AT1aR in the BKAT(tail) chimera. The IC2 (residues 166–175) of AT1aR was exchanged with that of the BKB2 receptor in the ATBK(IC2) mutant. The mutant receptors bound BK or AngII with approximately the same K_d and B_{max} values as their WT counterparts, Table IIB, whereas they displayed only minimal release of ARA (Gαi coupled) and PI turnover (Gαq coupled) when activated by AngII or BK, as illustrated in Fig. 2A,B. On the contrary, both mutants increased CTGF mRNA levels markedly (Fig. 2C). Interestingly, BKAT(Tail) proved considerably more active than its WT receptor counterpart. It increased CTGF mRNA to twice the level of increase seen by activation of wild-type BKB2R transfected cells.

Collagen 1 Type A1 mRNA Levels in Response to AngII and BK

CTGF has usually been observed to be linked to increased collagen production particularly in response to TGFβ stimulation [Leivonen et al., 2005]. However, microarray data showed that

TABLE I. DNA Microarray Analysis of Upregulated or Downregulated Gene Expression by BK or AngII in Stably Transfected Rat1 Cells With AT1a and BKB2 Receptor cDNAs

Gene	Gene symbol	FC BK	FC AngII
A disintegrin and MMP with TSP motifs 1	Adamts1	4.6	5.9
Activating transcription factor 3	Atf3	10.4	3.3
Amphiregulin	Areg	7.1	4.1
Amyloid beta precursor protein 3	Apbb3	−4.1	−3.5
Androgen receptor-related protein CBL27	Cbl27	−3	−2.2
ATP-binding cassette, sub-family A, member 2	Abca2	−3.9	−1.7
BMP activin membrane-bound inhibitor	Bambi	1.0	2.2
Calponin 1	Cnn1	28.5	13.1
Calreticulin	Calr	1.0	1.5
Caveolin	Cav	−2.9	−2.3
Cbp/p300-interacting transactivator, 2	Cited2	1.3	2.1
CCAAT/enhancer binding protein, beta	Cebpb	5.2	4.6
CD14 antigen	Cd14	−3.1	−2.3
Cell division cycle 2 homolog A (S. Pombe)	Cdc2a	−2.4	−1.3
Coagulation factor 3	F3	13.8	8.2
Collagen, type 1, alpha 1	Col1a1	−2.1	−1.2
Connective tissue growth factor	Ctgf	1.5	1.9
Cyclin-dependent kinase inhibitor 1B	Cdk7	−1.8	−1.2
Cysteine rich protein 61	Cyr61	9.4	9.9
Damage-specific DNA binding protein 1	Ddb1	−3.2	−1.5
DNA-damage inducible transcript 3	Ddit3	7.9	4.2
Dynamin 2	Dnm2	−7.5	−1.8
Early growth response 1	Egr1	6.3	6.7
Emerin	Emd	6.5	3.1
Ephrin A1	Efna1	1.7	−1.1
F-box protein FBL2	Fbxl2	−16.3	−2.4
Flap structure-specific endonuclease 1	Fen1	−1.2	−2.5
Fos-like antigen 1	Fosl1	14.9	9
Frizzled homolog 2 (*Drosophila*)	Fzd2	−3.3	−2.4
Gro	Gro1	9.9	6.3
Histone H1-0	H1f0	−2.2	−1.9
HMG-box containing protein 1	Hbp1	−2.7	−1.8
Immediate early gene transcription factor	Nr4a1	60.1	53
Immediate early response 5	Ier5	2.2	1.2
Inhibitor of DNA binding 3	Id3	5.2	4
Insulin-like growth factor II	Igf2	1.0	−3.4
Interferon-related developmental regulator 1	Ifrd1	15.5	7.9
Jun B proto-oncogene	Junb	6.1	4.7
Kruppel-like factor 4 (gut)	Klf4	8.1	4.7
Lysyl oxidase	Lox	−1.5	1.1
Mesenchyme homeo box 2	Meox2	−3	−1.1
Microtubule-associated protein 2	Mtap2	−1.8	−1.1
Myeloid differentiation response gene 116	Myd116	5.9	3.2
N-acetylglucosaminyltransferase I	Mgat1	−1.1	1.5
Neurochondrin	Ncdn	−3.7	−2.1
Nuclear factor, interleukin 3, regulated	Nfil3	13.4	10.4
Nuclear pore complex protein	Nup107	−3.1	−2.4
Nuclear receptor subfamily 2, group F2	Nr2f2	−1.9	1.3
Nuclear receptor subfamily 4, group A3	Nr4a3	827.2	460.5
OB-receptor gene related protein	Obrgrp	1.2	2.0
PDGF receptor, beta	Pdgfrb	−2.2	−1.1
Phosphatidylinositol 4-kinase	Pik4cb	−4.2	−1.7
Platelet-derived growth factor receptor alpha	Pdgfra	−2.6	−1.5
Polypeptide *N*-acetylgalactosaminyltransferase 7	Galnt7	−3	−23.2
Prostaglandin-endoperoxide synthase 2	Ptgs2	2.5	2.8
PX serine/threonine kinase	Pxk	−3.6	−3
Ras-like protein	Tc10	1.9	1.2
Reticulocalbin 2	Rcn2	1.0	1.7
Rhob gene	Arhb	1.8	−1.2
RT1 class Ib gene	RT1Aw2	2.2	−1.1
Runt related transcription factor 1	Runx1	1.1	2.0
Serine threonine kinase pim3	Pim3	−1.3	1.8
Serine/cysteine proteinase inhibitor 1	Serpine1	5.7	7.2
Smooth muscle alpha-actin	Acta2	5.1	4.3
Solute carrier family 20, member 1	Slc20a1	6	4.6
Synaptic vesicle glycoprotein 2b	Sv2b	−1.1	1.6
Transcription factor 2	Tcf2	−9.8	−1.2
Transcription factor MTSG1	Mtsg1	−1.8	1.3
Transgelin (smooth muscle 22 protein)	Tagln	6.7	5.7
Tumor-associated antigen 1	Taa1	6	4.6
Upregulated by 1,25-dihydroxyvitamin D-3	Vdup1	−5.8	−3.9

(*Continued*)

TABLE I. (*Continued*)

Gene	Gene symbol	FC BK	FC AngII
Vesicle-asso calmodulin-binding protein	1G5	3.2	1.5
V-jun sarcoma virus 17 homolog	Jun	1.8	1.0
Zinc finger protein 36	Zfp36	4.2	4.9
Zinc finger protein 386 (Kruppel-like)	Znf386	−1.9	1.1

Stably transfected Rat1-AT1/BKB2 cells were incubated with or without ligand (100 nM) for 2 h. Total RNA was isolated and DNA microarray was carried out using Affimatrix gene chips. Gene expressions shown are no lower than 1.5-fold of control and are expressed as FC: (fold change) gene expression in the presence of BK or AngII/control. +/−, Increase or decrease in gene expression in relation to control.

both AngII and BK lowered the expression of COL1A1 in the Rat1 cells (Table I). We used real time PCR to determine the effect of AngII and BK (100 nM) on COL1A1 expression in the Rat1-AT1/BKB2 cells. As illustrated in Figure 3, activation of the WT BKB2 or the AT1a receptors decreased COL1A1 mRNA levels markedly whereas TGFβ (5 ng/ml) had no effect on this parameter. On the other hand, neither the activation of the BKAT(Tail) nor of the ATBK(IC2) mutant receptors resulted in a statistically significant change from basal levels, contrary to their effect on CTGF expression.

Abundant Transcription-Factor Binding Motifs (TFBMs)

We performed combined sequence-based analysis of gene promoter regions with statistical prevalence analyses to identify transcription-factor binding motifs (TFBMs) that are prominent among genes regulated by BK or AngII. The transcription element listening system (TELiS), http://www.telis.ucla.edu/index.htm) was used for this analysis [Cole et al., 2005]. As shown in Table III, 15 TFBMs were much more abundant among the promoters of the regulated genes compared to the entire gene set presented in the Affymetrix GeneChip Rat Expression Set 230A, with a $P < 0.01$ for a 14% false discovery rate. Interestingly, several CREB and CREB related motifs were present in the list of overrepresented TFBMs. The observation that CREB responds to activation of the receptors for AngII or BK agrees with the results from previous studies showing that both BK and AngII activate the signaling cascade, PLA2 > ARA > PGE > cAMP > CREB [Han et al., 2004; Murakami and Kudo, 2004]. Another

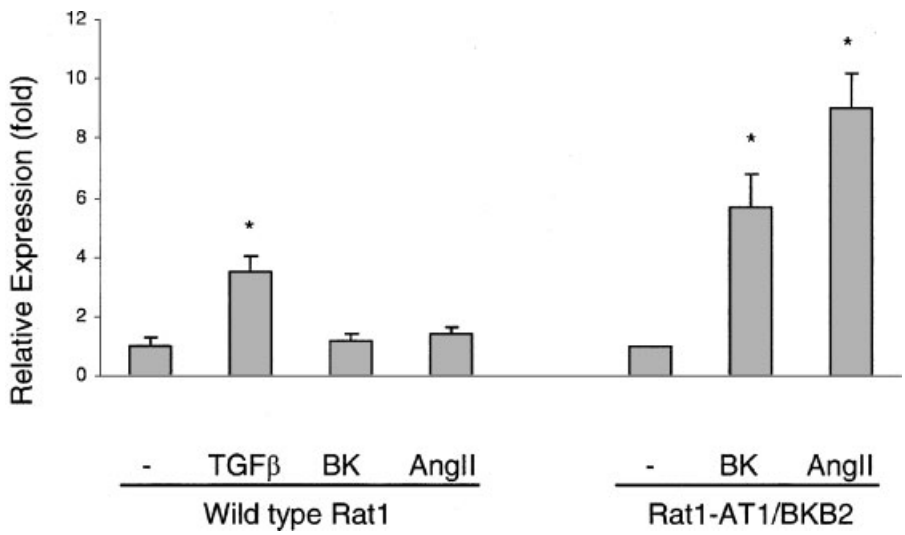

Fig. 1. Real-time PCR analysis of CTGF expression in Rat1 cells. Wild-type Rat1 cells and Rat1-AT1/BKB2 cells expressing both AT1a and BKB2 receptors were incubated at 37°C for 2 h with or without TGFβ (5 ng/ml), BK (100 nM), or AngII (100 nM). Total RNA was isolated and real-time PCR analysis for CTGF expression was carried out as described under Materials and Methods. CTGF mRNA expression was analyzed by real-time PCR and normalized with GAPDH levels. Data represent the mean ± SD of triplicate points. Data are representative of at least three separate experiments. *Change with ligand stimulation compared to control (C) with a $P < 0.05$.

TABLE IIA. Amino Acid Sequence Alignment

C-terminus BKAT mutants:			
rBKB2	...^{311}GKRFRKKSREVYQAICRKGGCMGESVQMENSMGTLRTSISVDRQIHKLQDWAGNKQ		
rAT1a	...^{306}**GKKFKKYFLQLLKYIPPKAKSHSSLSTKMSTLSYRPSDNMSSSAKKPASCFEVE**		
BKAT(Tail)	...^{311}**GKKFKKYFLQLLKYIPPKAKSHSSLSTKMSTLSYRPSDNMSSSAKKPASCFEVE**		
IC2 ATBK mutant:			
	TM3	IC2	TM4
RBKB2R	...^{159}DRYLALV	KTMSMGRMRG	VRWAK
rAT1aR	...^{125}DRYLAIV	HPMKSRLRRT	MLVAK
ATBK(IC2)	...^{125}DRYLAIV	**KTMSMGRMRG**	MLVAK

BKAT(Tail): the whole C-terminal region of BKB2 receptor exchanged with that of AT1a. ATBK(IC2): the IC2 of AT1a exchanged with that of the BKB2 receptor. Bold letter indicates the mutated residue.

prominent motif in this list is SRF and SRF-related transcription factors whose biological functions have been elucidated in embryogenesis and mesoderm development as well as in mediating cardiomyopathy [Arsenian et al., 1998; Zhang et al., 2001a,b]. SRF and CREB have been shown to be regulated by a combination of MEK/ERK and JNK signaling pathways [Gineitis and Treisman, 2001; Pathak et al., 2004; Tullai et al., 2004; Cho et al., 2005].

Protein Phosphorylation Within the AngII and BK Signaling Pathways

The cell lysates of non-stimulated and stimulated Rat1-AT1/BKB2 cells were subjected to proteomic screening with protein phosphate-specific antibodies (Kinexus, Canada; Table IV). Focal adhesion kinase was prominently phosphorylated in response to 100 nM BK but less so in response to AngII. Differential phosphorylation in response to BK vs. AngII was seen with Cyclin-dependent kinase 1 and IR/IGF. Phosphorylation of Src was decreased by 22% upon BK stimulation, but only decreased slightly in response to stimulation by AngII. Dramatic increases in phosphorylation of ERK2 and JNK occurred in response to BK and AngII, while phosphorylation of p38 MAPK increased about twofold above basal levels in response to both effectors. These effects of BK and AngII on ERK, JNK, and p38 MAPK phosphorylation were confirmed by Western blot analysis (Fig. 4). The Rat1-AT1/BKB2 cells responded to the two effectors with marked increases in phosphorylation of all three kinases.

TABLE IIB. Binding Parameters of [^{3}H] BK and [^{3}H] AngII in Transfected Rat-1 Cells

Receptor	B_{max} (receptors/cells $\times 10^3$)	K_d (nM)
BKB2R	53.9 ± 3.2	1.9
BKAT(Tail)	33.4 ± 2.0	2.5
AT1aR	71.8 ± 4.2	5.8
ATBK(IC2)	69.6 ± 5.1	4.7

Participation of ERK and JNK But Not p38 MAPK in AT1aR- and BKB2R-mediated Upregulation of CTGF

Specific kinase inhibitors were used to determine whether the activation of ERK1/2, JNK, or p38 MAPK is involved in the BK- and AngII-mediated upregulation of CTGF (Fig. 5A). CTGF expression increased in Rat1-AT1/B2R cells by 6.5- and 3.5-fold, in the presence of 100 nM AngII or BK, respectively. The induction of CTGF expression by BK or AngII was not affected by the p38 MAPK inhibitor SB202190. However, SP600125, the JNK inhibitor, decreased BK stimulation by approximately 50%, and AngII stimulation by 70%. The inhibitor of ERK activation, PD98059, was also effective in limiting both BK- and AngII-dependent increases of CTGF mRNA levels. None of these three inhibitors decreased levels of COL1A1 mRNA in response to BK or AngII, although the inhibitors of p38 MAPK and JNK marginally accentuated the inhibitory effect of AngII (Fig. 5B).

JNK and ERK Phosphorylation by Mutant Receptors

We then proceeded to confirm that the mutant receptors activate JNK and ERK, and their specific inhibitors are functional. Cells stably transfected with each mutant cDNA were exposed to either 100 nM AngII or BK. The effect of JNK and ERK inhibitors on the activation of these two kinases by AngII and BK was confirmed with Western blot analysis. This is illustrated in Fig. 6. In the presence of their effectors, both mutants exhibited increased phosphorylation of ERK (Fig. 6A). Only BKAT (Tail) activated JNK (Fig. 6B). In fact the BKAT(Tail) mutant exhibited a very strong signal for both ERK and JNK phosphorylation. Interestingly, this mutant also demonstrated

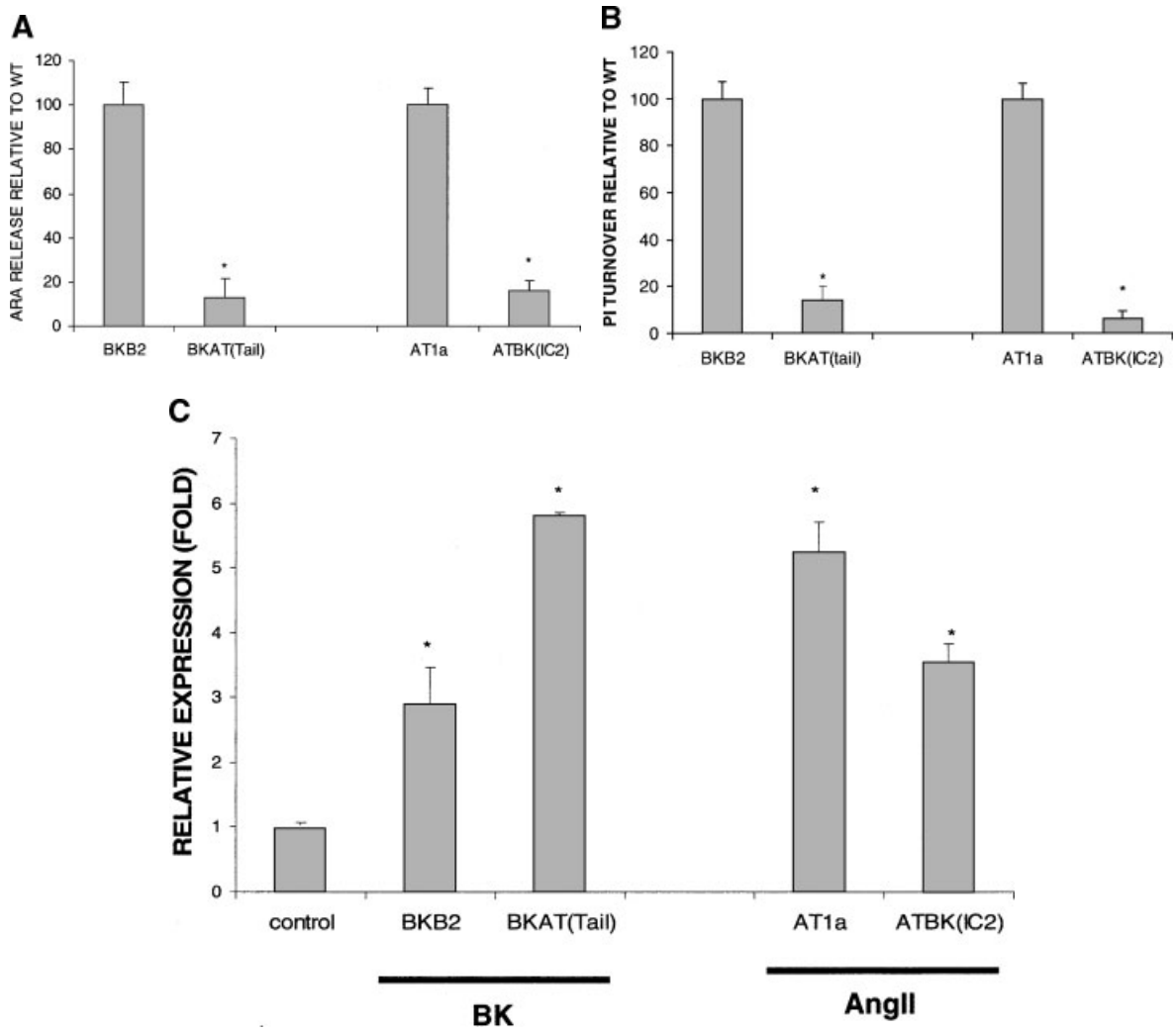

Fig. 2. Signaling by chimeric AT1a and BKB2 receptor, and CTGF expression. **A**: *ARA release in Rat-1 cells expressing wild-type and chimeric receptors.* ARA release was measured in [^{3}H]arachidonate labeled cells as described in Materials and Methods. Results are presented as effector stimulated ARA release minus basal ARA, normalized to that of wild-type receptors. Data represent the average of triplicate samples ± SD SD from a representative experiment of at least three separate experiments. **B**: *Inositol phosphate production in Rat-1 cells expressing wild-type and chimeric receptors.* PI turnover was measured in myo-[^{3}H]inositol-labeled cells as described in Materials and Methods. Results are presented as ligand stimulated IP production minus basal IP normalized to that of wild-type receptors. Data represent the average of triplicate samples ± SD from a representative experiment of at least three experiments. **C**: *CTGF expression in Rat1 cells transfected with wild-type or mutant AT1a and BKB2 receptors.* Stably transfected Rat1 cells, BKB2, and AT1a, BKAT(Tail) and ATBK(IC2), were treated with or without 100 nM AngII or BK at 37°C for 2 h. CTGF expression in these cells in response to ligand stimulation was analyzed by real-time PCR and normalized with GAPDH levels. The relative CTGF expression was compared to that of unstimulated cells. Each point was run in triplicate and represent the mean ± SD. The result is representative of at least two experiments. *Statistically significant changes compared to control (C) ($P < 0.05$).

very strong BK stimulated CTGF expression (Fig. 2C).

DISCUSSION

The regulation of CTGF expression in fibroblasts has been reported by number of laboratories [Frazier et al., 1996; Gupta et al., 2000; Leask et al., 2003; Papakrivopoulou et al., 2004; Zhao et al., 2004]. Angiotensin II has been shown to regulate CTGF expression in certain cell types where the AT1 receptor is endogenous [Finckenberg et al., 2003; Iwanciw et al., 2003; Ruperez et al., 2003; Wilkinson-Berka and Fletcher, 2004; He et al., 2005]. BK has also been reported to regulate CTGF expression in cells expressing endogenous BKB2R [Douillet et al., 2000; Yu et al., 2004; Tan et al., 2005]. At

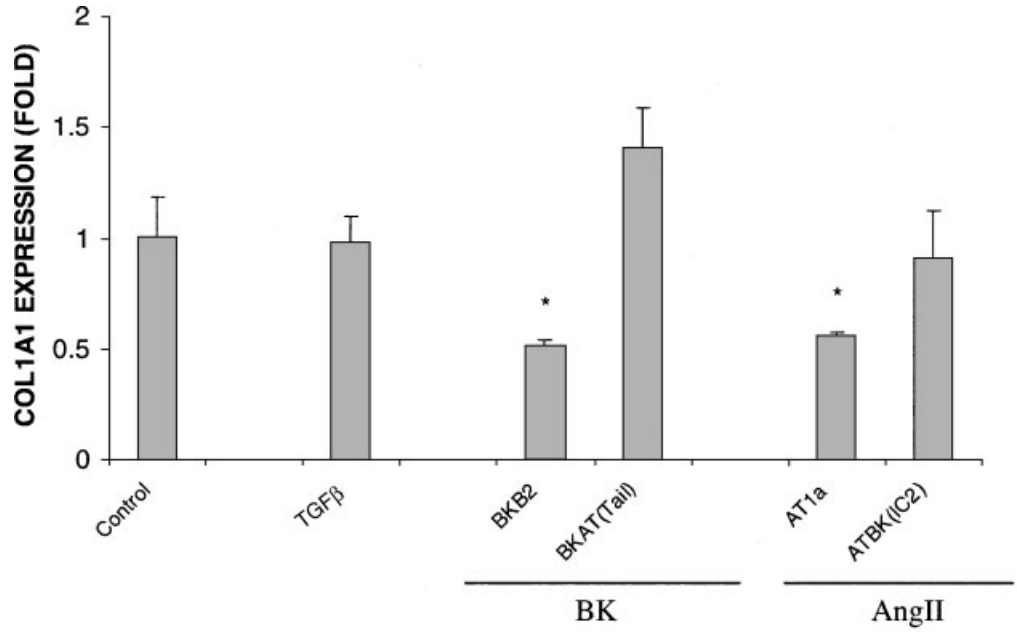

Fig. 3. COL1A1 expression in Rat1 cells stably transfected with wild-type or mutant AT1a and BKB2 receptors. Rat1 cells and Rat1 cells stably transfected with WT BKB2, WT AT1, BKAT(Tail), and ATBK(IC2) cDNAs were treated with the indicated effector at 37°C for 24 h. COL1A1 mRNA expression in these cells in response to ligand stimulation was analyzed by real-time PCR and normalized with GAPDH levels. The relative COL1A1 expression for each response is shown as a ratio with the corresponding, unstimulated culture having a value of one (shown as control). The concentration of BK and AngII was 100 nM, and TGFβ was 5 ng/ml. Each point is a result of triplicate incubation and represent the mean ± SD. The results are representative of at least two experiments. *, Significant changes compared to control (C) ($P < 0.05$).

this time, little is known about the cascades involved in the cooperative and antagonistic physiologic activities of the AT1 and BKB2 receptors. To begin to investigate this dilemma, Rat1 cells which lack demonstrable binding to either receptor were stably transfected with the cDNAs of both receptors. Rat-1 cells are a 3T3-like cell line developed by W.C. Topp. They are readily transfectable by exogenous DNA and are phenotypically normal [Topp, 1981]. Our previous studies have shown that both the AT1aR and BKB2R receptors mediated signaling normally via G protein (Gq and Gi) after being transfected into the Rat-1 cells [Prado et al., 1997; Yu et al., 2002]. At this time, however, the level of understanding is poor about the participating downstream regulators of either the AT1 or BKB2 receptors linking to CTGF expression in Rat-1 cell.

Upon successful expression of the two receptors, microarray analyses were carried out to assess and compare gene action in response to BK and AngII in the same cell line. A number of genes were detected to be differentially regulated by the AT1a and BKB2 receptors. For example, calreticulin which is upregulated by AngII but not BK has been linked to the acceleration of AT1 receptor mRNA decay [Nickenig et al., 2002]. Moreover, the insulin-like growth factor 2 receptor sizably (threefold) downregulated by AngII, while expression of Tc10, a small GTPase, is suppressed by BK. Tc10 has been reported to be active in Glut4 translocation [Chiang et al., 2001; Watson et al., 2001]. Microarray analyses of the Rat1 cells also showed that both receptors induce two CCN family genes (*CCN1* and *CCN2*). CCN is a newly defined family of immediate-early response genes of which six members have been identified thus far, Cyr61 (CCN1), CTGF (CCN2), Nov (CCN3) (CCCCN2), Wnt-inducible secretory protein-1 (WISP-1) (CCN4), WISP-2 (CCN5), and WISP-3 (CCN6). This family of genes appears to be involved in multifunctional signaling pathways and is expressed in wide variety of tissues during normal development. Cyr61 is stress related and is known to be involved in angiogenesis and wound repair [Jin et al., 2005]. The microarray determinations revealed that Cyr61 was upregulated tenfold while CTGF expression was doubled by both receptors. Both receptors, particularly BKB2R,

TABLE III. Representative Transcription Factor Binding Motifs (TFBMs) in the Promoter Regions of Genes Regulated by BK or AngII*

TFBM	TFBM description	Frequency[a]	Incidence[b]
SRF_C	Serum responsive factor	z = 5.80 P = 1.00E−10 Sample mean = 0.103 Population mean = 0.013 Ratio = 7.7	n = 4/78 P = 0.0154 Sample mean = 5.13% Population mean = 1.22% Ratio = 4.2
CREB_Q4	cAMP-response element binding protein	z = 5.33 P = 1.00E−10 Sample mean = 0.385 Population mean = 0.145 Ratio = 2.6	n = 26/78 P = 3.74E−6 Sample mean = 33.33% Population mean = 13.11% Ratio = 2.5
RSRFC4_01	Related to serum response factor, C4	z = 4.68 P = 2.86E−6 Sample mean = 0.064 Population mean = 0.010 Ratio = 6.4	n = 4/78 P = 0.0074 Sample mean = 5.13% Population mean = 0.98% Ratio = 5.2
CREB_02	cAMP-responsive element binding protein	z = 4.43 P = 9.22E−6 Sample mean = 0.423 Population mean = 0.193 Ratio = 2.2	n = 24/78 P = 0.0016 Sample mean = 30.77% Population mean = 16.74% Ratio = 1.8
CREB_01	cAMP-responsive element binding protein	z = 4.05 P = 5.18E−5 Sample mean = 0.269 Population mean = 0.112 Ratio = 2.4	n = 19/78 P = 0.0003 Sample mean = 24.36% Population mean = 10.44% Ratio = 2.3
CREBP1_Q2	CRE-binding protein 1	z = 3.86 P = 0.0001 Sample mean = 0.179 Population mean = 0.064 Ratio = 2.8	n = 13/78 P = 0.0008 Sample mean = 16.67% Population mean = 6.04% Ratio = 2.8
ATF_01	Activating transcription factor	z = 3.62 P = 0.0003 Sample mean = 0.462 Population mean = 0.247 Ratio = 1.9	n = 27/78 P = 0.0040 Sample mean = 34.62% Population mean = 21.09% Ratio = 1.6
CREBP1CJUN_01	CRE-binding protein 1/c-Jun heterodimer	z = 3.27 P = 0.0011 Sample mean = 0.128 Population mean = 0.047 Ratio = 2.7	n = 10/78 P = 0.0027 Sample mean = 12.82% Population mean = 4.51% Ratio = 2.8
CREB_Q2	cAMP-response element-binding protein	z = 3.16 P = 0.0016 Sample mean = 0.244 Population mean = 0.116 Ratio = 2.1	n = 17/78 P = 0.0028 Sample mean = 21.79% Population mean = 10.56% Ratio = 2.1
MEF2_02	Myogenic MADS factor MEF-2	z = 3.15 P = 0.0016 Sample mean = 0.051 Population mean = 0.012 Ratio = 4.2	n = 4/78 P = 0.0154 Sample mean = 5.13% Population mean = 1.22% Ratio = 4.2
MEF2_03	Myogenic MADS factor MEF-2	z = 3.00 P = 0.0027 Sample mean = 0.051 Population mean = 0.013 Ratio = 4.0	n = 4/78 P = 0.0186 Sample mean = 5.13% Population mean = 1.29% Ratio = 4.0
MZF1_01	MZF1	z = 2.98 P = 0.0029 Sample mean = 2.962 Population mean = 2.310 Ratio = 1.3	n = 69/78 P = 0.1683 Sample mean = 88.46% Population mean = 83.81% Ratio = 1.1
EVI1_04	Ectopic viral integration site 1 encoded factor	z = 2.97 P = 0.0029 Sample mean = 0.013 Population mean = 0.001 Ratio = 10.7	n = 1/78 P = 0.0890 Sample mean = 1.28% Population mean = 0.12% Ratio = 10.7
SRF_Q6	Serum response factor	z = 2.95 P = 0.0032 Sample mean = 0.103 Population mean = 0.036 Ratio = 2.8	n = 5/78 P = 0.1311 Sample mean = 6.41% Population mean = 3.44% Ratio = 1.9
USF_Q6	Upstream stimulating factor	z = 2.88 P = 0.0040 Sample mean = 0.859 Population mean = 0.594 Ratio = 1.4	n = 46/78 P = 0.0045 Sample mean = 58.97% Population mean = 43.59% Ratio = 1.4

*The transcription element listening system (TELiS), www.telis.ucla.edu/index.htm was used for the analysis and $P < 0.01$ was used for a 14% false discovery rate. The sample mean frequencies and the incidences were calculated based on 78 available promoter sequences (−1,000 to +200 bp) of 90 differentially regulated genes (87% of submission). The population mean frequencies and the incidences were calculated based on all available promoter sequences for the whole gene set presented on the Affymetrix Rat230A array. A total of 192 TFBMs (source: TRANSFAC 3.2) were scanned.

[a]Frequency analyses compare the average number of TFBMs detected in promoters of differentially expressed genes with the average number in all assayed genes (the "sampling frame"). These comparisons are carried out using a z-test.

[b]Incidence analyses determine whether a TFBM is present in a greater fraction of differentially expressed genes than in the sampling frame as a whole. This is a binary analysis (TFBM is present vs. not in each promoter), executed as an exact binomial test. With >1,000 genes, incidence analyses switch to the normal approximation to the binomial.

TABLE IV. Phospho-Specific Screening in Rat1 Cells Stably Transfected With Both AT1 and BKB2 Receptor cDNAs

Full name of protein	Abbreviations	Norm C.P.M.			% Change	
		Control	BK	AngII	BK	AngII
Cyclin-dependent kinase 1 (T14/Y15)		6,608	7,386	4,929	11.8	−25.4
Extracellular signal-regulated kinase 2 (T185/Y187)	ERK2	87	542	207	523.0	137.9
Focal adhesion kinase (S910)		852	1,670	1,297	96.0	52.2
Insulin/insulin-like growth factor-1 receptor (Y1162/Y1163)		1,889	1,697	2,574	−10.2	36.6
MAP kinase kinase 1 (S298) (41)	MEK1 S298	11,626	11,819	7,496	1.7	−35.5
MAP kinase kinase 1 (T386)		1,919	4,065	3,748	111.8	95.3
Oncogene SRC (Y529)		12,039	7,542	10,510	−37.4	−12.7
p38 alpha MAP kinase T180/Y182 (38)	p38 MAPK T180/Y182	4,044	8,282	7,709	104.8	90.6
Stress-activated protein kinase (JNK) T183/Y185) (39)	SAPK T183/Y185		694	405	Increase	Increase

Rat1-AT1/BKB2 cells were incubated for 10 min at 37°C with or without 100 nM AngII or BK. Total cell lysate was prepared and subjected to immuno-screening with radioactive labeled phospho-specific antibodies as described in the Materials and Methods section. Results are presented as CPM.

also suppressed COL1A1 expression. In this case the relatively small effect of AngII may be due to the relatively short incubation time used for the microarray analysis. Induction of the COL1A1 gene generally requires a longer time for induction [McGaha et al., 2003]. Real-time PCR confirmed these responses to the two receptors. The real time PCR results also showed that AngII and BK upregulate CTGF expression to a level equal to or greater than those induced by TGFβ. Traditionally, TGFβ has been linked to CTGF expression to upregu-

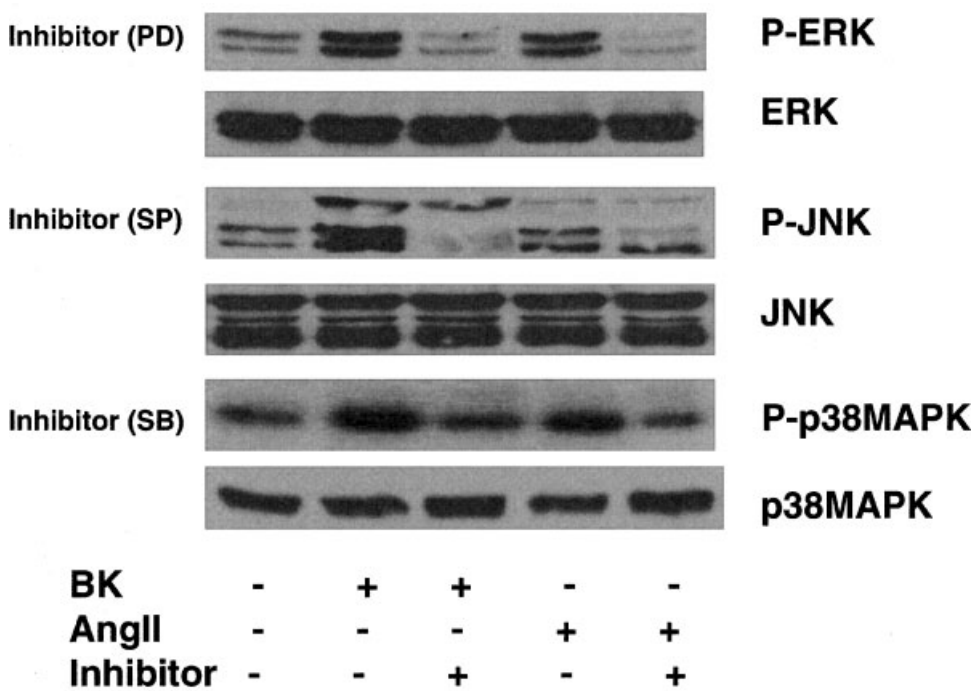

Fig. 4. AngII and BK regulated MAP kinase Phosphorylation. Western blot analysis of cell lysates of Rat1-AT1/BKB2 cells stimulated with 100 nM AngII or BK. Immunoreactive kinases were identified on membranes with specific antibodies against ERK1/2, JNK or p38 MAPK as described in the Materials and Methods section. The cells were pretreated with ERK inhibitor: PD98059 (50 μM), JNK inhibitor: SP600125 (20 μM) or p38 MAPK inhibitor: SB202190 (10 μM) at 37°C for 1 h. The results are representative of at least three experiments.

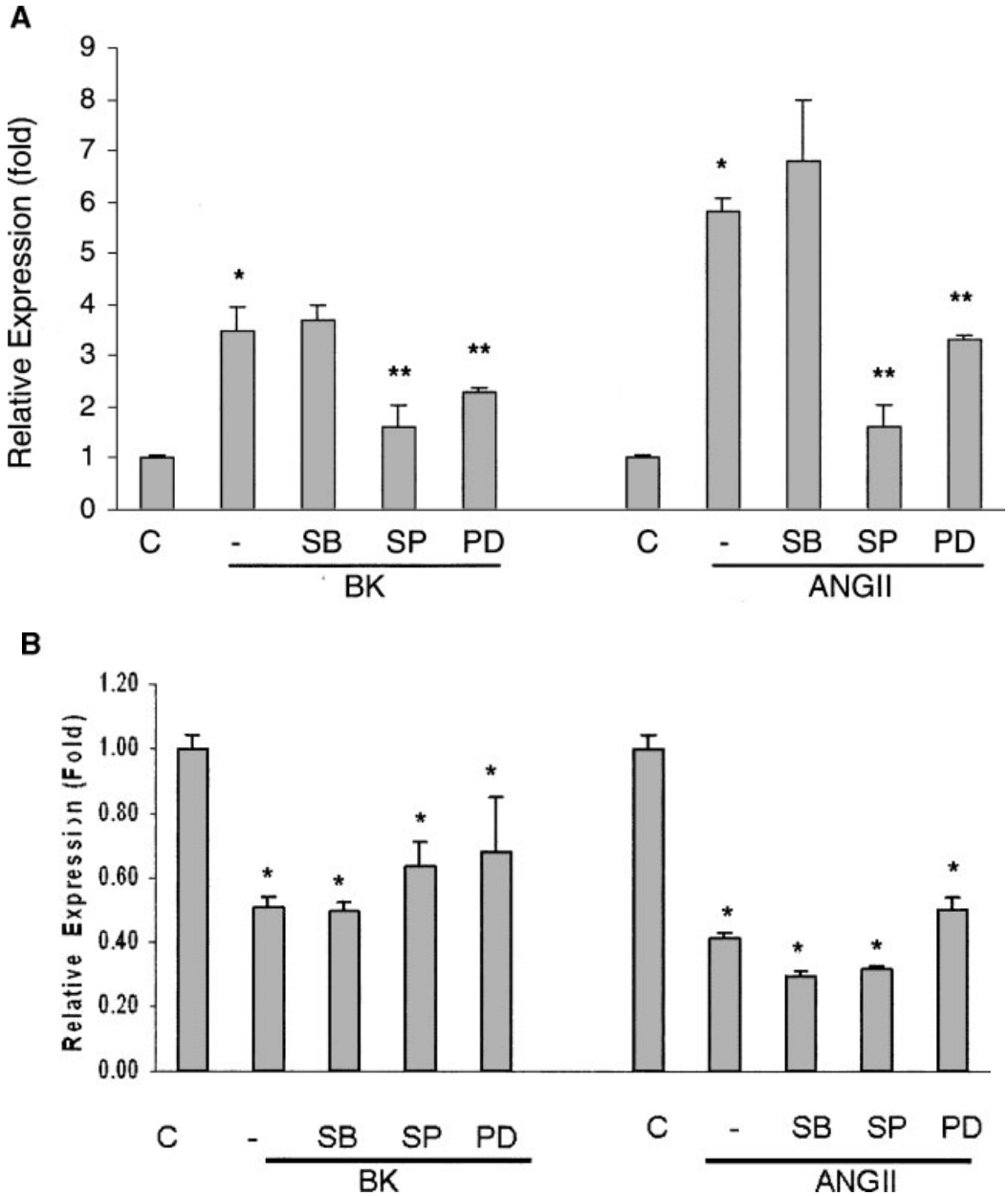

Fig. 5. ERK, JNK, and p38MAPK Phosphorylation Inhibitor effects on CTGF expression. **A**: Rat1-AT1/BKB2 cells were pretreated with ERK inhibitor: PD98059 (50 μM), JNK inhibitor: SP600125 (20 μM) or p38 MAPK inhibitor: SB202190 (10 μM) at 37°C for 1 h. The cells were then simulated with or without 100 nM AngII or BK at 37°C for 2 h. CTGF expression in response to the treatments was analyzed by real-time PCR and normalized with GAPDH levels. The relative CTGF expression for each response is shown as a ratio with the corresponding, unstimulated culture having a value of one (shown as control). **B**: Effects of the three inhibitors on COL1A1 mRNA levels were determined by real time PCR as in 5A. In both analyses data were collected in three separate incubations (triplicate) and represent the mean ± SD. The results shown are a representative experiment of at least two separate experiments carried out in triplicate. *Changes with ligand stimulation compared to control (C) with a $P < 0.05$. **Changes with inhibitor pretreatment compared to untreated with $P < 0.05$.

late collagen production [Leivonen et al., 2005]. Here we show that TGFβ increases CTGF mRNA levels, but has no effect on COL1A1 expression in the WT Rat1 cells (Fig. 1 and 3). At the same time, BK and AngII increase CTGF but decrease COL1A1 mRNA in these cells. Thus the link between CTGF and an increase of COL1A1 expression is missing in Rat1 cells.

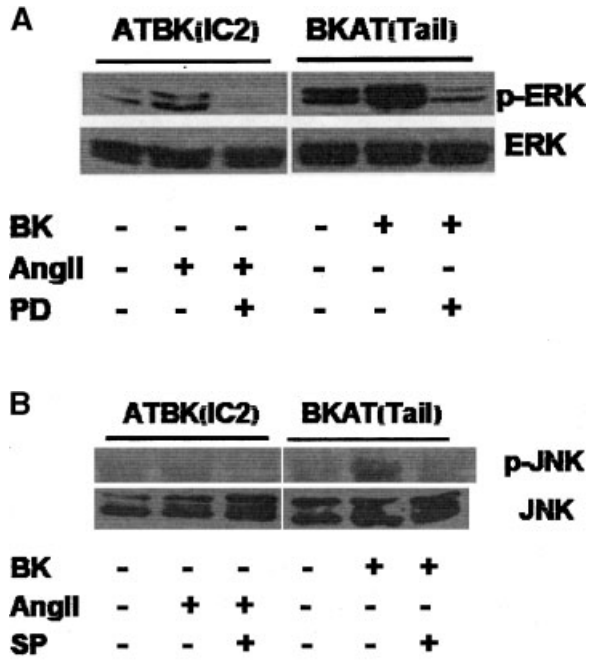

Fig. 6. Western blot analysis of Rat1 cells expressing mutant AT1a or BKB2 receptors. Rat1 cells stably transfected with mutant BKAT(Tail) and mutant ATBK(IC2) receptor cDNAs were preincubated with either the ERK inhibitor PD98059 (50 μM) or JNK inhibitor SP600125 (20 μM) at 37°C for 1 h. The cells were then stimulated with or without 100 nM AngII, or BK and analyzed by Western blot as described in Materials and Methods.

Recent studies have shown that genome-wide assessment of TFBM prevalence can be coupled with population-based statistical inference to provide accurate "reverse inference" of transcription factor activity based on microarray differential expression data. This method has been successfully applied for the detection of in vivo activations of NF-κB and the Type I interferon system by HIV-1 infection and pharmacologic activation of the glucocorticoid receptor in peripheral blood mononuclear cells [Cole et al., 2005]. We adapted this statistical approach to identify transcription factor target motifs, which are utilized by both the BKB2 and AT1 receptors. Our analysis shows that both receptors stimulate pathways leading to the expression of genes, which are regulated by transcription factors CREB, SRF, and ATF-1. Interestingly, a few published reports have now shown that CREB is indeed an important target of the AT1 receptor [Cook et al., 2004]. The action of CREB converges on cAMP function which has been linked to the control of connective tissue production [Liu et al., 2004]. SRF is involved in CTGF expression [Wunderlich et al., 2000; Goppelt-Struebe et al., 2001]. ATF-1 is associated with fibronectin and COL1A1 translation processes [Akiyama-Uchida et al., 2002; Chan et al., 2003].

The action of these factors has been shown to be regulated by the MEK/ERK and JNK signaling pathways [Gineitis and Treisman, 2001; Pathak et al., 2004; Tullai et al., 2004; Cole et al., 2005]. This agrees well with our phosphokinase activation analysis, showing that AngII and BK both markedly activate ERK2 as well as JNK. ERK, JNK, and p38MAPK have been linked to the induction of CTGF expression [Crean et al., 2002; Zhao et al., 2004] and appear to be involved in a number of fibrogenic processes [Frazier et al., 1996; Fan et al., 2000]. For example, mechanical load-induced procollagen α (I) gene expression requires ERK1/2 activation. The p38MAPK pathway appears to negatively regulate this gene expression in cardiac fibroblasts. These pathways may be key in events leading to matrix deposition during heart growth and remodeling induced by mechanical load [Papakrivopoulou et al., 2004]. In human lung fibroblasts JNK appears to be involved in TGFβ induced CTGF expression [Utsugi et al., 2003]. Both JNK and ERK participate in TGFβ upregulated CTGF expression in airway smooth muscle cells [Xie et al., 2005]. In fact, it is increasingly evident that a "proper" balance between the Ras/MEK/ERK and JNK/MAPK cascades is necessary for TGFβ induction of CTGF in human lung fibroblasts [Leask et al., 2002a].

Our results on the effects of specific kinase inhibitors demonstrate that ERK and JNK are involved in AngII and BK induced CTGF expression but not in the reduction of COL1a expression in the Rat1 fibroblasts. p38MAPK is not involved in the upregulation of CTGF expression effected by either receptor.

With use of chimeric exchanges of the BKB2R with corresponding AT1aR motifs and with use of point mutations, we had formerly demonstrated that the C-terminal region of the BKB2 receptor is crucial in G-protein interaction [Yu et al., 2004]. Despite their lack of ability to transduce signals via the PLC or PLA2 paths, the BKB2R mutants containing the entire C-terminal peptide of the AT1aR sequence or the proximal AT1aR (residues 303–322) responded to BK stimulation with an increase in CTGF mRNA suggesting that an additional signal

mechanism is taking place here that does not require significant involvement by BKB2R-associated Gαq, Gαi transduced pathways. We also observed that, unlike the BKB2R, the second intracellular loop region in the AT1aR is critical for G-protein coupling. Thus, two mutants of two separate GPCRs continued to signal despite only minimal G-protein association. This permitted us to evaluate the role of G-proteins in CTGF upregulation and in the suppression of COL1A1 gene expression. Although the expression of CTGF was induced in both the AT1a and BKB2 mutants even when PI turnover and ARA release are minimal, the inhibition of COL1A1 expression by both BK and AngII was lost. These results point to a separation of receptor action between the induction of CTGF expression and suppression of COL1A1 expression. The simplest explanation is that CTGF induction takes place outside the classic G-protein coupled signaling cascade while COL1A1 requires robust G-protein coupling.

AngII has previously been reported to activate JNK and ERK1/2 [Izawa et al., 2005], while BK has been reported to link to the phosphorylation of ERK [El-Dahr et al., 1998; Douillet et al., 2000]. The mutant receptor BKAT(Tail) phosphorylated both ERK and JNK. However, ATBK(IC2), the AT1a mutant, only marginally phosphorylated JNK in the Rat1 cells while it phosphorylated ERK markedly. The markedly stimulated phosphorylation of ERK and JNK in response to ligand binding to the mutant receptor, BKAT(Tail), relates well to the consequent high level of expression of CTGF. Interestingly, the mutant ATBK(IC2) effect on CTGF expression is less than that by the WT AT1a receptor. This supports the present result indicating that the regulation of CTGF expression in Rat1 cells is regulated through both kinases.

In sum, both receptors act very similarly to regulate CTGF and COL1A1 expression in the Rat1 cell. The degree to which these actions are species and/or cell-type specific remains to be determined. Nevertheless, it is clear that although the inhibition of COL1A1 expression is regulated through G-protein coupling, CTGF expression is regulated through a very different mechanism which is largely independent of G-protein coupling. It remains to be determined how the upregulation of CTGF and downregulation of COL1A1 expression are related. Also further work is required to determine what G-protein coupled cascades are utilized by the two receptors to downregulate COL1A1 expression in the Rat1 cells.

ACKNOWLEDGMENTS

We wish to thank Dr. Herbert Kagan for a critical reading of the manuscript.

REFERENCES

Akiyama-Uchida Y, Ashizawa N, Ohtsuru A, Seto S, Tsukazaki T, Kikuchi H, Yamashita S, Yano K. 2002. Norepinephrine enhances fibrosis mediated by TGF-beta in cardiac fibroblasts. Hypertension 40:148–154.

Arsenian S, Weinhold B, Oelgeschlager M, Ruther U, Nordheim A. 1998. Serum response factor is essential for mesoderm formation during mouse embryogenesis. EMBO J 17:6289–6299.

Bleau AM, Planque N, Perbal B. 2005. CCN proteins and cancer: Two to tango. Front Biosci 10:998–1009.

Brigstock DR. 2003. The CCN family: A new stimulus package. J Endocrinol 178:169–175.

Chan TM, Leung JK, Tsang RC, Liu ZH, Li LS, Yung S. 2003. Emodin ameliorates glucose-induced matrix synthesis in human peritoneal mesothelial cells. Kidney Int 64:519–533.

Chiang SH, Baumann CA, Kanzaki M, Thurmond DC, Watson RT, Neudauer CL, Macara IG, Pessin JE, Saltiel AR. 2001. Insulin-stimulated GLUT4 translocation requires the CAP-dependent activation of TC10. Nature 410:944–948.

Cho KN, Choi JY, Kim CH, Baek SJ, Chung KC, Moon UY, Kim KS, Lee WJ, Koo JS, Yoon JH. 2005. Prostaglandin E2 induces MUC8 gene expression via a mechanism involving ERK MAPK/RSK1/cAMP response element binding protein activation in human airway epithelial cells. J Biol Chem 280:6676–6681.

Chretien L, Richard DE, Poirier SN, Poitras M, Guillemette G. 1998. Bovine adrenal glomerulosa cells express such a low level of functional B2 receptors that bradykinin does not significantly increase their aldosterone production. J Endocrinol 156:449–460.

Cole SW, Yan W, Galic Z, Arevalo J, Zack JA. 2005. Expression-based monitoring of transcription factor activity: The TELiS database. Bioinformatics 21:803–810.

Cook JL, Re R, Alam J, Hart M, Zhang Z. 2004. Intracellular angiotensin II fusion protein alters AT1 receptor fusion protein distribution and activates CREB. J Mol Cell Cardiol 36:75–90.

Crean JK, Finlay D, Murphy M, Moss C, Godson C, Martin F, Brady HR. 2002. The role of p42/44 MAPK and protein kinase B in connective tissue growth factor induced extracellular matrix protein production, cell migration, and actin cytoskeletal rearrangement in human mesangial cells. J Biol Chem 277:44187–44194.

de Gasparo M, Catt KJ, Inagami T, Wright JW, Unger T. 2000. International union of pharmacology. XXIII. The angiotensin II receptors. Pharmacol Rev 52:415–472.

Douillet CD, Velarde V, Christopher JT, Mayfield RK, Trojanowska ME, Jaffa AA. 2000. Mechanisms by which

bradykinin promotes fibrosis in vascular smooth muscle cells: Role of TGF-beta and MAPK. Am J Physiol Heart Circ Physiol 279:H2829–H2837.

El-Dahr SS, Dipp S, Baricos WH. 1998. Bradykinin stimulates the ERK ≥ Elk-1 ≥ Fos/AP-1 pathway in mesangial cells. Am J Physiol 275:F343–F352.

Fan WH, Pech M, Karnovsky MJ. 2000. Connective tissue growth factor (CTGF) stimulates vascular smooth muscle cell growth and migration in vitro. Eur J Cell Biol 79:915–923.

Finckenberg P, Inkinen K, Ahonen J, Merasto S, Louhelainen M, Vapaatalo H, Muller D, Ganten D, Luft F, Mervaala E. 2003. Angiotensin II induces connective tissue growth factor gene expression via calcineurin-dependent pathways. Am J Pathol 163:355–366.

Frazier K, Williams S, Kothapalli D, Klapper H, Grotendorst GR. 1996. Stimulation of fibroblast cell growth, matrix production, and granulation tissue formation by connective tissue growth factor. J Invest Dermatol 107: 404–411.

Garcia-Sainz JA, Avendano-Vazquez SE. 1999. Activation of bradykinin B2 receptors increases calcium entry and intracellular mobilization in C9 liver cells. Biochem Mol Biol Int 47:927–933.

Gineitis D, Treisman R. 2001. Differential usage of signal transduction pathways defines two types of serum response factor target gene. J Biol Chem 276:24531–24539.

Gonzalez A, Lopez B, Querejeta R, Diez J. 2002. Regulation of myocardial fibrillar collagen by angiotensin II. A role in hypertensive heart disease? J Mol Cell Cardiol 34:1585–1593.

Goppelt-Struebe M, Hahn A, Iwanciw D, Rehm M, Banas B. 2001. Regulation of connective tissue growth factor (ccn2; ctgf) gene expression in human mesangial cells: Modulation by HMG CoA reductase inhibitors (statins). Mol Pathol 54:176–179.

Gupta S, Clarkson MR, Duggan J, Brady HR. 2000. Connective tissue growth factor: Potential role in glomerulosclerosis and tubulointerstitial fibrosis. Kidney Int 58:1389–1399.

Han C, Demetris AJ, Liu Y, Shelhamer JH, Wu T. 2004. Transforming growth factor-beta (TGF-beta) activates cytosolic phospholipase A2alpha (cPLA2alpha)-mediated prostaglandin E2 (PGE)2/EP1 and peroxisome proliferator-activated receptor-gamma (PPAR-gamma)/Smad signaling pathways in human liver cancer cells. A novel mechanism for subversion of TGF-beta-induced mitoinhibition. J Biol Chem 279:44344–44354.

He Z, Way KJ, Arikawa E, Chou E, Opland DM, Clermont A, Isshiki K, Ma RC, Scott JA, Schoen FJ, Feener EP, King GL. 2005. Differential regulation of angiotensin II-induced expression of connective tissue growth factor by protein kinase C isoforms in the myocardium. J Biol Chem 280:15719–15726.

Iwanciw D, Rehm M, Porst M, Goppelt-Struebe M. 2003. Induction of connective tissue growth factor by angiotensin II: Integration of signaling pathways. Arterioscler Thromb Vasc Biol 23:1782–1787.

Izawa Y, Yoshizumi M, Fujita Y, Ali N, Kanematsu Y, Ishizawa K, Tsuchiya K, Obata T, Ebina Y, Tomita S, Tamaki T. 2005. ERK1/2 activation by angiotensin II inhibits insulin-induced glucose uptake in vascular smooth muscle cells. Exp Cell Res 308:291–299.

Jin Y, Kim HP, Ifedigbo E, Lau LF, Choi AM. 2005. Cyr61 protects against hyperoxia-induced cell death via Akt pathway in pulmonary epithelial cells. Am J Respir Cell Mol Biol 33:297–302.

Leask A, Abraham DJ, Finlay DR, Holmes A, Pennington D, Shi-Wen X, Chen Y, Venstrom K, Dou X, Ponticos M, Black C, Bernabeu C, Jackman JK, Findell PR, Connolly MK. 2002a. Dysregulation of transforming growth factor beta signaling in scleroderma: Overexpression of endoglin in cutaneous scleroderma fibroblasts. Arthritis Rheum 46:1857–1865.

Leask A, Holmes A, Abraham DJ. 2002b. Connective tissue growth factor: A new and important player in the pathogenesis of fibrosis. Curr Rheumatol Rep 4:136–142.

Leask A, Holmes A, Black CM, Abraham DJ. 2003. Connective tissue growth factor gene regulation. Requirements for its induction by transforming growth factor-beta 2 in fibroblasts. J Biol Chem 278:13008–13015.

Leivonen SK, Hakkinen L, Liu D, Kahari VM. 2005. Smad3 and extracellular signal-regulated kinase 1/2 coordinately mediate transforming growth factor-beta-induced expression of connective tissue growth factor in human fibroblasts. J Invest Dermatol 124:1162–1169.

Liu X, Ostrom RS, Insel PA. 2004. cAMP-elevating agents and adenylyl cyclase overexpression promote an antifibrotic phenotype in pulmonary fibroblasts. Am J Physiol Cell Physiol 286:C1089–C1099.

McGaha TL, Le M, Kodera T, Stoica C, Zhu J, Paul WE, Bona CA. 2003. Molecular mechanisms of interleukin-4-induced up-regulation of type I collagen gene expression in murine fibroblasts. Arthritis Rheum 48:2275–2284.

Murakami M, Kudo I. 2004. Recent advances in molecular biology and physiology of the prostaglandin E2-biosynthetic pathway. Prog Lipid Res 43:3–35.

Nickenig G, Michaelsen F, Muller C, Berger A, Vogel T, Sachinidis A, Vetter H, Bohm M. 2002. Destabilization of AT(1) receptor mRNA by calreticulin. Circ Res 90:53–58.

Papakrivopoulou J, Lindahl GE, Bishop JE, Laurent GJ. 2004. Differential roles of extracellular signal-regulated kinase 1/2 and p38MAPK in mechanical load-induced procollagen alpha1(I) gene expression in cardiac fibroblasts. Cardiovasc Res 61:736–744.

Park PJ, Cao YA, Lee SY, Kim JW, Chang MS, Hart R, Choi S. 2004. Current issues for DNA microarrays: Platform comparison, double linear amplification, and universal RNA reference. J Biotechnol 112:225–245.

Pathak SK, Bhattacharyya A, Pathak S, Basak C, Mandal D, Kundu M, Basu J. 2004. Toll-like receptor 2 and mitogen- and stress-activated kinase 1 are effectors of Mycobacterium avium-induced cyclooxygenase-2 expression in macrophages. J Biol Chem 279:55127–55136.

Prado GN, Taylor L, Polgar P. 1997. Effects of intracellular tyrosine residue mutation and carboxyl terminus truncation on signal transduction and internalization of the rat bradykinin B2 receptor. J Biol Chem 272:14638–14642.

Prado GN, Taylor L, Zhou X, Ricupero D, Mierke DF, Polgar P. 2002. Mechanisms regulating the expression, self-maintenance, and signaling-function of the bradykinin B2 and B1 receptors. J Cell Physiol 193:275–286.

Rachfal AW, Brigstock DR. 2003. Connective tissue growth factor (CTGF/CCN2) in hepatic fibrosis. Hepatol Res 26:1–9.

Ruperez M, Lorenzo O, Blanco-Colio LM, Esteban V, Egido J, Ruiz-Ortega M. 2003. Connective tissue growth factor

is a mediator of angiotensin II-induced fibrosis. Circulation 108:1499–1505.
Szymanski PT. 2004. Calponin (CaP) as a latch-bridge protein—a new concept in regulation of contractility in smooth muscles. J Muscle Res Cell Motil 25:7–19.
Takehara K. 2003. Hypothesis: Pathogenesis of systemic sclerosis. J Rheumatol 30:755–759.
Tan Y, Wang B, Keum JS, Jaffa AA. 2005. Mechanisms through which bradykinin promotes glomerular injury in diabetes. Am J Physiol Renal Physiol 288:F483–F492.
Thomas WG, Qian H, Smith NJ. 2004. When 6 is 9: 'uncoupled' AT1 receptors turn signaling on its head. Cell Mol Life Sci 61:2687–2694.
Topp WC. 1981. Normal rat cell lines deficient in nuclear thymidine kinase. Virology 113:408–411.
Tullai JW, Schaffer ME, Mullenbrock S, Kasif S, Cooper GM. 2004. Identification of transcription factor binding sites upstream of human genes regulated by the phosphatidylinositol 3-kinase and MEK/ERK signaling pathways. J Biol Chem 279:20167–20177.
Utsugi M, Dobashi K, Ishizuka T, Masubuchi K, Shimizu Y, Nakazawa T, Mori M. 2003. c-Jun-NH2-terminal kinase mediates expression of connective tissue growth factor induced by transforming growth factor-beta1 in human lung fibroblasts. Am J Respir Cell Mol Biol 28:754–761.
Watson RT, Shigematsu S, Chiang SH, Mora S, Kanzaki M, Macara IG, Saltiel AR, Pessin JE. 2001. Lipid raft microdomain compartmentalization of TC10 is required for insulin signaling and GLUT4 translocation. J Cell Biol 154:829–840.
Wilkinson-Berka JL, Fletcher EL. 2004. Angiotensin and bradykinin: Targets for the treatment of vascular and neuro-glial pathology in diabetic retinopathy. Curr Pharm Des 10:3313–3330.
Wunderlich K, Senn BC, Todesco L, Flammer J, Meyer P. 2000. Regulation of connective tissue growth factor gene expression in retinal vascular endothelial cells by angiogenic growth factors. Graefes Arch Clin Exp Ophthalmol 238:910–915.
Xie S, Sukkar MB, Issa R, Oltmanns U, Nicholson AG, Chung KF. 2005. Regulation of TGF-beta 1-induced connective tissue growth factor expression in airway smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 288:L68–L76.
Yu J, Prado GN, Taylor L, Pal-Ghosh R, Polgar P. 2002. Hybrid formation between the intracellular faces of the bradykinin B2 and angiotensin II AT1 receptors and signal transduction. Int Immunopharmacol 2:1807–1822.
Yu J, Liu B, Eramian D, Mierke D, Taylor L, Polgar P. 2004. K317, R319, and E320 within the proximal C-terminus of the bradykinin B2 receptor form a motif important for phospholipase C and phospholipase A2 but not connective tissue growth factor related signaling. J Cell Biochem 92:547–559.
Zhang X, Azhar G, Chai J, Sheridan P, Nagano K, Brown T, Yang J, Khrapko K, Borras AM, Lawitts J, Misra RP, Wei JY. 2001a. Cardiomyopathy in transgenic mice with cardiac-specific overexpression of serum response factor. Am J Physiol Heart Circ Physiol 280:H1782–H1792.
Zhang X, Chai J, Azhar G, Sheridan P, Borras AM, Furr MC, Khrapko K, Lawitts J, Misra RP, Wei JY. 2001b. Early postnatal cardiac changes and premature death in transgenic mice overexpressing a mutant form of serum response factor. J Biol Chem 276:40033–40040.
Zhao Q, Chen N, Wang WM, Lu J, Dai BB. 2004. Effect of transforming growth factor-beta on activity of connective tissue growth factor gene promoter in mouse NIH/3T3 fibroblasts. Acta Pharmacol Sin 25:485–489.
Zhou X, Prado GN, Taylor L, Yang X, Polgar P. 2000. Regulation of inducible bradykinin B1 receptor gene expression through absence of internalization and resensitization. J Cell Biochem 78:351–362.