Bradykinin induces tubulin phosphorylation and nuclear translocation of MAP kinase in mesangial cells

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Jaffa, Ayad A., Bradley S. Miller, Steven A. Rosenzweig, Padma S. Naidu, Victoria Velarde, and Ronald K. Mayfield. Bradykinin induces tubulin phosphorylation and nuclear translocation of MAP kinase in mesangial cells. Am. J. Physiol. 273 (Renal Physiol. 42): F916–F924, 1997.—Glomerular hypertension and glomerular hypertrophy act early and synergistically to promote glomerular injury in diabetes. We have previously shown that increased renal kinin production contributes to the glomerular hemodynamic abnormalities associated with diabetes. Glomerulosclerosis, characterized by mesangial cell proliferation and matrix expansion, is the final pathway leading to renal failure. The signal(s) initiating mesangial cell proliferation is ill defined. In the present study, we utilized immunofluorescence, immunoprecipitation, and immunoblotting techniques to identify substrates that are tyrosine phosphorylated in response to bradykinin in action in mesangial cells. Immunofluorescence microscopy of mesangial cells stained with anti-phosphotyrosine (anti-PY) antibodies following bradykinin treatment (10−9–10−6 M) revealed a dose-dependent increase in the labeling of cytoplasmic and nuclear proteins. Immunoprecipitation with anti-PY, followed by immunoblot revealed bradykinin-induced tyrosyl phosphorylation of tubulin and mitogen-activated protein kinase (MAPK). Confocal microscopy of mesangial cells stained for MAPK indicated that bradykinin stimulation resulted in translocation of MAPK from the cytoplasm to the nucleus by 2 h. These data demonstrate that bradykinin action results in the tyrosine phosphorylation of cellular proteins in mesangial cells and suggest a role for tubulin and MAPK in the signaling cascade of bradykinin leading to altered mesangial function.

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diabetic glomerulosclerosis, characterized by mesangial cell proliferation and extracellular matrix expansion, is thought to be the final pathway leading to end-stage renal failure (5, 23). This destructive pathway leads to focal and eventually diffuse glomerulosclerosis, independent of the initial pathogenic mechanism (5). Since hyperperfusion and hyperfiltration are associated with some of the conditions that predispose to development of these glomerular abnormalities, it was postulated that hemodynamic factors are responsible for the initiation and progression of glomerular injury (6). A number of recent observations suggest that glomerular hypertension is closely linked to the development of glomerulosclerosis (18, 39). Although glomerular hypertrophy frequently accompanies glomerular hypertension, some evidence suggests that mesangial cell expansion is the predominant factor in the development of glomerulosclerosis (11, 29, 43). Although glomerular hypertrophy and glomerular hypertension may exert independent effects, recent evidence indicates that they may also act synergistically to cause glomerular injury and accelerated sclerosis (44).

The initiating and sustaining signals that may link increased glomerular pressure and mesangial cell hypertrophy are not fully understood. With regard to the hemodynamic changes that may initiate renal injury, we have accumulated considerable evidence that supports a role for the renal kallikrein-kinin system as a mediator of hemodynamic changes occurring in diabetes. Diabetic rats with moderate hyperglycemia show increased renal and urinary excretion of active kallikrein and kinins, in conjunction with reduced renal vascular resistance and increased glomerular filtration rate (GFR) and renal plasma flow (RPF) (15). Acute treatment of these hyperfiltering diabetic rats with aprotinin, a kallikrein inhibitor, or with a B2-kinin receptor antagonist, reduced GFR and RPF and increased renal vascular resistance (15, 19). Moreover, type 1 diabetic patients with hyperfiltration show increased excretion of active kallikrein compared with diabetic patients with normal GFR (14).

A role for kinins as growth promoting factors in mesangial cell proliferation was only recently explored. Although bradykinin was shown to directly stimulate DNA synthesis and cell number in mesangial cells, the signaling mechanisms leading to mesangial cell proliferation in response to bradykinin are still undefined (9). The B2-kinin receptor is a member of the seven transmembrane G protein-coupled receptor superfamily that lacks the tyrosine kinase domain (31). Since protein tyrosine phosphorylation is one of the primary signaling events utilized by growth factors to initiate cellular responses, we initiated studies to identify substrates that are tyrosine phosphorylated in response to bradykinin in mesangial cells.

METHODS

Rat glomerular mesangial cells were prepared by a modification of the method of Lovett et al. (27). Kidneys from male Sprague-Dawley rats (75–100 g body wt) were removed under sterile conditions, and cortices were isolated, minced finely, and passed through consecutive sterilized stainless-steel sieves with pore sizes of 180, 150, 90, and 75 µm. Glomeruli collected on top of the 75-µm sieve are ≥95% pure, as assessed by light microscopy. The glomeruli were then incubated in Hanks’ balanced salt solution plus N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), pH 7.4, containing 0.3% collagenase at 37°C for 2 h to remove epithelial cells, leaving the glomerular cores containing mesangial and endo-
cultural dishes were incubated with [32P]orthophosphate (1 M, n = 6) for various times (0–30 min). Following treatment, the cells were washed with cold phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde plus 0.1% glutaraldehyde for 10 min at room temperature and permeabilized using serial dilutions of ethanol. Nonspecific binding was blocked by incubating cells for 10 min in PBS containing 1% bovine serum albumin (BSA). In studies examining the effects of bradykinin on tyrosyl phosphorylation, mesangial cells were incubated overnight at 4°C with a 1:50 dilution of primary anti-rabbit polyclonal anti-phosphotyrosine (anti-PY) antibody (33), followed by washing with PBS containing 1% BSA and incubation at room temperature for 1 h with a rhodamine-conjugated goat anti-rabbit immunoglobulin G (IgG) secondary antibody diluted 1:50 (Chemicon International). Cells were then stimulated with bradykinin at different concentrations (10⁻⁹–10⁻⁶ M, n = 6) for various time intervals (0–30 min, n = 6). Following treatment, the cells were washed with cold phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde plus 0.1% glutaraldehyde for 10 min at room temperature and permeabilized using serial dilutions of ethanol. Nonspecific binding was blocked by incubating cells for 10 min in PBS containing 1% bovine serum albumin (BSA). In studies examining the effects of bradykinin on tyrosyl phosphorylation, mesangial cells were incubated overnight at 4°C with a 1:50 dilution of primary anti-rabbit polyclonal anti-phosphotyrosine (anti-PY) antibody (33), followed by washing with PBS containing 1% BSA and incubation at room temperature for 1 h with a rhodamine-conjugated goat anti-rabbit immunoglobulin G (IgG) secondary antibody diluted 1:50 (Chemicon International). Cells were washed three times with cold PBS, 10 min each, and mounted with Fluorsave (Calbiochem, San Diego, CA) on glass slides. The stained cells were viewed under epifluorescence microscopy.

Confocal microscopy. In studies examining the effects of bradykinin on mitogen-activated protein kinase (MAPK) also known as extracellular signal-regulated kinases (ERK) nuclear translocation, mesangial cells were incubated overnight at 4°C with a 1:50 dilution of primary monoclonal anti-Pan ERK antibody (Transduction Laboratories, Lexington, KY), followed by incubation at room temperature for 1 h with a rhodamine-conjugated goat anti-mouse IgG secondary antibody diluted 1:50. The cells were washed, mounted onto slides using Fluorsave, and viewed by laser-scanning confocal microscopy on a Zeiss Axioscope (MRC 1000, Bio-Rad) using a ×63 objective.

[^32P]Phosphate incorporation and immunoprecipitation. Quiescent mesangial cells grown to subconfluence in 60-mm culture dishes were incubated with [32P]orthophosphate (1 mCi/60-mm dish) for 3 h at 37°C. After the incubation period, cells were washed with phosphate-free buffer and stimulated with bradykinin (10⁻⁷ M, n = 3) for 5 min. The cells were then washed with cold PBS and immediately frozen in liquid nitrogen. The frozen cell monolayer was scraped and solubilized in 400 µl of solubilization buffer (50 mM HEPES, pH 7.4, 10 mM sodium pyrophosphate, 100 mM NaF, 4 mM sodium orthovanadate, 1% (vol/vol) Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), 100 U/ml aprotinin), incubated for 15 min at 4°C and centrifuged at 3,000 revolutions/min (rpm) for 10 min. The supernatant was harvested as the cytosolic fraction that was subjected to immunoprecipitation.

The cytosolic fraction was treated with 50 µl of Pansorbin (Calbiochem) suspension for 1 h at 4°C and centrifuged at 13,000 rpm for 3 min, and the resulting supernatant was incubated with anti-tubulin and or anti-Pan ERK antibodies for 18 h at 4°C. The immunocomplex was recovered by addition of 50 µl of Pansorbin at 4°C for 1 h, followed by centrifugation at 13,000 rpm for 3 min. The pellet obtained was resuspended in 500 µl of low-salt wash [50 mM HEPES, pH 7.4, 0.1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA, 0.15 M NaCl, 0.05% NaN₃], centrifuged at 13,000 rpm for 3 min, followed by sequential high-, low-, and near-maximal salt washes. After the final wash and centrifugation, the antigen was dissociated from the antibody by boiling for 3 min in sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions.

Immunoblotting. Quiescent mesangial cells stimulated with bradykinin (10⁻⁸ M, n = 3) for 5 min were suspended in 250 µl of lysis buffer [20 mM tris(hydroxymethyl)aminomethane, 130 mM NaCl, 10% glycerol, 10 mM 3-[3-cholamidopropyl]di-methylammonio]-1-propanesulfonate, 1 mM PMSF, 2 mM sodium vanadate, 100 µM/ml aprotinin, 0.15 mg/ml benzamidine, pH 8.0] sonicated for 10 s and centrifuged at 13,000 g for 10 min. A quantity of 25–30 µg of the cytosolic fraction was analyzed by SDS-PAGE, and the separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes and immunoblotted with either a recombinant anti-PY RC 20 H antibodies (1:5,000 dilution, Transduction Laboratories) or with anti-phospho-MAPK polyclonal antibody (1:1,000 dilution; New England BioLabs, Beverly, MA). Immunoreactive bands were visualized using the enhanced chemiluminescence reagent (ECL, Amersham) according to the procedure described by the supplier. The protein concentration in the cytosolic fractions was determined by the assay of Lowry et al. (28).

RESULTS

Bradykinin stimulates tyrosyl phosphorylation of mesangial cells: immunocytochemical studies. Protein phosphorylation reactions play a pivotal role in amplifying and disseminating incoming signals throughout the cell. To examine the pattern of tyrosyl phosphorylation in response to bradykinin, quiescent mesangial cells grown on coverslips were stimulated with bradykinin (10⁻⁶ M) for various times (0–30 min). The cells were fixed, permeabilized, and incubated overnight at 4°C with a 1:50 dilution of primary anti-PY antibody, followed by incubation at room temperature for 1 h with a rhodamine-conjugated secondary antibody diluted 1:50. Cells were viewed under epifluorescence microscopy with a ×63 objective. Bradykinin produced a rapid and intense tyrosyl phosphorylation of cytoplasmic and nuclear proteins within 1 min of stimulation, decreasing in intensity over 30 min (Fig. 1). Tyrosine-phosphorylated filaments spanning the entire cytoplasmic domain were observed in the bradykinin-stimulated cells. Bradykinin produced a dose-dependent increase in tyrosyl phosphorylation with a significant immunofluorescence occurring with 10⁻⁷ M and near maximal response at 10⁻⁶ M (Fig. 2, B–E). No fluorescent staining was detected using secondary antibodies in the absence of primary antibodies (Fig. 2, F).

To identify the tyrosine-phosphorylated filaments observed in response to bradykinin, double immunolabeling experiments were carried out. Since microtubules are mainly composed of tubulin polymers, mesangial cells treated with bradykinin (10⁻⁸ M) for various times were stained with polyclonal anti-PY and monoclonal anti-tubulin primary antibodies. Primary anti-
bodies were then detected with rhodamine-conjugated goat anti-rabbit (PY) and fluorescein-conjugated goat anti-mouse (tubulin) secondaries. As shown in Fig. 3, bradykinin stimulated the tyrosine phosphorylation of filamentous structures. The same cytoskeletal elements stained with anti-PY were also stained with anti-tubulin antibodies, indicating microtubules are tyrosyl phosphorylated in response to bradykinin stimulation.

Immunoprecipitation and immunoblotting studies. To further characterize the cytoplasmic and nuclear proteins that were tyrosyl phosphorylated in response to bradykinin, quiescent mesangial cells grown in 10-cm dishes were stimulated with bradykinin ($10^{-8}$ M) for 5 min. Cell lysates (500 µg protein) were preincubated for 5 min with Pansorbin, followed by an overnight immunoprecipitation with 5 µg of anti-PY antibodies at 4°C. The immunocomplex was resolved by SDS-PAGE, and the separated proteins were transferred to PVDF membranes and immunoblotted with recombinant anti-PY RC 20 H antibodies. The labeled proteins...
were visualized using the ECL reagent. Addition of bradykinin to mesangial cells resulted in a rapid increase in tyrosine phosphorylation of several proteins (Fig. 4).

To identify whether the 56- and 42-kDa protein bands that were tyrosine phosphorylated in response to bradykinin were tubulin and MAPK, respectively, mesangial cells were labeled with [32P]orthophosphate and were stimulated with bradykinin (10⁻⁷ M) for 5 min. Cytoplasmic and membrane fractions were prepared as described above and subjected to selective immunoprecipitation with anti-MAPK antibodies and anti-tubulin antibodies, respectively. Bradykinin stimulated the phosphorylation of both the 42- and 44-kDa forms of MAPK in the cytoplasmic fraction and tubulin in the membrane fraction, indicating that both MAPK and tubulin may serve as substrates for bradykinin-stimulated tyrosine phosphorylation in mesangial cells (Fig. 5, A and B).
Activation and nuclear translocation of MAPK by bradykinin. The MAPK signal transduction pathway represents an important mechanism by which growth factors regulate cell growth. To examine whether bradykinin stimulates the tyrosyl phosphorylation of MAPK, mesangial cells were treated with bradykinin (10^(-8) M) for 5 min (Fig. 6). Bradykinin resulted in tyrosyl phosphorylation and, hence, activation of MAPK.

The nuclear consequences of ERK translocation include activation of the transcription factor Elk-1, which results in production of c-fos leading to activation of the AP-1 complex (37, 12). For this to occur, ERK must translocate to the nucleus. We utilized immunocytochemistry and confocal microscopy to examine whether bradykinin induces nuclear translocation of ERK. Figure 7, A and B, represents an optical section through formation of fibrous filaments. The same cytoskeletal structures that stain for anti-phosphotyrosine also stain for anti-tubulin, identifying tubulin as one of the microtubule proteins that is phosphorylated in response to bradykinin. Experiment was repeated 3 times with similar results.

Fig. 3. Tyrosyl phosphorylation of microtubules by bradykinin. Double immunofluorescence labeling study. Mesangial cells grown on coverslips were stimulated with bradykinin (1 µM) for various 5 min (C and D) and 30 min (E and F) (A and B are 5 min controls). Cells were fixed and incubated overnight with primary anti-phosphotyrosine (left: A, C, and E) and anti-tubulin antibodies (right: B, D, and F), followed by incubation with rhodamine- and fluorescein-conjugated secondary antibodies. Addition of bradykinin resulted in tyrosyl phosphorylation of formation of fibrous filaments. The same cytoskeletal structures that stain for anti-phosphotyrosine also stain for anti-tubulin, identifying tubulin as one of the microtubule proteins that is phosphorylated in response to bradykinin. Experiment was repeated 3 times with similar results.
the nucleus of mesangial cells treated with vehicle or bradykinin (10^{-7} M) for 2 h, respectively. In unstimulated cells, ERK localization was entirely cytoplasmic and concentrated in the perinuclear region of the cells. Treatment of mesangial cells with bradykinin resulted in the nuclear translocation of MAPK, based on the

Fig. 4. Immunoprecipitation and immunoblotting studies. To characterize the proteins that are tyrosyl phosphorylated in response to bradykinin, quiescent mesangial cells were stimulated with bradykinin (BK, 10^{-8} M; C, control) for 5 min. Cell lysate was analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were transferred to polyvinylidene difluoride membranes and immunoblotted with recombinant anti-PY RC 20 H antibodies. The tyrosine-phosphorylated bands were visualized using the enhanced chemiluminescence (ECL, Amersham) reagent. Addition of bradykinin to mesangial cells resulted in a rapid increase in tyrosine phosphorylation of several protein substrates. Arrows, positions of mitogen-activated protein kinase (MAPK) and tubulin. Blot is representative of 3 separate experiments.

Fig. 5. Tubulin (A) and MAPK (B) are phosphorylated by bradykinin. Mesangial cells labeled with ^{32}P were stimulated with bradykinin (10^{-8} M) for 5 min. Cell lysates were selectively immunoprecipitated with anti-tubulin and anti-MAPK antibodies, and immunocomplexes were resolved by SDS-PAGE. Bradykinin significantly increased phosphorylation of tubulin and MAPK compared with control or insulin-like growth factor-I (IGF-I)-stimulated cells. Blots are representative of 3 separate experiments.

Fig. 6. Bradykinin stimulates tyrosine phosphorylation of MAPK. Cell lysates (30 µg) from mesangial cells treated with vehicle (control) or bradykinin (BK, 10^{-7} M) for 5 min were immunoblotted using an anti-phospho-MAPK antibody. Bradykinin produced a 2.5-fold increase in MAPK phosphorylation. Blot is a representative of 3 separate experiments. *P < 0.05 vs. control.
intense immunofluorescent staining within the nucleus (Fig. 7B). No fluorescence was detected in mesangial cells treated with bradykinin in the absence of primary antibody (Fig. 7C).

DISCUSSION

Growth factor receptor activation initiates biochemical cascades or signal transduction pathways that ultimately lead to changes in gene expression and induction of the mitogenic response. Identification of the signaling pathways is crucial to understanding the regulatory mechanisms involved in cell growth. Protein phosphorylation and dephosphorylation appears to be a primary signaling mechanism utilized by many growth factor receptor families that are linked to protein tyrosine kinases and those that are coupled to G proteins (34, 42). In the present study, we demonstrate that bradykinin stimulates the tyrosyl phosphorylation of a number of proteins in mesangial cells. Immunocytochemical and biochemical evidence revealed that microtubules are tyrosyl phosphorylated on tubulin in response to bradykinin. Bradykinin also induces the activation and nuclear translocation of MAPK in mesangial cells. These findings provide the initial evidence that bradykinin stimulates early growth events in mesangial cells.

The mechanism(s) through which bradykinin stimulates tyrosyl phosphorylation is as yet undefined. The B2-kinin receptor is a member of the seven transmembrane G protein-coupled receptor superfamily that lacks the tyrosine kinase homology region (31). Upon binding to its receptors in mesangial cells, bradykinin activates phospholipase C via a heterotrimeric GTP-binding protein and induces a marked increase in inositol 1,4,5-trisphosphate, leading to increases in intracellular calcium concentrations (2, 24). Rises in intracellular calcium have been shown to modulate bradykinin-induced mesangial cell contraction (3). In this regard, contraction of mesangial cells in vitro has been shown to activate specific signaling events leading to induction of early growth gene expression and activation of protein kinase C and S6 kinase (1, 17). Moreover, increases in intracellular calcium have been shown to activate MAPK via a calcium/calmodulin-dependent and-independent mechanisms (10). Whether the activation of MAPK we observed in mesangial cells in response to bradykinin stimulation is a result of mechanical forces leading to increases in intracellular calcium and, hence, activation of calmodulin is yet to be determined.

A number of recent observations have shown that bradykinin induces tyrosyl phosphorylation of proteins in a variety of cells (8, 25, 26). Our results demonstrate that bradykinin stimulates the tyrosyl phosphorylation of tubulin and MAPK in mesangial cells. The initiating cytoplasmic tyrosine kinase that promotes tyrosine phosphorylation of tubulin and MAPK in response to bradykinin is as yet undefined. However, our preliminary data in vascular smooth muscle cells indicate that bradykinin results in the activation and association of

Fig. 7. Confocal immunofluorescence microscopy showing bradykinin-induced MAPK nuclear translocation. Mesangial cells grown on coverslips were stimulated with bradykinin \((10^{-7} \text{ M})\) for 2 h. Cells were fixed, permeabilized, and incubated overnight with a 1:50 dilution of primary monoclonal anti-Pan ERK antibody, followed by incubation with a rhodamine-conjugated secondary antibody diluted 1:50. Stained cells were viewed by laser-scanning confocal microscopy. Optical sections are through the nucleus. Vehicle-treated (A), bradykinin-treated (B), and bradykinin-treated cells with no primary antibody added (C). MAPK staining in A is entirely cytoplasmic and perinuclear. However, treatment of mesangial cells with bradykinin resulted in nuclear translocation of MAPK within 2 h. This is evidenced by intense immunofluorescence staining observed within the nucleus (B). No immunofluorescence was detected in absence of primary antibody (C).
pp60-src with focal adhesion kinase and growth factor receptor binding protein-2 (40). Activation of pp60-src in response to growth factor stimulation results in its redistribution from the plasma membrane to the cytosol, where it may colocalize with microtubule-associated structures (21, 41). Recently it was shown that pp60-src phosphorylates tubulin in vivo (30). Tubulin is the main component of microtubules which form the mitotic spindle, and once phosphorylated, it is thought to play a regulatory role in cell growth and division (13, 30).

A large body of evidence indicates that MAPK plays a pivotal role in transmitting mitogenic signals in response to growth factors (4, 7). In the present study, we have shown that bradykinin induces tyrosyl phosphorylation and nuclear translocation of MAPK in mesangial cells, thus providing a link in the signal transduction pathway from the cytoplasm to the nucleus. Once in the nucleus, MAPK can phosphorylate and activate transcription factors such as TCF/ELK-1 resulting in c-Fos production, which in turn modulates transcription of other target proteins via AP-1 complex formation (12, 22).

The mechanisms through which G protein-coupled receptors like the bradykinin B₂-receptor regulate MAPK are under intense investigation. MAPK activation by bradykinin B₂-receptors may occur via a Ras-dependent or independent pathway by utilizing a pertussis toxin-sensitive or-insensitive G protein. In the case of receptors with intrinsic tyrosine kinase activity, several steps in the signal transduction leading to MAPK activation have been elucidated. Interaction of growth factors with their respective receptors activates intracellular tyrosine kinase domains, which result in the phosphorylation of Shc followed by its association with adaptor protein GRB2, which in turn promotes the interaction of guanine nucleotide exchange factor mSOS with Ras leading to its activation. Activated Ras in turn activates Raf-1, which in turn phosphorylates and activates MEK, ultimately leading to phosphorylation and activation of MAPK (36). Whether bradykinin activates MAPK via protein kinase C or by Ras activation or via the calcium/calmodulin pathway remains to be established.

The mechanism by which bradykinin induces nuclear translocation of MAPK is not clearly identified. Since MAPK lacks a canonical nuclear localization sequence, a number of alternative mechanisms have been proposed to explain this phenomenon (14). In addition, recent evidence indicates that the cytoskeletal microtubules may play a role in nuclear translocation of MAPK. First, MAPK was shown to be associated with cytoskeletal microtubules, and separation of the microtubule-bound MAPK from the cytoskeleton prevented its nuclear translocation in response to agonist stimulation (35). Second, stabilization of microtubules by taxol treatment prevented bradykinin-induced nuclear translocation of MAPK (20).

In summary, the findings of the present study demonstrate that bradykinin results in tyrosyl phosphorylation of a number of proteins in mesangial cells and suggest a role for tubulin and MAPK in the bradykinin signal transduction pathway leading to altered mesangial cell function. The significance of these findings to the in vivo actions of bradykinin on glomerular function is unclear at the present time. Since the in vivo actions of bradykinin on glomerular function are modulated by various autacoids such as nitric oxide and eicosanoids (38), the contribution of these autacoids on the signaling mechanisms initiated by bradykinin in mesangial cells needs to be addressed.

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