Rapid Transactivation of the Vascular Endothelial Growth Factor Receptor KDR/Flk-1 by the Bradykinin B2 Receptor Contributes to Endothelial Nitric-oxide Synthase Activation in Cardiac Capillary Endothelial Cells*

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Bradykinin (BK) and vascular endothelial growth factor (VEGF)-165 stimulate vasodilatation, microvascular permeability, and angiogenesis via the activation of the B2-type and KDR/Flk-1 receptors. To delineate the signal transduction pathways distal to the receptor activation in microvascular permeability, we compared their effects on two downstream targets, i.e. endothelial nitric-oxide (NO) synthase (eNOS) and F-actin, in primary cultures of cardiac capillary endothelial cells. The two mediators induced a similar cytoskeletal reorganization and both the translocation and activation of eNOS, leading to NO release within the first minutes of cell exposure. At the same time, BK produced the tyrosine phosphorylation and internalization of KDR/Flk-1 as did VEGF itself. This transactivation was blocked by the selective inhibitor of VEGF receptor tyrosine kinase activity but not by inhibitors of epidermal growth factor receptor or protein kinase C activity. The selective inhibitor of VEGF receptor tyrosine kinase activity totally prevented the effects of VEGF but only partially inhibited NO release induced by BK without affecting the concomitant cytoskeletal reorganization. Thus, BK transactivated KDR/Flk-1 through an intrinsic kinase activity of KDR/Flk-1, resulting in a further eNOS activation in endothelial cells. This represents a novel mechanism whereby a G protein-coupled receptor activates a receptor tyrosine kinase to generate biological response.

Activation of phospholipase C generates inositol trisphosphate and diacylglycerol, causing intracellular Ca2+ mobilization and protein kinase C (PKC) activation. The consecutive productions of NO and prostacyclin (PGI2) mediate vasopermeability, angiogenesis, and anti-hyperplasia of neointimal endothelial cells. In addition to this well recognized Gq/11-activating feature, B2R induces several signaling events commonly evoked by growth factor receptors, such as activation of mitogen-activated protein kinases (MAPK/ERK) and expression of nuclear proto-oncogenes, c-fos and c-jun (5). Although B2R lacks intrinsic tyrosine kinase activity, it stimulates rapid tyrosine phosphorylation of multiple signaling proteins including focal adhesion kinases, paxillin, phosphatidylinositol 3-kinase (PI3K) and phospholipase Cγ (6–10). Interestingly, most of these signals are known to mediate diverse cellular actions of VEGF via the KDR/Flk-1 receptor, a member of the receptor tyrosine kinase (RTK) family (2, 11–17). KDR/Flk-1 mediates most biological functions of VEGF such as cell survival, proliferation, the generation of NO and prostacyclin, increased microvascular permeability, and angiogenesis (2, 11, 13, 18–20). However, important aspects of KDR/Flk-1 signal transduction remain to be elucidated. For instance, KDR/Flk-1 induces a rapid/transient form of microvascular hyperpermeability as does BK, but it also promotes a chronic form of hyperpermeability, which has been reported to be a prerequisite for angiogenesis (11, 18). No specific intracellular mechanism has yet been found to mediate one or the other form of regulation. Interestingly, NO is also reported to modulate vascular tone, permeability, and capillary EC growth (21–24). Furthermore, VEGF up-regulates eNOS enzyme and elicits both an acute and chronic stimulation of NO production (23, 24), which in turn activates the MAPK/ERK cascade and up-regulates VEGF expression, indicating the existence of a positive feedback loop between these two factors. In addition, the eNOS activity is regulated by Ca2+-calmodulin binding and diverse protein kinases of the signaling cascades following KDR/Flk-1 and B2R activation (25).

Here we compared the respective effects of BK and VEGF on two downstream targets regulating EC barrier, i.e. cytoskeleton and eNOS, in primary cultures of cardiac capillary EC that express B2R and only one type of VEGF receptor, KDR/Flk-1 (26). Our results indicated that some but not all actions of BK were mediated by transactivation of the KDR/Flk-1 receptor for VEGF.

EXPERIMENTAL PROCEDURES
This work conforms to institutional guidelines (License 03387 from the Ministère Français de l’Agriculture).
Materials—VEGF-165 was purchased from Upstate Biotechnology. BK was from Sigma. Specific inhibitors of EGFR receptor (tyrphostin or AG1478) and VEGF receptor tyrosine kinases (4-[(4′-chloro-2′-fluoro)
phenyllalimeno-6,7-dimethoxy-quinazoline; TKi), and inhibitors of PI3K (wortmannin) and PKC (Ro31–8220) activities, were obtained from Calbiochem. All inhibitors were used at concentrations known to block completely these activities in various cell types.

Cell Culture—Left ventricular capillary cells were isolated from adult rat hearts by retrograde perfusion of an enzymatic solution (collagenase type II; Worthington) as described previously (26). Cells were plated onto laminin (5 μg/ml)-coated glass coverslips in the standard culture medium M199 containing 10% fetal calf serum (Bio-Media), 200 units/ml streptomycin-penicillin, and 100 units/ml kanamycin (Invitrogen). Cultures were used 10 days post-isolation and were incubated for 1 day in 0% fetal calf serum culture medium before testing mediators and inhibitors. These standard culture conditions were also used for protein extraction and nitrite dosage.

**Nitrite Release in Cell-conditioned Medium**—Culture supernatants were assayed for nitrite using the Griess reaction. Media were centrifuged at 1500 × g for 15 min at 4 °C to remove cellular debris and added to a 1:1 (v/v) Griess reagent (Sigma). A standard curve was constructed by use of known concentrations of sodium nitrite over the linear range (0.003 to 300 μM nitrite).

**Immunofluorescence Microscopy**—Cells were fixed in 4% formaldehyde and then permeabilized with 0.1% Triton X-100 at 23 °C. Actin microfilaments were stained with 50 μg/ml tetramethylrhodamine isothiocyanate-phalloidin for 40 min. For other staining, cells were incubated with the primary antibody for 1 h at 23 °C or overnight at 4 °C and then with the secondary antibody (Fab′)2 fragment of IgG conjugated with Texas Red or fluorescein isothiocyanate (Jackson Immunoresearch Laboratories) for another 1-h period. The primary antibodies were a polyclonal anti-eNOS antibody (1:100; Sigma), a monoclonal anti-KDR/Flk-1 antibody (1:1000; Upstate Biotechnology), and a polyclonal anti-caveolin-1 antibody (1:1000; Transduction Laboratories). Specificity of labeling was demonstrated by performing cell labeling with non-immune IgG instead of the primary antibodies. Observations were performed on a Leica laser confocal microscope. Series of eight optical sections were collected using a standard scanning mode format (1024 × 1024 pixels).

**Detection of KDR/Flk-1 Tyrosine Phosphorylation**—After a 1-day period in 0% fetal calf serum culture medium, cell cultures were preincubated with or without inhibitors for 30 min, stimulated with mediator for 5 min at 37 °C, and then scraped in a sonication buffer containing (in mM): 20 Tris, 20 EDTA, 1 phenylmethylsulfonyl fluoride, 10 sodium fluoride, 100 sodium vanadate, plus 1 μg/ml each of leupeptin and pepstatin (pH 8). Total homogenate was centrifuged for 10 min at 3000 × g and 4 °C. Protein content of the resulting supernatant (i.e. cellular fraction without nucleus) was determined using a Bio-Rad assay kit. Equal amounts of protein (200 μg/ml) were boiled for 5 min before use. For immunoprecipitation, samples were precleared with protein G-Sepharose beads and incubated with the monoclonal anti-phosphotyrosine (clone 4G10; Upstate Biotechnology) conjugated to Sepharose beads overnight at 4 °C. The samples were analyzed on 8% SDS-PAGE, electrophoretically transferred onto HybondTM ECL (Amersham Biosciences, Inc.). After blocking with 5% bovine serum albumin for 1 h at 23 °C, membranes were reacted with the monoclonal anti-KDR/Flk-1 (2 μg/ml; Transduction Laboratories) overnight at 4 °C. The blots were washed and then incubated with horseradish peroxidase-conjugated secondary antibodies (1:1000; Jackson Immunoresearch Laboratories) for 1 h at 23 °C. After washing, the signal was detected using a chemiluminescence ECL detection kit (Amersham Biosciences, Inc.).

**Statistical Analysis**—Optical densities of the dominant (220 kDa) band corresponding to the mature form of receptor KDR/Flk-1 were quantified using NIH Image 1.61. The optical density values of protein bands obtained from drug-treated samples were normalized to their respective control values. Results were expressed as mean ± S.E. from n different experiments. Tests of significance were made using analysis of variance (ANOVA; Statview Software). p values < 0.05 were considered significant.

**RESULTS AND DISCUSSION**

**Cytoskeletal Reorganization and Fenestration of Capillary EC Monolayers**—Cultured capillary EC express the BK receptor B2R and only one type of VEGF receptor, KDR/Flk-1 (26). Cell treatment with BK and VEGF resulted in a time-dependent reorganization of the cytoskeleton. Fig. 1A shows the F-actin pattern of EC treated for various times with BK (10 nM). Untreated cells exhibited a distinct band of actin at the cell periphery. By 3 min of BK treatment, the density of peripheral bands increased, and some intercellular openings appeared between adjacent cells as reported in EC in vivo. Indeed, agonist-elicted hyperpermeability results in a contraction process occurring at the margins of cells rather than in their general contraction (27). By 10 min of BK treatment, a large increase in the amount of F-actin is evident at the cell periphery. By 30 min of BK treatment, actin fibers (stress fibers) were prominent throughout the cell and often appeared to abut with colinear filaments from adjacent cells. These effects were insensitive to cell pre-treatment with pertussis toxin (200 ng/ml) for 1 day (not shown). VEGF (50 ng/ml) rapidly induced cytoskeletal changes and formation of intercellular openings between adjacent cells, as did BK within the first 10 min of cell exposure (see Fig. 1A). However, the increase in peripheral actin band was maintained for at least 2 h with VEGF (Fig. 1B).

**Translocation and Activation of eNOS**—Numerous reports demonstrate a role for NO not only in agonist-elicted microvascular hyperpermeability (3, 18) but also in both migration and proliferation promoted by VEGF (16, 20, 24, 28). The potency of BK and VEGF to activate eNOS is compared in Fig. 2A. Exposure of confluent cultures to BK (10 nM) or to VEGF (50 ng/ml) led to a rapid increase in eNOS activity as assessed by measuring nitrite contents of cell-conditioned media. Within the first 10 min of cell exposure, the BK-induced nitrite production was about 2-fold higher than that induced by VEGF (from 0.09 ± 0.04 μM in control to 1.53 ± 0.08 μM in BK and 0.76 ± 0.34 μM in VEGF; p < 0.01 control versus agents; n = 10). Following 2 h of cell exposure, no significant difference in the nitrite content was observed between media from BK- and VEGF-treated cells (from 0.13 ± 0.04 μM in control to 1.93 ± 0.11 μM in BK and to 2.02 ± 0.24 μM in VEGF; p < 0.05 10 min versus 2 h for each condition; n = 10; not shown). Our data support the view that BK causes a rapid and transient activation of eNOS leading to a short-term NO production, whereas VEGF initiates a delayed and sustained NO production in association with its prolonged hyperpermeabilizing effect on EC monolayers. Short-term NO production involves activation of eNOS by PKC and Ca2+ mobilization (1, 11, 25, 29, 30). Recent evidence indicates that B2R and KDR/Flk-1 may also activate eNOS via the PI3K/Akt pathway (10, 14, 16, 31, 32). Therefore, we used specific inhibitors of PKC and PI3K to define their respective contribution to the initial phase of nitrite release. As shown in Fig. 2A, wortmannin (10 nM), a specific inhibitor of PI3K, attenuated slightly but significantly...
the nitrite production after 10 min of cell stimulation by either BK or VEGF ($p < 0.5; n = 10$). Ro31–8220 (30 μM), an inhibitor of PKC, completely suppressed this production ($p < 0.01; n = 10$). Thus, undefined isoforms of PKC contribute to a major part to the initial eNOS activation induced by both BK and VEGF in our cell model. The contribution of PI3K is less.

eNOS activity is known to be regulated by its interaction with the caveolar structural protein caveolin-1 (13, 29, 30, 33). We therefore documented actions of BK and VEGF on the subcellular distribution of eNOS and caveolin-1 (Fig. 2B). Under basal conditions, a weak labeling for eNOS was observed at the cell periphery and in the nuclear region. The caveolin-1 labeling was found within plasma membrane and intracellular compartments. Exposure to BK or VEGF induced the translocation of both eNOS and caveolin-1 to the perinuclear region of EC within 10 min. Following 1 h of stimulation with BK, both eNOS and caveolin-1 rapidly cycled between plasma and Golgi membranes as reported previously by others (30). In contrast, eNOS remained around cell nuclei after treatment with VEGF (not shown). Thus, BK and VEGF induce the translocation of both eNOS and caveolin-1 inside EC. Their actions, however, differ in that the effects of BK were transient whereas those of VEGF were sustained. Note that VEGF specifically maintained eNOS in the perinuclear region (13, 24, 28, 34) in association with its prolonged contracting effect on EC (Fig. 1B). Such a differential regulation may reflect the rapid/transient and chronic forms of VEGF-induced microvascular permeability to promote inflammatory and growth-promoting signals, respectively (11, 18).

Transactivation and Internalization of KDR/Flk-1 by B2R—Caveolae are enriched in various signaling molecules such as B2R, KDR/Flk-1, and PKC (9, 33). We therefore analyzed the subcellular distribution of KDR/Flk-1 (Fig. 3A). Under basal conditions, most of KDR/Flk-1 labeling was located in the plasma membrane and nuclear region, forming a fluorescent pattern similar to that observed with eNOS in quiescent cells (see Fig. 2B and Fig. 3A). Stimulation for 10 min with either VEGF or BK resulted in the total disappearance of KDR/Flk-1 labeling at the cell periphery. A weak KDR/Flk-1 labeling was found in the nuclear region. Western blotting of whole-cell extracts (without nucleus) from treated EC revealed a 30% decrease in the amount of KDR/Flk-1. The optical density values relative to controls were 0.66 with BK and 0.68 with VEGF ($p < 0.05$ control versus agonists; $n = 6$). Thus, BK and VEGF induced internalization of KDR/Flk-1, in addition to eNOS and caveolin-1 (13, 29, 35). KDR/Flk-1 is activated through ligand-stimulated receptor dimerization and phosphorylation of tyrosine residues in the cytoplasmic kinase domain. Fig. 3B shows that VEGF and BK induced the tyrosine phosphorylation of KDR/Flk-1. The immunoprecipitation with 4G10 followed by blotting with the anti-KDR/Flk-1 antibody revealed a dominant (230 kDa) band corresponding to the mature form of the phosphorylated receptor in BK- and VEGF-treated cells. The phosphorylation of KDR/Flk-1 was detectable after 1 min of stimulation (peaking at 5–10 min) and was increased by higher concentrations of BK (>100 nM). It must be noticed that no band (at 230 kDa) was
observed in control conditions excluding a basal activation by endogenous VEGF (n = 8). Recently, it has been reported that VEGF expression is up-regulated by Ras and Src, through PKC and PI3K pathways (36). Because BK activates these pathways, it may be suspected that the BK induces KDR/Flk-1 phosphorylation secondarily to secretion of VEGF. However, the transcriptional regulation of VEGF is reported to release VEGF only after 20 min of cell stimulation whereas the BK-induced transactivation of KDR/Flk-1 occurs within 1 min. In addition, we observed that the cell pretreatment for 15 min with 10 μM anti-VEGF monoclonal antibody (Genentech) to prevent the possible release of VEGF (37) did not suppress the effect of BK (n = 2; not shown). Thus, it is unlikely that BK induces KDR/Flk-1 phosphorylation via an autocrine loop.

Recently, ligand-independent tyrosine phosphorylation of RTK, such as EGFR or platelet-derived growth factor receptor, has been suggested to mediate MAPK/ERK activation by GPCR (38–40). The molecular mechanism is still poorly understood, and it remains unclear whether PI3K and PKC stimulate the kinase activity of EGFR or suppress a tyrosine phosphatase activity. Therefore, we used specific inhibitors of PI3K, PKC and KDR/Flk-1 tyrosine kinase, to determine which of those intermediates might be involved in BK-induced phosphorylation of KDR/Flk-1. The VEGF-induced KDR/Flk-1 phosphorylation was blocked by submicromolar concentrations of TKi, a selective inhibitor of VEGF RTK activity (41). The BK-induced KDR/Flk-1 phosphorylation was also blocked by TKi, but complete inhibition required 10-fold higher concentrations (10 μM; see Fig. 3B). A similar phenomenon was described for the inhibitory effect of AG1478, a selective inhibitor of EGFR RTK activity, on the EGFR phosphorylation by GPCR agonists (5, 42–45). This could be attributed to an autophosphorylation-independent mechanism induced by GPCR and distinct from RTK signaling itself. Cell pretreatment by wortmannin (10 nM) induced a slight but significant reduction of the BK-induced transactivation of KDR/Flk-1 suggesting contribution of PI3K (not shown). However, neither AG1478 (100 nM) nor Ro31–8220 (30 μM) had any effect on BK-induced transactivation. Thus, our data suggest that BK induces tyrosine phosphorylation of KDR/Flk-1 mainly through an intrinsic kinase activity of KDR/Flk-1.

BK-induced eNOS Activation Involves Both PKC and KDR/Flk-1 Tyrosine Kinase Activities—Contribution of KDR/Flk-1 transactivation to nitrite production and/or F-actin rearrangement induced by BK was examined (Fig. 4). Whereas AG1478 (100 nM) had no inhibitory effect on NO release, TKi (10 μM) reduced the effect of BK by about 30% (from 1.53 ± 0.08 to 1.00 ± 0.22; n = 8; p < 0.05). Because TKi totally inhibited effects of VEGF, the incomplete inhibition of BK-induced NO release indicates that eNOS activation is not exclusively mediated by activation of KDR/Flk-1. The alternative activation signal(s) may involve other transducers such as PI3K/Akt and PKC. Indeed, the BK-induced NO release was inhibited slightly by wortmannin (10 nM) and was totally prevented by Ro31–8220 (30 μM; see Fig. 2A). Furthermore, Ro31–8220 (30 μM) totally blocked the VEGF-induced NO release but did not affect tyrosine phosphorylation of KDR/Flk-1 induced by VEGF itself. We concluded that PKC activity occurs downstream of the receptor activation. Our data are in agreement with the literature concerning the VEGF-stimulated eNOS expression that occurs via activation of KDR/Flk-1 and is inhibited by selective PKC inhibitors within the first 10 min of cell exposure (11, 25).

As shown in Fig. 1, exposing cells to BK or VEGF for 10 min leads to F-actin rearrangement and openings between adjacent cells. With 10 μM TKi pretreatment for 30 min, this actin rearrangement was totally prevented in VEGF-treated cells whereas it still occurred in response to BK (Fig. 4B). Thus, BK-induced tyrosine phosphorylation of KDR/Flk-1 was not involved in the cytoskeletal remodeling and cell shape changes. Such a selective inhibition has been reported previously for AG1478, which inhibited the MAPK activation and protein synthesis related to GPCR-induced transactivation of EGFR but did not affect the intracellular Ca2+ mobilization or the tyrosine phosphorylation of focal adhesion kinase and paxillin by GPCR agonists (42, 43). Our data suggest that B2R and KDR/Flk-1 use separate pathways to regulate EC permeability (4, 27), although VEGF activation of KDR/Flk-1 leads to rapid changes in cytoskeleton similar to those elicited by the BK activation of B2R.

Conclusions—our study demonstrates that the bradykinin receptor B2R transactivates the VEGF receptor KDR/Flk-1 in cardiac capillary EC. Although our pharmacological results suggest the involvement of an intrinsic kinase activity and a small contribution of PI3K/Akt pathway, the detailed mechanism whereby BK-activated B2R causes tyrosine phosphorylation of KDR/Flk-1 remains unknown. This KDR/Flk-1 transactivation results in rapid internalization of KDR/Flk-1 as does VEGF itself and a supplementary NO production mainly targeted into the nuclear compartment. Previously, the signals generated by RTK and GPCR were thought to be neatly com-

![Fig. 4. BK-induced transactivation of KDR/Flk-1 contributes to NO release. Shown are contributions of KDR/Flk-1 transactivation by BK to NO production and cytoskeletal reorganization. Cultures were incubated for 1 day in 0% fetal calf serum and then pretreated for 30 min with or without the inhibitor of VEGF receptor kinase activity (TKi; 10 μM) prior to stimulation by 10 nM BK or 50 ng/ml VEGF for 10 min. A, bar graph showing the nitrite accumulation in culture supernatants induced by the two mediators. Data are mean ± S.E. (n = 8; *, p < 0.05 control versus agonists). Shown are confocal images of capillary EC stimulated with agonists for 10 min and then labeled with antibody against eNOS (bar, 15 μM). TKi totally prevents the VEGF-induced translocation of eNOS whereas it partially inhibits that induced by BK. Confocal micrographs are representative of ten separate experiments. B, TKi totally prevent the cytoskeletal reorganization induced by VEGF but did not affect that induced by BK. F-actin was visualized with tetramethylrhodamine isothiocyanate-phalloidin (bar, 15 μM). Confocal micrographs are representative of ten separate experiments. Note the similar pattern of immunoreactivity in control and VEGF-treated cells after pretreatment with TKi.](http://www.jbc.org/content/386/4/2031/F4)
partmentalized, with very little cross-talk between these pathways. Here we show that a GPCR possess a high capacity for cross-talk with an RTK, other than EGFR or PDGFR, by using the second receptor itself as a signaling platform to amplify cell response. To our knowledge, this represents a novel mechanism whereby a GPCR activates an RTK to generate a biological activity different from the mitogenic action of GPCR.

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