Bradykinin activates the Janus-activated kinase/signal transducers and activators of transcription (JAK/STAT) pathway in vascular endothelial cells: localization of JAK/STAT signalling proteins in plasmalemmal caveolae

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Bradykinin (BK) is an important physiological regulator of endothelial cell function. In the present study, we have examined the role of the Janus-activated kinase (JAK)/signal transducers and activators of transcription (STAT) pathway in endothelial signal transduction through the BK B2 receptor (B2R). In cultured bovine aortic endothelial cells (BAECs), BK activates Tyk2 of the JAK family of tyrosine kinases. Activation results in the tyrosine phosphorylation and subsequent nuclear translocation of STAT3. BK also activates the mitogen-activated p44 and p42 protein kinases, resulting in STAT3 serine phosphorylation. Furthermore, Tyk2 and STAT3 form a complex with the B2R in response to BK stimulation. Under basal conditions, Tyk2, STAT3 and the B2R are localized either partially or entirely in endothelial plasmalemmal caveolae. Following BK stimulation of BAECs, however, the B2R and STAT3 are translocated out of caveolae. Taken together, these data suggest that BK activates the JAK/STAT pathway in endothelial cells and that JAK/STAT signalling proteins are localized in endothelial caveolae. Moreover, caveolar localization of the B2R and STAT3 appears to be regulated in an agonist-dependent manner.

Key words: B2 receptor, caveolin-1, mitogen-activated protein kinase, STAT3, Tyk2.

INTRODUCTION

The peptide hormone bradykinin (BK) is an important regulator of vascular tone. BK acts as a vasodilator by stimulating vascular endothelial cells to release nitric oxide, prostacyclin and endothelial-derived hyperpolarizing factor [1]. When released, each of these substances exerts effects on vascular smooth-muscle cells (VSMCs) to promote vasodilatation. Endothelial BK signal transduction is mediated by a cell-surface receptor termed the B2 receptor (B2R). The B2R has been classified as a member of the seven-transmembrane-domain G-protein-coupled receptor family [2,3]. B2 post-receptor signalling in endothelial cells, as well as in many other cell types, involves activation of phospholipase C and phospholipase A_2 [1]. Activation of phospholipase C results in hydrolysis of inositol-containing membrane lipids and a transient increase in the formation of inositol 1,4,5-trisphosphate and diacylglycerol. BK stimulation also produces a transient elevation of intracellular Ca^{2+} due to both inositol 1,4,5-trisphosphate-mediated Ca^{2+} release from intracellular stores and Ca^{2+} influx across the plasma membrane [4]. Elevated intracellular Ca^{2+} activates the Ca^{2+}/calmodulin-dependent nitric oxide synthase resulting in increased nitric oxide release. Elevated intracellular Ca^{2+} also activates the Ca^{2+}-sensitive phospholipase A_2. Phospholipase A_2 hydrolyses membrane phospholipids to liberate arachidonic acid which, in endothelial cells, is the rate-limiting step in the synthesis of prostacyclin [5]. In addition, Ca^{2+} together with diacylglycerol activates various isoforms of the serine/threonine-specific protein kinase, protein kinase C [6].

BK post-receptor signal-transduction pathways have been investigated extensively, not only in endothelial cells, but also in a number of fibroblast and neuronal cell lines. Only recently, however, has it been shown that these signalling pathways include a role for protein tyrosine phosphorylation. We and others, for example, have reported that a number of different proteins are tyrosine-phosphorylated in endothelial cells in response to BK stimulation [7–10]. Three of these proteins have been identified thus far. These include mitogen-activated protein kinase (MAPK) [7], phospholipase Cγ1 [9] and the B2R [10]. The tyrosine kinases responsible for these phosphorylation events have not been identified but may include the Pyk2 and Src tyrosine kinases, as these two kinases are known to be activated by BK in neuronal PC12 cells [11,12].

One of the signal-transduction pathways that utilizes tyrosine phosphorylation is the Janus-activated kinase (JAK)/signal transducers and activators of transcription (STAT) pathway. In this pathway, ligand binding to cell-surface receptors leads to the activation of tyrosine kinases of the JAK family. JAK kinases then phosphorylate latent cytoplasmic transcription factors termed STATs. The phosphorylated STAT proteins move to the nucleus, bind specific DNA elements, and direct transcription of specific genes. Initially, it was believed that signalling by the JAK/STAT pathway was restricted to cytokine or growth-factor receptors [13]. However, it is now recognized that the JAK/STAT cascade is also activated upon ligand binding to certain G-protein-coupled receptors including the angiotensin II (Ang II) AT1 receptor in VSMCs [14] and cardiac myocytes [15], the thrombin receptor in platelets [16] and VSMCs [17], the serotonin 5-HT2A receptor in skeletal muscle myoblasts [18] and the endothelin-1 ETA receptor in Chinese hamster ovary K1 cells transfected with the receptor cDNA [19].

Whether ligand binding to the B2R involves activation of the JAK/STAT pathway has not yet been reported for any cell type. In the present study, therefore, we have examined whether BK signalling in cultured bovine aortic endothelial cells (BAECs)

Abbreviations used: BK, bradykinin; VSMC, vascular smooth-muscle cell; B2R, B2 receptor; MAPK, mitogen-activated protein kinase; JAK, Janus-activated kinase; STAT, signal transducers and activators of transcription; Ang II, angiotensin II; BAEC, bovine aortic endothelial cell; MEK, MAPK kinase; SH2, Src homology 2; PP1, 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine.

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involves activation of the JAK/STAT pathway. In addition, because the B2R has been shown to be localized in plasmalemmal caveolae in DDT_1 MF-2 smooth-muscle cells [20], we have investigated whether the B2R and potential downstream JAK/STAT effector proteins are localized in the caveolae of endothelial cells. We have also examined whether these proteins are translocated into or out of caveolae in response to BK stimulation.

MATERIALS AND METHODS

Materials

Non-phospho-specific antibodies to JAK1, JAK3, Tyk2, STAT1, STAT2, STAT3, STAT4, STAT5A/B and STAT6, anti-B2R antibody, anti-caveolin-1 antibody and anti-phosphotyrosine (PY20) antibody were obtained from Transduction Laboratories. Phosphoserine-specific anti-STAT3 antibody, phosphotyrosine-specific anti-STAT5A/B antibody and anti-phosphotyrosine (4G10) antibody were purchased from Upstate Biotechnology. Non-phospho-specific anti-JAK2 antibody came from Santa Cruz Biotechnology and phosphotyrosine-specific anti-JAK1 antibody came from Biosource International. Phosphotyrosine-specific anti-STAT1 antibody, phosphotyrosine-specific anti-STAT3 antibody, non-phospho-specific anti-MAPK antibody and phosphothreonine/phosphotyrosine-specific anti-MAPK antibody were obtained from New England Biolabs. 4-Amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP1), AG-490 and PD98059 were purchased from Calbiochem. Genistein was obtained from Life Technologies. BK was obtained from Sigma. Protein molecular-mass standards, detergent-compatible protein assay kit and peroxidase-conjugated anti-IgG antibodies were purchased from Bio-Rad. Cell-culture media and supplements were from Mediatech (Herndon, VA, U.S.A.). Chemiluminescence detection kits were obtained from Amersham or Pierce.

Cell culture

BAECs were passaged from primary cultures and used for experiments during passages 2–6. Cultures were maintained in M199 medium supplemented with 10% fetal bovine serum, 5% iron-supplemented calf serum, 20 μg/ml L-glutamine, 1× minimal-essential-medium amino acid and vitamin solutions, 0.6 μg/ml thymidine, 500 IU/ml penicillin and 500 μg/ml streptomycin. Serum-containing medium was replaced by serum-free medium 24 h prior to each experiment.

Immunoprecipitation and immunoblotting

Immunoprecipitation and immunoblotting were carried out essentially as described previously [9,21]. Briefly, BAECs were either treated or not treated with BK (1 μM) for various times. Cells were washed twice with ice-cold Tris-buffered saline containing 1 mM Na_3VO_4. Cells were then lysed in ice-cold buffer containing 20 mM Tris/HCl, pH 7.4, 2.5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM NaF, 10 mM $Na_4P_2O_7$, 1 mM Na_3VO_4 and 1% PMSF. Lysates were then subjected to immunoprecipitation with various antibodies or were immunoblotted with phospho-specific antibodies.

Isolation of cell nuclei and caveolae

Nuclei were isolated by the method of Dignam et al. [22].

Caveolar membranes were isolated from BAEC cultures on discontinuous sucrose-density gradients by the method of Song et al. [23] with minor modifications, as described previously [24].

RESULTS AND DISCUSSION

BK activates Tyk2 in endothelial cells

Whether BK activates the JAK/STAT pathway in endothelial cells or any other cell type has not been reported previously. In order to investigate the possible occurrence of BK-stimulated JAK/STAT signal-transduction events in cultured endothelial cells, we first identified the JAKs and the STATs that are expressed in BAECs. Cells were lysed and immunoblotted with specific anti-JAK and anti-STAT antibodies. The JAK family of tyrosine kinases consists of four known members, termed JAK1, JAK2, JAK3 and Tyk2. The STAT family of latent cytoplasmic transcription factors includes seven known members, termed STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6 [25]. Immunoblotting of BAEC lysates with antibodies to each of these proteins detected the presence of JAK1 and Tyk2 (but not JAK2 or JAK3) as well as STAT1, STAT3, STAT5A/B and STAT6 (but not STAT2 or STAT4; results not shown). We next examined whether BK treatment of BAECs produces tyrosine phosphorylation of either JAK1 or Tyk2, as it is known that tyrosine phosphorylation of these two enzymes leads to their activation. BAECs were treated with BK (1 μM) for 0, 1, 5, 10, 15 and 30 min. Cells were then lysed and JAK1 and Tyk2 were immunoprecipitated from lysates with anti-JAK1 and anti-Tyk2 antibodies. Immunoprecipitated proteins were subsequently immunoblotted with an anti-phosphotyrosine antibody. No BK-stimulated tyrosine phosphorylation of JAK1 was detected in these experiments (results not shown). However, BK did stimulate a rapid and transient increase in the tyrosine phosphorylation of Tyk2 (135-kDa band). Although the enzyme was already partially tyrosine-phosphorylated under basal conditions, BK stimulated an increased phosphorylation of Tyk2 that was maximal at 5 min (Figure 1A). These data therefore suggest that BK signalling in

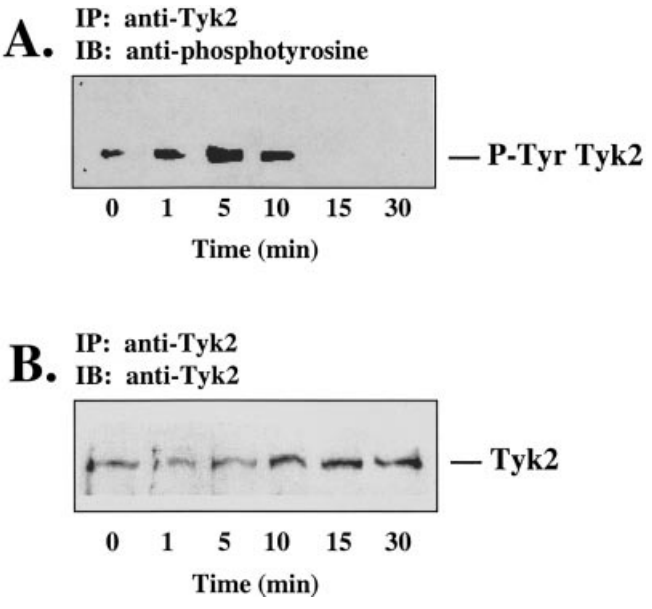

Figure 1 BK stimulation of tyrosine phosphorylation of Tyk2 in endothelial cells

BAECs were treated for the times indicated with BK (1 μM). Cells were lysed and lysates were immunoprecipitated (IP) with anti-Tyk2 antibody. Immunoprecipitated proteins were then immunoblotted (IB) with either anti-phosphotyrosine antibody (**A**) or anti-Tyk2 antibody (**B**). Results shown are representative of three experiments.

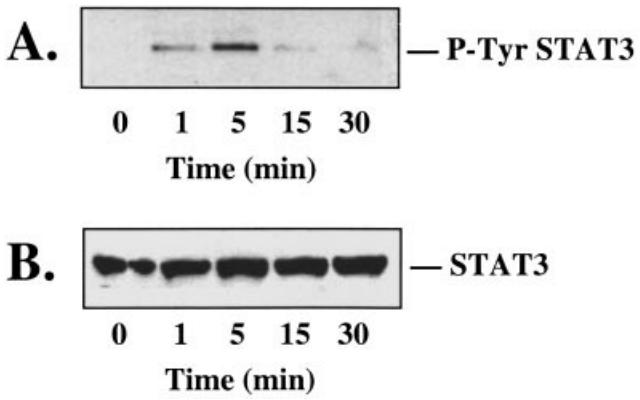

Figure 2 BK stimulation of tyrosine phosphorylation of STAT3 in endothelial cells

BAECs were treated for the times indicated with BK (1 μM). Cells were lysed and lysates were immunoblotted with a phosphotyrosine-specific anti-STAT3 antibody (**A**) or non-phospho-specific anti-STAT3 antibody (**B**). Similar results were obtained in three experiments.

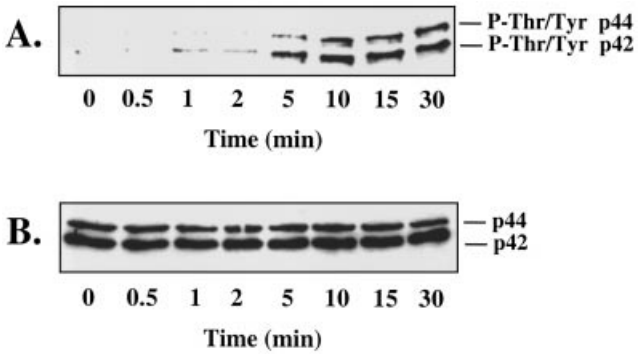

Figure 3 BK stimulation of phosphorylation of MAPK in endothelial cells

BAECs were treated for the times indicated with BK (1 μM). Cells were lysed and lysates were immunoblotted with a phospho-specific anti-MAPK antibody (**A**) or a non-phospho-specific anti-MAPK antibody (**B**). Similar immunoblots were obtained in three experiments.

endothelial cells involves activation of Tyk2. Differences in the amounts of immunoreactivity detected in immunoblots (Figure 1A) were not due to differences in the amounts of Tyk2 that were immunoprecipitated, because when immunoprecipitates were immunoblotted with anti-Tyk2 antibody equal amounts of Tyk2 were detected for all time points (Figure 1B). Similar results to those shown in Figure 1 were also obtained in experiments in which Tyk2 was immunoprecipitated from lysates with an anti-phosphotyrosine antibody and immunoprecipitates were immunoblotted with an anti-Tyk2 antibody (results not shown). When the experiments examining tyrosine phosphorylation of Tyk2 were repeated following pre-exposure of the cells to the broad-spectrum tyrosine kinase inhibitor genistein (20 μg/ml for 30 min) [26], BK-stimulated tyrosine phosphorylation of Tyk2 was completely blocked (results not shown).

BK stimulates the tyrosine phosphorylation of STAT3 in endothelial cells

To determine whether BK activation of Tyk2 results in tyrosine phosphorylation of any of the STATs expressed in BAECs (STATs 1, 3, 5A/B and 6) we utilized phospho-specific antibodies that recognize the tyrosine-phosphorylated, but not the non-phosphorylated, forms of these STATs. BAECs were exposed to BK (1 μM) for 0, 1, 5, 15 and 30 min. Cells were lysed and equal amounts of lysate (50 μg of protein) from each time point were immunoblotted with phosphotyrosine-specific anti-STAT antibodies. No tyrosine phosphorylation of either STAT1 or STAT6 was detected in these experiments (results not shown). A weak tyrosine phosphorylation was detected for STAT5A/B (results not shown). A more robust tyrosine phosphorylation (on residue 705) was observed for STAT3 (92-kDa band; Figure 2A). Phosphorylation was transient and maximal at 5 min, a time point that coincides with that at which Tyk2 is maximally tyrosine-phosphorylated and hence maximally activated. These data suggest, therefore, that BK-stimulated phosphorylation of STAT3 is mediated by Tyk2. A lack of a change in the total amount of STAT3 protein during the time course of these experiments was confirmed by immunoblotting with an anti-STAT3 antibody that recognizes both non-phosphorylated and phosphorylated forms of STAT3 (Figure 2B). When the experiments were repeated following pre-exposure of the cells to genistein (20 μg/ml for 30 min), BK-stimulated tyrosine phosphorylation of STAT3 was blocked completely (results not shown).

STAT3 has been shown previously to be tyrosine-phosphorylated, not only by JAK tyrosine kinases, but also by the Src family of tyrosine kinases [27,28]. Therefore, in order to confirm that it is Tyk2 and not a Src family tyrosine kinase that is responsible for BK stimulation of STAT3 tyrosine phosphorylation in BAECs, we utilized the Src family-selective inhibitor, PP1 [29]. We have shown previously that pretreatment of VSMCs with 1 μM PP1 for 30 min completely blocks Ang II-induced tyrosine phosphorylation of STAT3 [28]. In the present study, the same conditions of pretreatment had no effect on BK-induced tyrosine phosphorylation of STAT3 in BAECs (results not shown), suggesting that the Src family tyrosine kinases are not involved in the phosphorylation. We have also shown previously that the JAK2 inhibitor known as AG-490 or tyrphostin B42 (pretreatment for 1 h with 10 μM of the inhibitor) completely blocks the Ang II-induced STAT1 tyrosine phosphorylation mediated by JAK2 in VSMCs [30]. This inhibitor appears to be highly selective for JAK2 (and possibly also for JAK1 and JAK3) and has been reported to have no effect on Tyk2 [31]. Likewise, in the present study, we found that AG-490 had no effect on BK stimulation of STAT3 tyrosine phosphorylation in BAECs (results not shown), confirming the previous conclusion that AG-490 does not inhibit Tyk2.

BK activates MAPK in endothelial cells

Many of the cytokines and hormones that stimulate STAT3 tyrosine phosphorylation also induce serine phosphorylation of the protein, an event catalysed by MAPK [28,32–34]. Serine phosphorylation is required for maximal transcriptional activity of the factor [32,35]. Activation of MAPK occurs through phosphorylation of both threonine 202 and tyrosine 204 of the p44 and p42 MAPKs at the sequence motif TEY by a single upstream dual-specificity MAPK kinase (MEK). Both the p44 and the p42 isoforms of MAPK (Erk1 and Erk2) have been shown previously to be activated in response to BK stimulation of human umbilical vein endothelial cells [7]. To confirm that BK activates MAPK in BAECs we utilized a phospho-specific antibody that recognizes only the threonine-202- and tyrosine-204-phosphorylated, but not the non-phosphorylated, forms of MAPK. BAEC were treated with BK (1 μM) for 0, 0.5, 1, 2, 5,

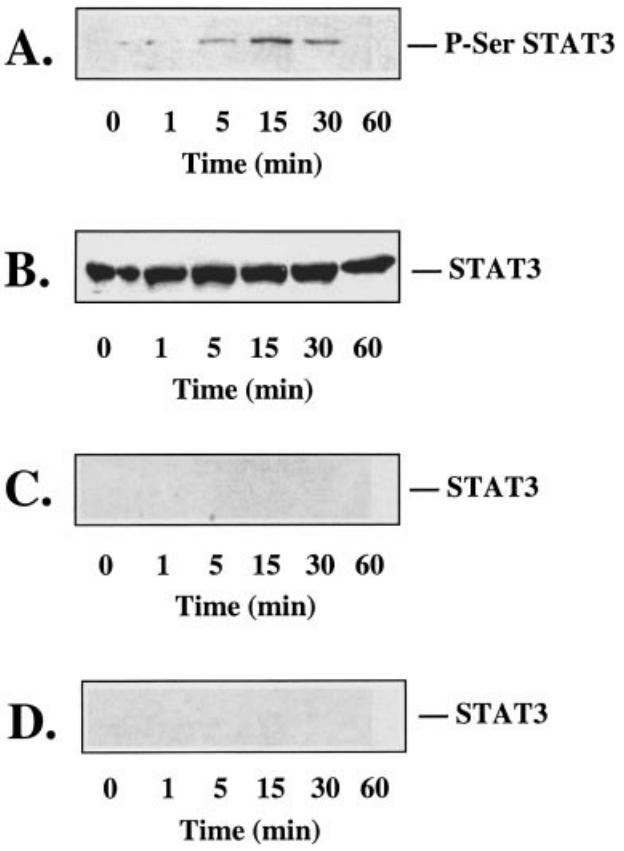

Figure 4 BK stimulation of serine phosphorylation of STAT3 in endothelial cells

BAECs were treated with BK (1 μM) for the times indicated and cells were lysed. Lysates were immunoblotted with a phosphoserine-specific anti-STAT3 antibody (**A**) or a non-phospho-specific anti-STAT3 antibody (**B**). In parallel experiments, BAECs were treated with BK (1 μM) following preincubation with PD98059 (30 μM for 1 h; **C**) or genistein (20 μg/ml for 30 min; **D**). Cells were then lysed and lysates were immunoblotted with phosphoserine-specific anti-STAT3 antibody. Results shown are representative of three experiments.

10, 15 and 30 min, cells were lysed, and equal amounts of lysate (50 μg of protein) from each time point were immunoblotted with the phosphospecific anti-MAPK antibody. As shown in Figure 3(A), BK activated both the p44 and p42 forms of MAPK (44- and 42-kDa bands) with maximal activation occurring after 10–15 min. Equal amounts of p44 and p42 MAPKs in lysates from all time points were confirmed by immunoblotting with an antibody that recognizes both non-phosphorylated and phosphorylated forms of the enzyme (Figure 3B).

BK stimulates the serine phosphorylation of STAT3 in endothelial cells

To determine whether BK-stimulated MAPK activation is accompanied by MAPK phosphorylation of STAT3, BAECs were treated with BK (1 μM) for 0, 1, 5, 15, 30 and 60 min in either the absence or the presence of the MEK inhibitor (and hence MAPK inhibitor) PD98059 (30 μM for 1 h) [36,37] or the broad-spectrum tyrosine kinase inhibitor genistein (20 μg/ml for 30 min). Cell lysates were prepared and equal amounts of lysate (50 μg of protein) from each condition were immunoblotted with an antibody that recognizes the serine-727-phosphorylated, but not the non-phosphorylated, form of STAT3. As shown in Figure 4(A), BK stimulated a transient serine phosphorylation of STAT3 (92-kDa band) with maximal phosphorylation occurring at 15 min, a time point that coincides with the time point at which MAPK is maximally activated. Equal amounts of total STAT3 protein for all time points were confirmed by immunoblotting with the non-phospho-specific anti-STAT3 antibody (Figure 4B). No BK-stimulated serine phosphorylation of STAT3 was observed in the presence of PD98059 (Figure 4C), indicating that serine phosphorylation of STAT3 is probably mediated by MAPK. Serine phosphorylation was also blocked by genistein (Figure 4D), suggesting that an upstream tyrosine-phosphorylation event (or events) is required for downstream activation of MAPK in the BK-MAPK signalling cascade.

BK stimulates complex formation between the B2R and Tyk2 and between the B2R and STAT3 in endothelial cells

Cytokine activation of the JAK/STAT pathway is generally initiated by cytokine-receptor dimerization. Under basal conditions, cytokine receptors are physically associated with non-covalently bound JAK tyrosine kinases. In the absence of cytokine stimulation, these kinases have a very low tyrosine kinase activity. However, when the receptors undergo ligand-induced dimerization, the JAK kinases are brought together in close physical proximity which allows for JAK enzyme trans-phosphorylation. Tyrosine phosphorylation of the JAK kinases significantly increases their catalytic activity, resulting in phosphorylation by the JAKs of tyrosine residues in the cytoplasmic domains of the receptors. Phosphotyrosine residues in the receptors function as docking sites for binding by the Src homology 2 (SH2) domains of various effector proteins including the STAT proteins. Following binding of the STATs to the receptors, they become phosphorylated by the receptor-bound JAKs, an event that is a prerequisite for STAT homo- and/or heterodimerization and translocation of STAT dimers to the cell nucleus. A variation to this general paradigm for JAK/STAT signalling is one described previously for the G-protein-coupled Ang II AT1 receptor. AT1 receptors do not bind significant amounts of JAK kinase under basal conditions. However, stimulation of the AT1 receptor in VSMCs activates the JAK/STAT pathway by inducing a rapid and transient tyrosine phosphorylation, activation and association of JAK2 with the receptor [14,34]. JAK2-receptor association is mediated by a YIPP motif in the C-terminal intracellular domain of the receptor [38] that is identical to an SH2-domain-binding site identified previously in the platelet-derived-growth-factor receptor. Because JAK2 does not contain any SH2 domains, the molecular basis for the interaction of JAK2 with an SH2-domain-binding site was initially unclear. We have shown recently, however, that JAK2 associates with the AT1 receptor through the SH2-domain-containing SHP-2 phosphotyrosine phosphatase acting as an adaptor protein for JAK2 association [39]. The AT1 receptor thus forms a complex with both JAK2 and SHP-2 in a ligand-dependent manner. A recent report has also shown that the AT1 receptor forms a complex with STAT3 in an Ang II-dependent manner [40].

To examine whether BK induces complex formation of the B2R with either Tyk2 or STAT3 in BAECs, cells were exposed to BK (1 μM) for 0, 1, 5 or 10 min and then lysed. Lysates were immunoprecipitated with either anti-Tyk2 or anti-STAT3 antibodies. Immunoprecipitated proteins were then immunoblotted with an anti-B2R antibody in order to quantify the extent of complex formation between Tyk2 and the B2R and between STAT3 and the B2R. We have reported previously that immunoblotting of BAEC lysates with an anti-B2R antibody detects two

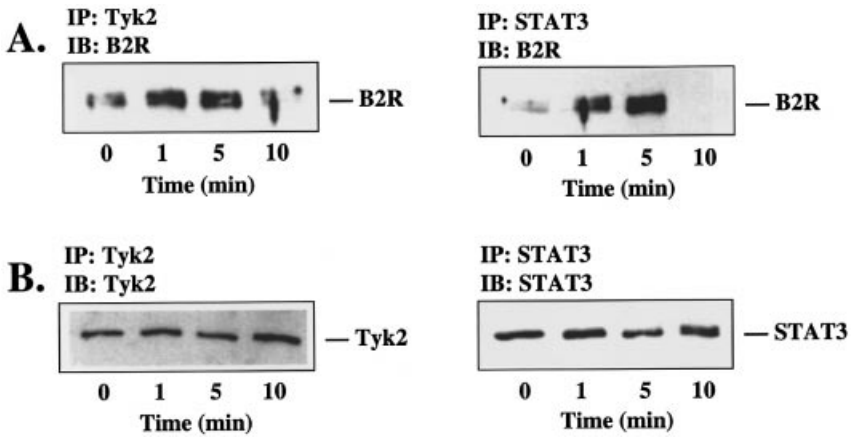

Figure 5 BK stimulation of complex formation between the B2R and Tyk2 and between the B2R and STAT3 in endothelial cells

BAECs were exposed to BK (1 μM) for the times indicated, cells were lysed, and Tyk2 and STAT3 were immunoprecipitated (IP) from lysates with anti-Tyk2 and anti-STAT3 antibodies. Immunoprecipitated proteins were then immunoblotted (IB) with an anti-B2R antibody (**A**). Blots were also stripped and reprobed with the same antibody used in the immunoprecipitation (**B**). Similar results were obtained in three experiments.

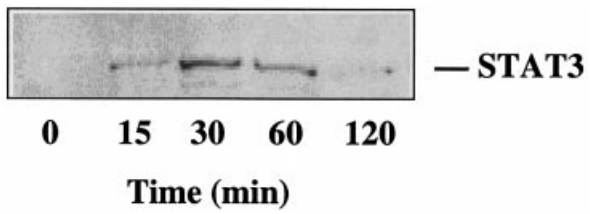

Figure 6 BK stimulation of nuclear translocation of STAT3 in endothelial cells

BAECs were treated with BK (1 μM) for the times shown and cell nuclei were isolated. Relative amounts of STAT3 in nuclear fractions for each time point were quantified by immunoblotting with a non-phospho-specific anti-STAT3 antibody. Three separate experiments gave similar results.

forms of the B2R with apparent molecular masses of 70 and 45 kDa, which correspond to the glycosylated and non-glycosylated forms of the receptor, respectively [9,10,21]. As shown in Figure 5(A), both Tyk2 and STAT3 formed a complex with the glycosylated B2R (70-kDa band) in a ligand-dependent manner. A small amount of complex formation was detected for both proteins at time zero. Following BK treatment, however, the amount of complex formation was dramatically and transiently increased for both proteins and was maximal at 5 min, a time point at which Tyk2 activation and STAT3 tyrosine phosphorylation were also maximal, suggesting that simultaneous binding of Tyk2 and STAT3 to the B2R may facilitate Tyk2 phosphorylation of STAT3. The differences observed in the amounts of the B2R co-immunoprecipitated for the various time points were not due to differences in the amounts of Tyk2 and STAT3 that were immunoprecipitated in these experiments because, when blots were stripped and reprobed with anti-Tyk2 and anti-STAT3 antibodies, equal amounts of the proteins were detected for all time points (Figure 5B). A consensus sequence of YXXQ has been identified in cytokine receptors that is absolutely required for binding of STAT3 [13,25]. Binding of STAT3 is promoted by phosphorylation of the tyrosine residue within this motif, which creates a docking site for the SH2 domain of STAT3. Binding of STAT3 to the B2R, however, may involve a different binding motif because, unlike cytokine receptors and the AT1 receptor [41], none of the B2Rs that have been cloned (from mouse, human and rat) contains a YXXQ sequence [2,3,42].

BK-stimulated tyrosine phosphorylation of STAT3 is followed by its translocation to the nucleus

Tyrosine phosphorylation of STAT proteins results in their homo- and heterodimerization and subsequent translocation from the cytosol to the cell nucleus. We therefore examined whether BK stimulation of BAECs is followed by translocation of STAT3 to the nucleus. Cells were treated with BK for 0, 15, 30, 60 and 120 min. Cells were then lysed and cell nuclei were isolated by the method of Dignam et al. [22]. Relative amounts of STAT3 in nuclear fractions were then quantified by immunoblotting with a non-phospho-specific anti-STAT3 antibody. As shown in Figure 6, BK stimulated a significant time-dependent increase in the amount of STAT3 localized in endothelial-cell nuclei with maximal translocation occurring after 30 and 60 min. Localization of STAT3 in the nucleus was transient, as expected, and was significantly decreased between 60 and 120 min. BK-stimulated tyrosine phosphorylation of STAT3 is maximal at 5 min whereas translocation to the nucleus is not maximal until at least 30 min. Thus it appears that translocation following phosphorylation occurs through a process that requires up to 25 min to complete. A similar time course (30 min to reach a maximum) has been reported previously for Ang II-stimulated nuclear translocation of STAT1 in VSMCs [14].

Tyk2, STAT3 and the B2R are localized in endothelial plasmalemmal caveolae

Plasmalemmal caveolae are small invaginations of the plasma membrane that function in both transmembrane transport and signal transduction. The major structural proteins of caveolae are the caveolins, a family of scaffolding proteins that organize 'preassembled signalling complexes' at the membrane [43]. Signalling proteins shown previously to be localized in caveolae include G-protein α subunits, Ha-Ras, Src family tyrosine kinases, protein kinase C isoforms, nitric oxide synthase isoforms, receptors containing intrinsic tyrosine kinase activity and certain

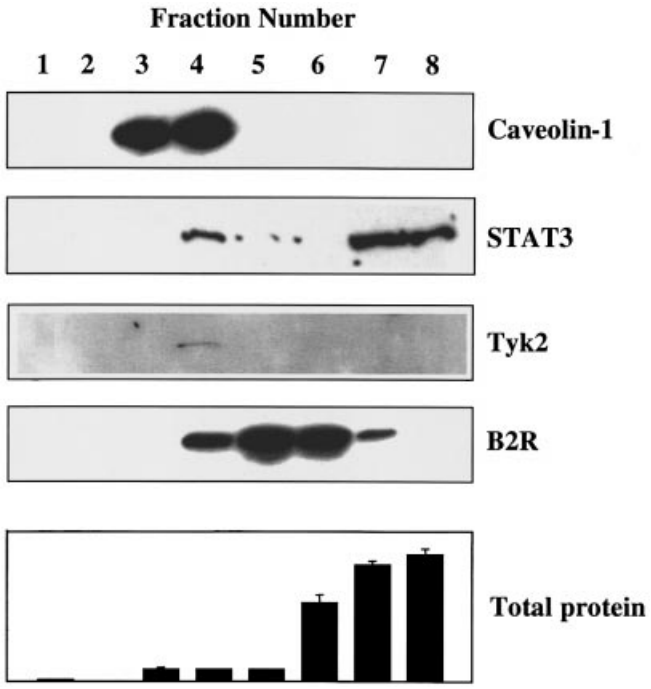

Figure 7 Distribution of caveolin-1, STAT3, Tyk2, B2R and total protein from BAECs in sucrose-density-gradient fractions

BAEC homogenates were fractionated on a discontinuous sucrose-density gradient. Eight fractions were collected from the top of each tube and the distributions of caveolin-1, STAT3, Tyk2 and the B2R among the fractions were detected by immunoblotting. Relative amounts of protein were quantified by protein assay. Results shown are representative of three experiments. Error bars represent S.E.M.

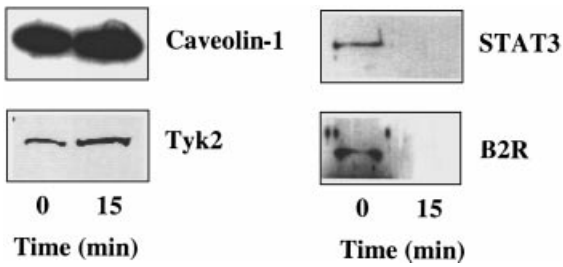

Figure 8 Effect of BK treatment of endothelial cells on caveolar localization of caveolin-1, Tyk2, STAT3 and the B2R

BAECs were treated or not treated with BK (1 μM) for 15 min. Caveolae were isolated by sucrose-density-gradient ultracentrifugation and the relative amounts of caveolin-1, Tyk2, STAT3 and the B2R in caveolar fractions before and after BK treatment were quantified by immunoblotting. Similar results were obtained in three experiments.

G-protein-coupled receptors including the B2R [43]. It has also been reported that binding of BK to the B2R in DDT_1 MF-2 smooth-muscle cells promotes the sequestration of the occupied receptors within caveolae [20]. In the present study, therefore, in order to determine whether the B2R, Tyk2 or STAT3 are localized under basal conditions in endothelial caveolae, we isolated caveolae from BAECs by the detergent-free purification method of Song et al. [23]. In this procedure caveolae membranes are separated from other cellular membranes and $> 95\%$ of cellular proteins based on their unique light buoyant density during ultracentrifugation on a discontinuous gradient of 5, 35 and 45% sucrose. BAEC homogenates were subfractionated on sucrose gradients and eight fractions were collected starting from the top of the gradient. The distributions of the B2R, Tyk2 and STAT3 in gradient fractions were then determined by immunoblotting with anti-B2R, anti-Tyk2 and anti-STAT3 antibodies. The relative amounts of total protein in each fraction were also quantified by protein assay. Fractions were also immunoblotted with caveolin-1, which was used as a caveolar marker. As shown in Figure 7, all of the caveolin-1 was found in fractions 3 and 4 of the gradient, a position corresponding to the interface between the 5 and 35% sucrose layers. The fractions of the gradient containing caveolae were thus identified as fractions 3 and 4. These caveolar fractions also contained a portion of the total cellular STAT3, all of the cellular Tyk2 and a portion of the total cellular B2R. Under basal conditions, therefore, STAT3 and the B2R appear to be partially localized in the plasmalemmal caveolae of BAECs whereas Tyk2 is localized exclusively in caveolae. However, it should be noted that, because the B2R is also found in fractions 5, 6 and 7 of the gradient, that caveolin-1 and the B2R could be localized in different membrane fractions that have similar but distinct densities, but which overlap in their distribution on the sucrose gradient.

BK stimulates the translocation of the B2R and STAT3 out of endothelial caveolae

To determine whether STAT3, Tyk2, the B2R or caveolin-1 move into or out of caveolae in response to BK stimulation, BAECs, were treated with BK (1 μM) for 0 or 15 min and caveolae were isolated on sucrose-density gradients. Caveolar fractions (fractions 3 and 4 of the gradient) were collected and pooled. Relative amounts of the four proteins of interest in the caveolar fractions for time 0 and after 15 min of BK exposure were then quantified by immunoblotting. As shown in Figure 8, relative amounts of caveolin-1 and Tyk2 in caveolae were unaltered by BK treatment. However, STAT3 and the B2R were translocated out of caveolae in response to BK. Movement of STAT3 out of caveolae is likely to be the first step in translocation of the factor to the nucleus. It is not clear why the B2R moves out of endothelial caveolae in response to BK. However, the receptor probably does not function as a chaperone for translocation of STAT3 to the nucleus because, as shown in Figure 5, the B2R–STAT3 complex is completely dissociated following only 10 min of BK exposure, a time at which very little nuclear translocation of STAT3 can be detected (Figure 6). Our observation of B2R movement out of plasmalemmal caveolae in response to BK treatment contrasts with what has been reported for smooth-muscle cells where BK promotes movement of the B2R into caveolae [20]. These differential effects of BK on caveolar localization of the B2R may reflect differences in BK signal transduction in endothelial versus smooth-muscle cells.

In summary, the results of the present study provide several new insights into B2 post-receptor signal-transduction pathways in endothelial cells. A first novel observation is that BK activates the JAK/STAT pathway in endothelial cells and, therefore, possibly in other cell types as well. BK signalling is generally thought of in terms of events that occur within seconds to minutes. BK activation of the JAK/STAT pathway, however, suggests that the peptide may also have longer-term transcriptional effects. A second finding of our study is that of BK-stimulated complex formation between the B2R and Tyk2 and between the B2R and STAT3. The only G-protein-coupled receptor shown previously to form a complex with a JAK kinase

or a STAT transcription factor is the Ang II AT1 receptor [14,40]. The results of the present study suggest that ligand-dependent binding of JAKs and STATs occurs with other G-protein-coupled receptors as well. Our results also demonstrate that the Tyk2 tyrosine kinase and the STAT3 transcription factor are localized in endothelial plasmalemmal caveolae. Koshelnick et al. [44] have shown previously that JAK/STAT signalling proteins are also localized in the caveolae of the kidney epithelial tumour cell line TCL-598. Taken together with the results of Koshelnick et al., our data suggest that caveolar localization of JAKs and STATs is not a unique feature of TCL-598 cells. Finally, our results demonstrate that caveolar localization of the B2R and STAT3 is regulated in an agonist-dependent manner.

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