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## Signal transduction of betacellulin in growth and migration of vascular smooth muscle cells

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<sup>1</sup>Cardiovascular Research Center and <sup>6</sup>Department of Physiology, Temple University School of Medicine, Philadelphia, Pennsylvania 19140; <sup>2</sup>Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee 37232; <sup>3</sup>Department of Pathology, Wakayama Medical University, Wakayama 641-8509, Japan; <sup>4</sup>Pacific Northwest Research Institute, Seattle 98112; and <sup>5</sup>Department of Medicine, University of Washington, Seattle, Washington 98195

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**Mifune, Mizuo, Haruhiko Ohtsu, Hiroyuki Suzuki, Gerald D. Frank, Tadashi Inagami, Hirotoshi Utsunomiya, Peter J. Dempsey, and Satoru Eguchi.** Signal transduction of betacellulin in growth and migration of vascular smooth muscle cells. *Am J Physiol Cell Physiol* 287: C807–C813, 2004. First published May 26, 2004; 10.1152/ajpcell.00436.2003.—Epidermal growth factor (EGF) family ligands have been implicated in cardiovascular diseases because of their enhanced expression in vascular lesions and their promoting effects on growth and migration of vascular smooth muscle cells (VSMCs). Betacellulin (BTC), a novel EGF family ligand, has been shown to be expressed in atherosclerotic lesions and to be a potent growth factor of VSMCs. However, the molecular mechanisms downstream of BTC involved in mediating vascular remodeling remain largely unknown. Therefore, the aim of this study was to examine the effects of BTC on signal transduction, growth, and migration in VSMCs. We found that BTC stimulated phosphorylation of EGF receptor (EGFR) at Tyr1068, which was completely blocked by an EGFR kinase inhibitor, AG-1478. BTC also phosphorylated ErbB2 at Tyr877, Tyr1112, and Tyr1248 and induced association of ErbB2 with EGFR, suggesting their heterodimerization in VSMCs. In post-receptor signal transduction, BTC stimulated phosphorylation of extracellular signal-regulated kinase (ERK)1/2, Akt, and p38 mitogen-activated protein kinase (MAPK). Moreover, BTC stimulated proliferation and migration of VSMCs. ERK and Akt inhibitors suppressed migration markedly and proliferation partially, whereas the p38 inhibitor suppressed migration partially but not proliferation. In addition, we found the presence of endogenous BTC in conditioned medium of VSMCs and an increase of BTC on angiotensin II stimulation. In summary, BTC promotes growth and migration of VSMCs through activation of EGFR, ErbB2, and downstream serine/threonine kinases. Together with the expression and processing of endogenous BTC in VSMCs, our results suggest a critical involvement of BTC in vascular remodeling.

epidermal growth factor receptors; ErbB2; migration; signal transduction

THE EPIDERMAL GROWTH FACTOR (EGF) family consists of EGF, heparin-binding EGF-like growth factor (HB-EGF), transforming growth factor- $\alpha$ , epiregulin, amphiregulin, epigen, neuregulins, and betacellulin (BTC) (12, 14). All of these ligands are synthesized as transmembrane precursors that are proteolytically cleaved to release biologically active mature soluble growth factors (12). These ligands bind and activate members

of the ErbB receptor tyrosine kinase family, which includes EGF receptor (EGFR, ErbB1), ErbB2, ErbB3, and ErbB4 (34). The EGF family and the ErbB receptors are pivotal participants in development, differentiation, proliferation, and survival (34). These cellular responses are considered to be orchestrated by specific ligand-receptor combinations and their selective expression (14, 34). The EGF family has long been implicated in cardiovascular diseases (3). For example, HB-EGF is known to be expressed in human atherosclerosis, pulmonary hypertension, and restenosis after balloon injury (24). In addition, EGF, epiregulin, and HB-EGF have strong mitogenic and chemotactic effects on VSMCs (3, 24). These accumulating data strongly suggest a critical involvement of the EGF family and their receptors in mediating vascular remodeling.

BTC, a novel member of the EGF family, was originally isolated as a growth-promoting factor in conditioned medium derived from a mouse pancreatic beta cell line (29). BTC is expressed in a wide range of tissues, including kidney, liver, and small intestine, and is particularly high in the pancreas (7). Interestingly, BTC was shown to be a potent mitogen for cultured VSMCs (29). BTC precursor protein is expressed in intimal and medial VSMCs of human atherosclerotic lesions (32). However, the exact function of BTC in mediating vascular remodeling is not completely characterized, and the activation of ErbB receptors and postreceptor signaling by BTC in VSMCs is poorly defined. In light of this, we hypothesized that there is a plausible participation of this new EGF ligand and its signaling in vascular pathological conditions. Therefore, the aim of the present study was to characterize the signal transduction of BTC in mediating growth and migration of VSMCs.

In this study, we demonstrate that BTC activates EGFR, ErbB2, and their postreceptor signal transduction pathways, leading to growth and migration responses in VSMCs. Moreover, we show the presence and processing of endogenous BTC in VSMCs, suggesting that BTC acts as an autocrine and/or paracrine growth factor in mediating vascular remodeling.

### MATERIALS AND METHODS

**Materials.** Recombinant human BTC, EGF, and HB-EGF were purchased from R&D Systems. Anti-BTC antibody was from Gro Pep. Anti-phosphotyrosine antibody (4G10) was from Upstate Biotechnology; phospho-specific antibody for Tyr1068-phosphorylated

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EGFR was from Biosource International; antibodies for EGFR, Tyr204-phosphorylated extracellular signal-regulated kinase (ERK)1/2, ERK2, p38 mitogen-activated protein kinase (MAPK), and anti-ErbB2 were from Santa Cruz Biotechnology; antibodies for Tyr877-, Tyr1112-, and Tyr1248-phosphorylated ErbB2, Akt, Ser473-phosphorylated Akt, and Thr180/Tyr182 dual-phosphorylated p38 MAPK were from Cell Signaling. AG-1478, PD-98059, SB-203580, and an Akt inhibitor (AktI) were obtained from Calbiochem. Angiotensin II was from Sigma.

**Cell culture.** VSMCs were prepared from thoracic aorta of Sprague-Dawley rats by the explant method. All animal procedures conformed to the "Guiding Principles for Research Involving Animals and Human Beings" of the American Physiological Society. Subcultured cells from passages 3–12 were used and showed 99% positive immunostaining with smooth muscle  $\alpha$ -actin antibody (9). For the experiments, VSMCs at 80–90% confluence were used after serum depletion with 0% serum Dulbecco's modified Eagle's medium for 2–3 days.

**Immunoprecipitation.** After stimulation at 37°C, cells were lysed with ice-cold immunoprecipitation buffer as previously described (9). The cell lysates or the conditioned medium was centrifuged, and the supernatant was immunoprecipitated with the antibody and protein A/G plus agarose at 4°C for 16 h.

**Western blotting.** Cell lysates or immunoprecipitation lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were electrophoretically transferred to a nitrocellulose membrane as previously described (9). The membranes were then exposed to primary antibodies overnight at 4°C. After incubation with the peroxidase-linked secondary antibody for 1 h at room temperature, immunoreactive proteins were visualized with ECL reagent (Amersham Life Sciences).

**DNA synthesis.** DNA synthesis was assessed by incorporation of  $^3\text{H}$ -labeled thymidine into cells as previously described (21). In brief,

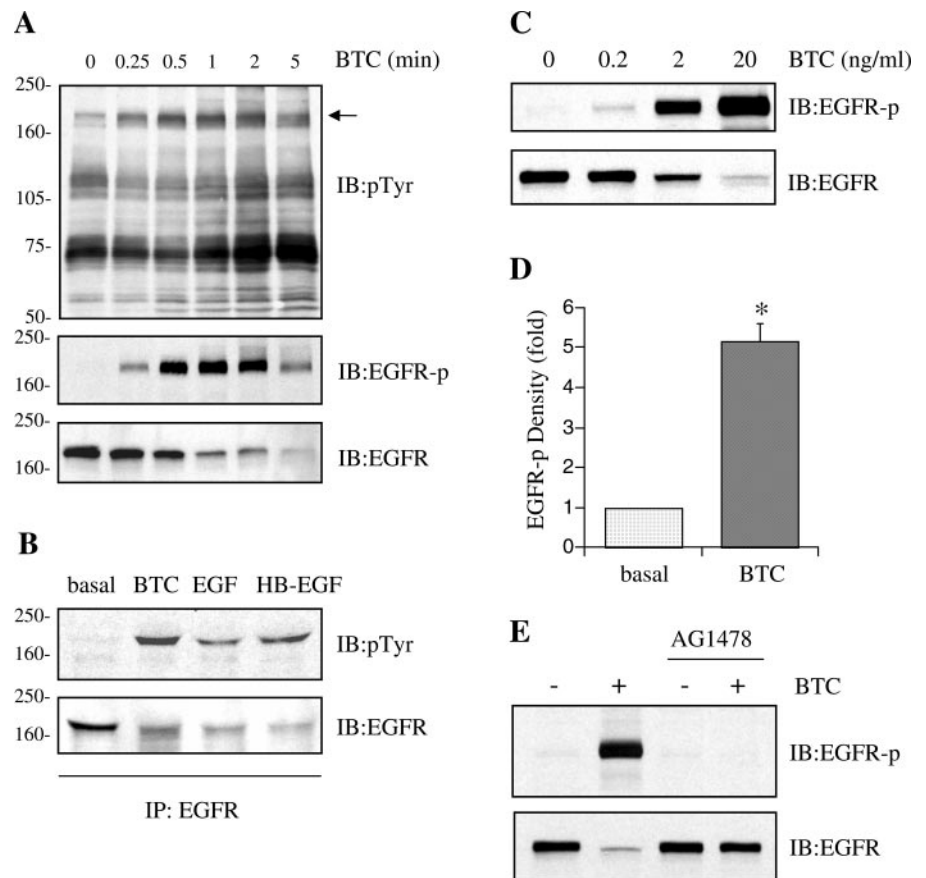
quiescent VSMCs were incubated with BTC for 24 h and 1  $\mu\text{Ci}$  of [ $^3\text{H}$ ]thymidine was added for the last 8 h. Trichloroacetic acid-insoluble radioactivity was measured in a liquid scintillation counter.

**Proliferation assay.** Cell proliferation was measured by using the CellTiter 96 Aqueous cell proliferation assay kit (Promega) according to the manufacturer's protocol. In brief, quiescent VSMCs in 96-well plates were pretreated with or without inhibitors for 1 h and then stimulated with BTC (20 ng/ml) for 48 h. After incubation with the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt solution provided by the kit, viable cells were determined at 490-nm absorbance with a 96-well plate reader.

**Migration assay.** Cell migration was performed with the Boyden chamber method as previously described (26). Briefly, the lower well of the chemotaxis chamber was filled with 600  $\mu\text{l}$  of serum-free medium that contained BTC (20 ng/ml) with or without inhibitors. An 8- $\mu\text{m}$  polycarbonate filter was placed between the upper and lower chambers. VSMCs were trypsinized and resuspended in serum-free medium to a concentration of  $10^5$  cells/100  $\mu\text{l}$ . This volume was then added to the upper chamber. After incubation for 8 h at 37°C in a 5%  $\text{CO}_2$  incubator, filters were removed and the cells on the top surface of the membrane were removed with a cotton swab. The membranes were washed with phosphate-buffered saline, fixed with methanol, and stained with Giemsa stain. Cells were counted with the use of a light microscope.

**RT-PCR.** Expression of BTC mRNA was analyzed by the RT-PCR method as previously described (21). Primers for BTC were 5'-GTCCTGGGTCTTGTGATTC-3' and 5'-GAAGAGGATGACAGCAGGT-3', which correspond to bases 106–124 and 466–484 of the rat BTC sequence (31), respectively. By using rat BTC cDNA (31) as a template, we have confirmed that the BTC primers used in this study yield an expected single band (376 bp). The amplification was done with PCR System 2700 (Applied Biosystems) with a Gene Amp RNA

Fig. 1. Effect of betacellulin (BTC) on epidermal growth factor (EGF) receptor (EGFR) tyrosine phosphorylation. **A:** vascular smooth muscle cells (VSMCs) were stimulated with BTC (20 ng/ml) for the indicated time periods, and the cell lysates were immunoblotted (IB) with anti-phosphotyrosine antibody, anti-phospho-EGFR, and anti-EGFR antibodies. Arrow denotes  $\sim 170$ -kDa tyrosine-phosphorylated protein. **B:** VSMCs were stimulated for 1 min by BTC (20 ng/ml), EGF (20 ng/ml), and heparin-binding EGF-like growth factor (HB-EGF; 20 ng/ml). The cell lysates were immunoprecipitated (IP) with anti-EGFR antibody and immunoblotted with anti-phosphotyrosine and anti-EGFR antibodies. **C:** VSMCs were stimulated with the indicated concentrations of BTC for 1 min and immunoblotted with anti-phospho-EGFR and anti-EGFR antibodies. **D:** phosphorylation of EGFR at Tyr1068 was measured by densitometry and shown as means  $\pm$  SE ( $n = 3$ ).  $*P < 0.05$  compared with the basal control. **E:** cells were pretreated with or without AG-1478 (500 nM) for 30 min and stimulated with BTC (20 ng/ml) for 1 min. The cell lysates were immunoblotted with anti-phospho-EGFR and anti-EGFR antibodies.



PCR core kit (Roche). Reaction products were resolved by electrophoresis through 2% agarose gels and were visualized with a laser image analyzer.

**Statistical analysis.** Data were analyzed by Student's *t*-test. Means  $\pm$  SE were determined with a significance level of  $P < 0.05$ . The results shown in blots are representative of at least three separate experiments using at least two distinct batches of VSMCs.

## RESULTS

**Effect of BTC on EGFR activation in VSMCs.** The effect of BTC on protein tyrosine phosphorylation in VSMCs is shown in Fig. 1A. BTC rapidly phosphorylated several distinct proteins in a time-dependent manner. The protein around 170 kDa was markedly phosphorylated as early as 30 s, whereas other proteins around 110, 70, and 50–60 kDa were maximally phosphorylated at 2 min. Because BTC has been shown to bind and activate the ErbB receptor family (7), the rapidly phosphorylated protein(s) around 170 kDa may likely represent the EGFR or other ErbBs. This result was confirmed by examining BTC-induced phosphorylation of EGFR at Tyr1068, an auto-phosphorylation site known to recruit the adaptor protein Grb2 (17). BTC induced phosphorylation of Tyr1068 in a time-dependent manner, with maximal phosphorylation occurring at 0.5–2 min. As EGFR phosphorylation increased, the total amount of EGFR was degraded by BTC in a reciprocal manner. In addition, immunoprecipitation analysis revealed that BTC stimulated tyrosine phosphorylation of the EGFR in a manner comparable to that of EGF and HB-EGF (Fig. 1B). Maximum phosphorylation of EGFR at Tyr1068 induced by BTC occurred at 20 ng/ml, with a fivefold increase above basal levels (Fig. 1, C and D). EGFR phosphorylation by BTC was blocked by an EGFR kinase inhibitor (20), AG-1478 (500 nM), confirming the BTC-induced EGFR activation in VSMCs (Fig. 1E).

**Effect of BTC on ErbB2 activation in VSMCs.** We next examined the effect of BTC on ErbB2 activation. BTC stimulated phosphorylation of ErbB2 at Tyr877 (a Src phosphorylation site), Tyr1112 (a Cbl binding site), and Tyr1248 (a Shc binding site) (18, 22) in a time-dependent manner (Fig. 2A). The maximum phosphorylation of ErbB2 at Tyr877, Tyr1112, and Tyr1248 was ~7-, 3.5-, and 3-fold, respectively, above basal levels (Fig. 2B). Because ErbB2 is known only to heterodimerize with other ErbB receptors (22), we examined whether ErbB2 associates with EGFR. On BTC stimulation, association of EGFR with tyrosine-phosphorylated ErbB2 was detected (Fig. 2C), suggesting a heterodimer formation between EGFR and ErbB2 in VSMCs.

**Effect of BTC on postreceptor signaling in VSMCs.** As shown in Fig. 3, A–D, we examined the postreceptor signal transduction induced by BTC in VSMCs. BTC stimulated ERK1/2 phosphorylation in a concentration-dependent manner, with maximum phosphorylation occurring at 20 ng/ml (Fig. 3, A and B). This phosphorylation was completely blocked by AG-1478 (Fig. 3C). BTC also stimulated phosphorylation of Akt and p38 MAPK maximally at 5 and 10 min, respectively (Fig. 3, D and E).

**BTC stimulates DNA synthesis and migration in VSMCs.** The effects of BTC on DNA synthesis and migration in VSMCs are shown in Fig. 4, A and B, respectively. BTC markedly stimulated DNA synthesis in VSMCs as determined by [<sup>3</sup>H]thymidine incorporation and migration as measured by

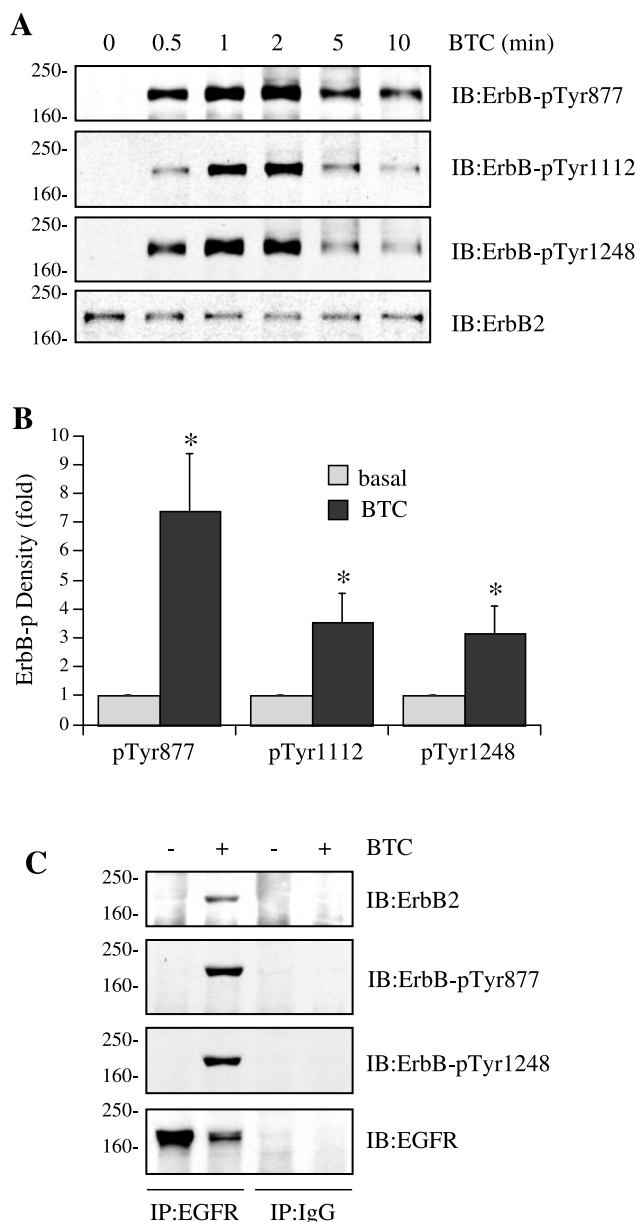
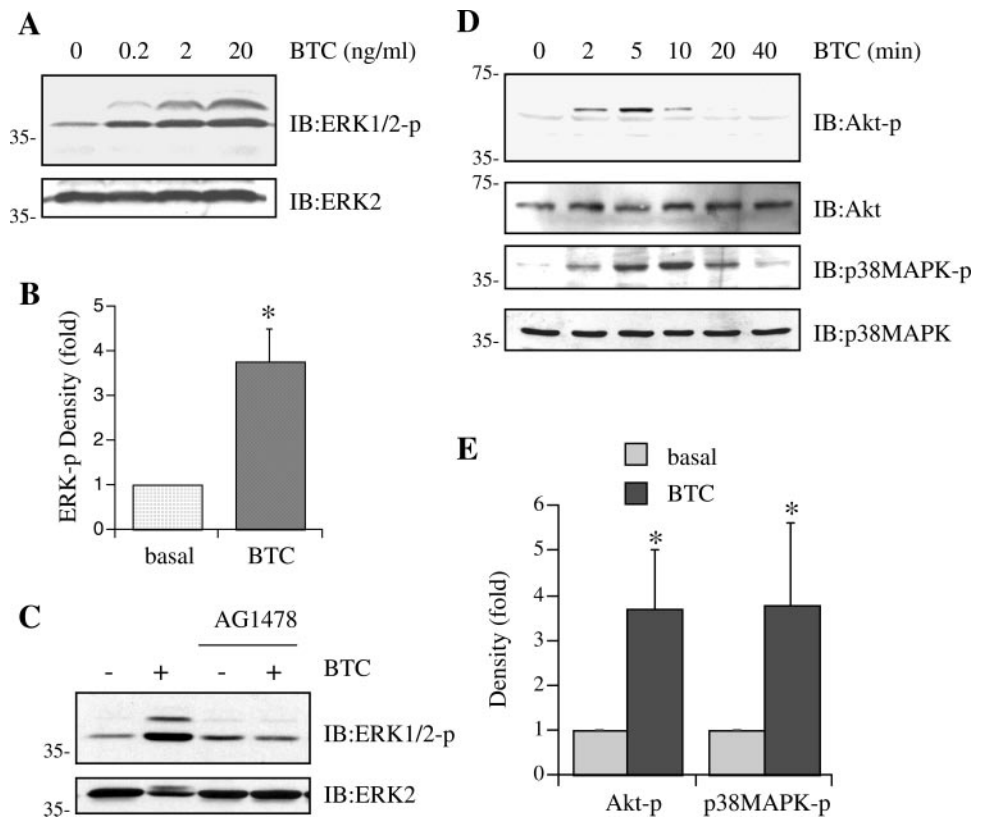


Fig. 2. Effect of BTC on ErbB2 tyrosine phosphorylation and its association with EGFR in VSMCs. A: VSMCs were stimulated with BTC (20 ng/ml) for the indicated time periods. The cell lysates were immunoblotted with anti-phospho-ErbB2 (Tyr877, Tyr1112, and Tyr1248) and anti-ErbB2 antibodies. B: phosphorylation of ErbB2 (Tyr877, Tyr1112, and Tyr1248) was measured by densitometry and shown as means  $\pm$  SE ( $n = 3$ ). \* $P < 0.05$  compared with the basal control. C: after stimulation with BTC (20 ng/ml) for 1 min, VSMC lysates were immunoprecipitated with anti-EGFR antibody or control rabbit IgG and immunoblotted with anti-phospho-ErbB2 (Tyr877 and Tyr1248), anti-ErbB2, and anti-EGFR antibodies.

the Boyden chamber assay with VSMCs. Both DNA synthesis and cell migration were completely blocked by pretreatment with AG-1478.

**Role of postreceptor signaling in BTC-induced proliferation and migration.** To study the possible participation of postreceptor signaling in VSMC proliferation and migration induced by BTC, the effects of a MEK inhibitor, PD-98059 (which inhibits the downstream ERK1/2; Ref. 6), a p38 MAPK inhibitor, SB-203580 (6), and an Akt inhibitor, AktI (15), on these

Fig. 3. Effect of BTC on postreceptor signal transduction in VSMCs. **A:** VSMCs were stimulated with indicated concentrations of BTC for 10 min and immunoblotted with anti-phospho-extracellular signal-regulated kinase (ERK)1/2 and anti-ERK2 antibodies. **B:** phosphorylation of ERK1/2 was measured by densitometry and shown as means  $\pm$  SE ( $n = 3$ ). \* $P < 0.05$  compared with the basal control. **C:** VSMCs were pre-treated with or without AG-1478 (500 nM) for 30 min and stimulated with BTC (20 ng/ml) for 10 min. The cell lysates were immunoblotted with anti-phospho-ERK1/2 and anti-ERK2 antibodies. **D:** VSMCs were stimulated with BTC (20 ng/ml) for the indicated time periods. The cell lysates were immunoblotted with anti-phospho-Akt, anti-Akt, anti-phospho-p38 mitogen-activated protein kinase (MAPK), and anti-p38 MAPK antibodies. **E:** phosphorylation of Akt and p38 MAPK was measured by densitometry and shown as means  $\pm$  SE ( $n = 3$ ). \* $P < 0.05$  compared with the basal control.



responses were studied. The concentration of these inhibitors was chosen according to previous publications addressing their specificity (6, 15). BTC-induced proliferation of VSMCs was partially blocked by pretreatments with PD-98059 and AktI but not with SB-203580 (Fig. 5A). We confirmed that AG-1478 (500 nM) completely blocked BTC-induced VSMC proliferation (data not shown). In contrast to proliferation, BTC-induced migration of VSMCs was inhibited markedly by PD-98059 and AktI and partially by SB-203580 (Fig. 5B).

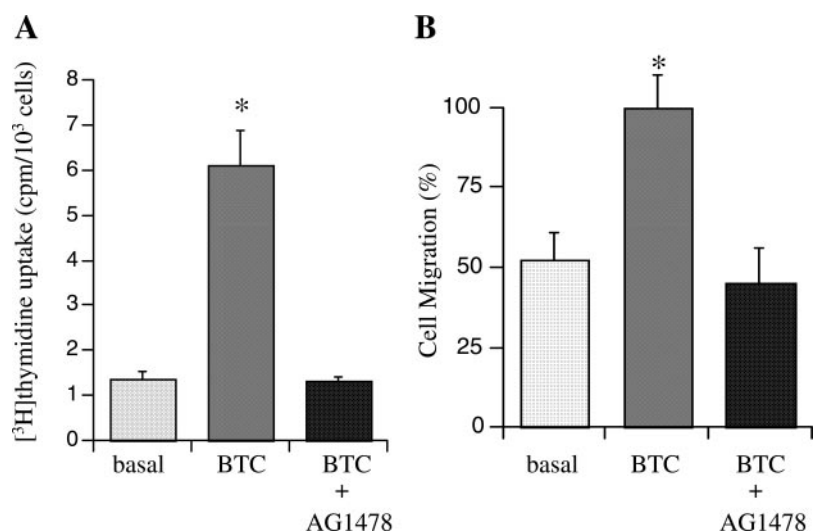
**Presence of endogenous BTC and processing by angiotensin II in VSMCs.** To demonstrate the presence of endogenous BTC in VSMCs, we performed RT-PCR. We observed a single

expected band representing a BTC transcript (Fig. 6A). We also examined the release of BTC in VSMCs after angiotensin II stimulation. Angiotensin II stimulation enhanced a single band around 20 kDa, as detected by anti-BTC antibody in the conditioned medium of VSMCs representing processed mature BTC from its membrane anchored precursor (Fig. 6B; Ref. 30).

## DISCUSSION

In this study, we have demonstrated that BTC activates EGFR and ErbB2 and subsequently induces postreceptor signaling such as ERK1/2, Akt, and p38 MAPK in VSMCs.

Fig. 4. Effect of BTC on DNA synthesis and migration in VSMCs. **A:** [ $^3$ H]thymidine incorporation assay was used to measure DNA synthesis after stimulation with BTC (20 ng/ml) in the presence or absence of AG-1478 (500 nM). Data are means  $\pm$  SE ( $n = 3$ ). \* $P < 0.05$  compared with the control. **B:** Boyden chamber method was used to examine migration after stimulation of BTC (20 ng/ml) in the presence or absence of AG-1478 (500 nM). Data represent %changes relative to basal control ( $n = 12$ ; means  $\pm$  SE). \* $P < 0.05$  compared with the stimulated control.



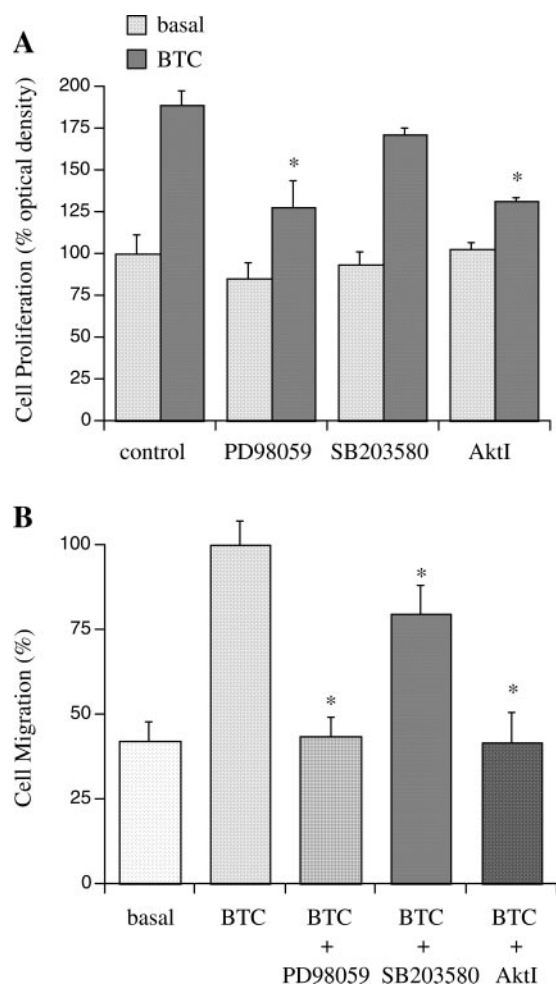


Fig. 5. Role of postreceptor signaling in BTC-induced proliferation and migration of VSMCs. **A**: cell proliferation was measured by using a CellTiter 96 AQueous cell proliferation assay kit. In brief, quiescent VSMCs in 96-well plates were pretreated with or without PD-98059 (50  $\mu$ M), SB-203580 (50  $\mu$ M), or AktI (40  $\mu$ M) for 1 h and then stimulated with BTC (20 ng/ml) for 48 h. Data represent %changes relative to basal control ( $n = 6$ ; means  $\pm$  SE). \* $P < 0.05$  compared with the control. **B**: Boyden chamber method was used to examine migration after stimulation with BTC (20 ng/ml) in the presence or absence of PD-98059 (50  $\mu$ M), SB-203580 (50  $\mu$ M), or AktI (40  $\mu$ M). Data represent %changes relative to the stimulated control ( $n = 12$ ; means  $\pm$  SE). \* $P < 0.05$  compared with the control.

BTC-induced migration of VSMC is dependent on ERK and Akt and partially on p38 MAPK, whereas BTC-induced proliferation partially requires ERK and Akt but not p38. Moreover, we showed the presence of endogenous BTC in VSMCs, suggesting its pathophysiological role in the vasculature. Quite recently, Shin et al. (28) showed that BTC stimulates DNA synthesis of human VSMCs that is accompanied by the activation of the Akt pathway. Their findings are in line with our present study. However, this is the first study showing BTC-stimulated migration through EGFR/ErbB2 activation as well as production of mature BTC in VSMCs.

The biological actions of EGF family ligands are mediated through a member of receptor tyrosine kinases, ErbB receptors (34). Our results showed that BTC stimulates tyrosine phosphorylation of various proteins in VSMCs. Among these proteins,  $\sim$ 170-kDa proteins were identified as EGFR and ErbB2. Although we have not characterized the other tyrosine-phos-

phorylated proteins (110–120, 70–75, and 50–60 kDa) induced by BTC, we assume that these proteins represent substrate and adaptor proteins of EGFR and ErbB2, such as Src (60 kDa), Cbl (120 kDa), SHP-2 (66 kDa), and Shc (52 and 46 kDa). As shown in Fig. 1C, EGFR phosphorylation by BTC occurred at a maximum concentration of 20 ng/ml, which is compatible with a recent study reported by Shin et al. (28) in VSMCs. Also, our results show that EGFR phosphorylation by BTC was equivalent to the phosphorylation induced by EGF or HB-EGF, suggesting similar potency of BTC in its growth-promoting effects on VSMCs.

Recent studies suggested that BTC is able to induce all possible combinations of homo- and heterodimer ErbB receptors, which could be cell type and tissue dependent (7). In this study, we showed that BTC stimulates marked phosphorylation of ErbB2 at three intrinsic tyrosine residues in VSMCs. Among these, Tyr1112 and Tyr1248 are considered as autophosphorylation sites, indicating that BTC stimulates the kinase activity of ErbB2. Because ErbB2 is known to heterodimerize with all other ErbB receptors (34), the association of EGFR and ErbB2 observed in this study suggests a heterodimerization of EGFR and ErbB2 induced by BTC in VSMCs. These data suggest that, in addition to its critical involvement in heart development (19) and cancer cell growth (1), ErbB2 may also play an

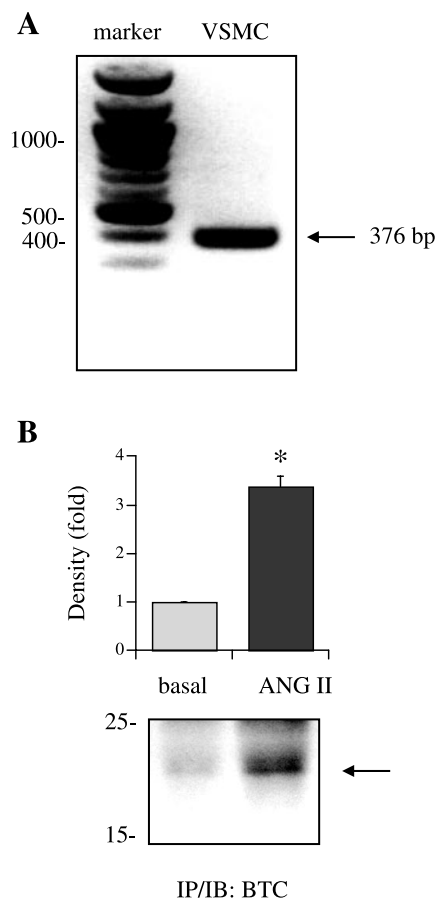


Fig. 6. Presence of endogenous BTC in VSMCs. **A**: 1  $\mu$ g of total RNA from cultured VSMCs was analyzed by RT-PCR using rat BTC-specific primers. **B**: VSMCs were stimulated by 100 nM angiotensin II for 1 h, and the conditioned medium was immunoprecipitated and immunoblotted with anti-BTC antibody. Arrow denotes  $\sim$ 20-kDa mature BTC. \* $P < 0.05$  vs. basal control.

important pathological role in vascular remodeling. AG-1478 is a well-recognized EGFR/ErbB1-selective kinase inhibitor. In the present study, the EGFR/ErbB1 kinase inhibitor AG-1478, which acts by competing with ATP (11), completely blocked ERK1/2 activation, DNA synthesis, and migration of VSMCs induced by BTC. However, because AG-1478 was reported to inhibit ErbB2 function by inducing an inactive heterodimer of EGFR/ErbB2 (2, 11), we could not distinguish the actions of these two receptors in mediating BTC function in VSMCs at this point. Shin et al. (28) reported that BTC induces tyrosine phosphorylation of ErbB3 and ErbB4 in addition to ErbB1 and ErbB2 in human aortic VSMCs in culture. Although we could detect transcripts of ErbB3 and ErbB4, we could not detect their protein expression in our VSMCs (Dempsey PJ and Eguchi S, unpublished observation). This may involve a difference in species; however, this may also indicate that ErbB3 and ErbB4 could be dispensable for BTC-induced growth and migration of VSMCs. The functional significance of each ErbB receptor in vascular diseases requires further examination.

Regarding postreceptor signal transduction, we showed that BTC activates ERK1/2, Akt, and p38 MAPK in VSMCs. Activation of each of these kinases by various agonists, such as angiotensin II, has been shown to be critically involved in growth promotion and/or migration of cultured VSMCs (8, 13, 25, 27, 33). In human aortic VSMCs, BTC-induced DNA synthesis and cell cycle progression were markedly inhibited by a phosphatidylinositol 3-kinase inhibitor, wortmannin, but not by an ERK inhibitor, PD-98059 (28), indicating a dominant role of phosphatidylinositol 3-kinase/Akt pathway in mediating VSMC growth by BTC. In contrast, our data showed a partial involvement of Akt as well as ERK in BTC-induced proliferation of rat VSMCs. The difference in these results might involve an Akt-independent growth pathway (ERK and/or other phosphatidylinositol 3-kinase downstream) operated by BTC in rat VSMCs as well as different responses to the PD-98059 concentration utilized. In addition, we have further addressed postreceptor signaling mediating VSMC migration induced by BTC. Our data indicate the requirement of ERK, Akt, as well as p38 MAPK to fully establish migration of VSMCs induced by BTC. This is consistent with the requirement of ERK, p38 MAPK, and phosphatidylinositol 3-kinase in VSMC migration induced by platelet-derived growth factor (16, 35).

In the present study, we demonstrated a transcript of membrane-anchored BTC (proBTC) by RT-PCR and release of mature BTC into the conditioned medium after angiotensin II stimulation. Together with the expression of BTC in human atherosclerotic lesions (28, 32), this result suggests that endogenous BTC produced from VSMC may be an important growth and migration factor involved in vascular disease states. It is now recognized that several G protein-coupled receptor ligands exert their growth-promoting action largely through the "trans"-activation of the EGFR (5, 9). In this signaling, metalloprotease-dependent cleavage of membrane-anchored EGF family ligand appears to be crucial (23). Our group recently reported that there is a major contribution of a metalloprotease-dependent EGFR ligand production in EGFR transactivation in VSMCs stimulated by angiotensin II (8) and reactive oxygen species (10). Very recently, EGFR transactivation by cleaved

BTC was reported in pancreatic beta cells (4). Together, our results suggest that BTC could be a ligand involved in the EGFR/ErbB transactivation in VSMCs.

In summary, this study found that BTC promotes proliferation and migration of VSMCs by activating EGFR/ErbB2 and downstream serine/threonine kinases. Together with the evidence of endogenous BTC expression and processing by angiotensin II in VSMCs, our findings suggest that production and signal transduction of BTC may mediate a critical pathological step of vascular disease. Therefore, our data provide a new mechanistic insight by which several risk factors induce vascular remodeling by possibly inducing BTC-mediated EGFR/ErbB2 signaling pathways.

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