Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 1307–1311, February 1995
Cell Biology

Angiogenesis: Role of calcium-mediated signal transduction

ELISE C. KOHN, RICCARDO ALESSANDRO, JOSEPH SPOONSTER, ROBERT P. WERSTO, AND LANCE A. LIOTTA

Signal Transduction and Prevention Unit, Laboratory of Pathology, National Cancer Institute, Bethesda, MD 20892

Communicated by George F. Vande Woude, Advanced BioScience Laboratories–Basic Research Program, Frederick, MD, September 28, 1994

ABSTRACT **During angiogenesis, endothelial cells react to stimulation with finely tuned signaling responses. The role of calcium-regulated signaling in angiogenesis has not been defined. This study investigated the calcium dependency of endothelial cell proliferation and invasion by using an inhibitor of ligand-stimulated calcium influx, CAI (carboxyamidotriazole). Incubation with CAI significantly inhibited proliferation of human umbilical vein endothelial cells (HUVECs) in response to serum (IC_{50} = 1 μM) or basic fibroblast growth factor (FGF2; $P_2 < 0.005$ at 10 μM). Statistically significant inhibition of HUVEC adhesion and motility to basement membrane proteins laminin, fibronectin, and type IV collagen was demonstrated (adhesion, $P_2 < 0.004$–0.01; motility, $P_2 < 0.009$–0.018). Marked inhibition of native and FGF2-induced gelatinase activity was shown by zymogram analysis and was confirmed by Northern blot analysis. CAI inhibited HUVEC tube formation on Matrigel and inhibited *in vivo* angiogenesis in the chicken chorioallantoic membrane assay, 67% at 20 μM and 56% at 10 μM compared with 16% for an inactive CAI analog or 9% for 0.1% dimethyl sulfoxide control. Incubation of HUVECs with CAI and/or FGF2 followed by immunoprecipitation with anti-phosphotyrosine antibody showed inhibition of FGF2-induced tyrosine phosphorylation of proteins in the range 110–150 kDa. These results suggest that calcium-regulated events are important in native and FGF2-stimulated HUVEC proliferation and invasion, perhaps through regulation of FGF2-induced phosphorylation events, and indicate a role for calcium in the regulation of angiogenesis *in vivo*.**

Angiogenesis, the formation of new blood vessels, occurs in a variety of normal and pathologic conditions (1). In physiological states such as embryonic growth and wound healing, neovascularization is strictly delimited and finely tuned by a balance of stimulatory and inhibitory angiogenic factors. These controls may fail and result in formation of a pathologic capillary network during the development of many diseases including cancer, diabetic retinopathy, hemangiomata, and vasculitides (2, 3). The angiogenic response is composed of the triad of protease secretion to facilitate basement membrane remodeling, proliferation of endothelial cells, and endothelial cell migration to form capillary sprouts and for lumen closure (2, 4).

Cell surface receptor stimulation may result in the activation of a variety of transmembrane signal transduction pathways, such as protein phosphorylation (5), ion flux (6), and phospholipid metabolism (7), which may be interconnected. For example, activation of cytosolic phospholipase A_2 requires influx of calcium in the range that occurs in response to activation of receptor-operated calcium channels (8). Calcium regulation of receptor-mediated phosphorylation events has also been described, such as downstream phosphorylation of phospholipase C-γ (PLC-γ) (9). The calcium requirement for tyrosine phosphorylation of PLC-γ also regulates the production of other second messengers, such as inositol trisphosphate and diacylglycerol, further interconnecting the signaling pathways. Thus, calcium homeostasis may regulate important cellular functions which are the end result of activation of these signal transduction pathways, such as proliferation, invasion, and differentiation (10–13). The role of calcium in regulation of angiogenesis both *in vivo* and *in vitro* has not been defined previously.

Carboxyamidotriazole (CAI) is an inhibitor of non-voltage-gated calcium channels including ionophore channels (14), receptor-operated channels (9, 14, 15), and refilling channels (E.C.K., unpublished observations) but does not chelate calcium (15). The calcium-sensitive pathways modulated by CAI include phospholipase A_2-induced generation of arachidonic acid and tyrosine phosphorylation of PLC-γ (9, 14, 15). No effect of CAI has been observed on non-calcium-sensitive production of inositol trisphosphate through PLC-β or on non-calcium-sensitive cAMP production (14). Modulation of calcium influx by CAI, and thus these receptor-activated transmembrane signaling pathways, has been shown to result in cytostatic inhibition of proliferation and invasive behavior of malignant cells *in vitro* and *in vivo*, including inhibition of matrix metalloproteinase 2 (MMP-2) gene expression (15). We have linked these biological properties in tumor cells to the inhibition of calcium-mediated pathways in tumor cells by using a family of CAI-related compounds (16). This family of compounds provides an important tool for elucidation of the role of calcium regulation in endothelial cell proliferation and invasion required for angiogenesis. The present study investigates the potential role of CAI and calcium-mediated signaling events in neovascularization.

MATERIALS AND METHODS

Reagents. Precast SDS/10% polyacrylamide gels containing gelatin (1 mg/ml) and 4–20% gradient polyacrylamide gels were purchased from Novex (San Diego). Recombinant basic fibroblast growth factor (FGF2) and transforming growth factor β_1 were from R&D Systems. Endothelial cell growth supplement, fibronectin, laminin, type IV collagen, thrombospondin, and Matrigel were obtained from Collaborative Research. Anti-phosphotyrosine monoclonal antibody (4G10) was from Upstate Biotechnology and anti-human FGF2 antibody was from Transduction Laboratories (Lexington, KY). Fertilized chicken eggs were from Truslow Farms (Chestertown, MD). Equipment and membranes for motility assays were from Neuroprobe (Cabin John, MD). All other reagents were of analytical or molecular grade.

Cell Culture. Human umbilical vein endothelial cells (HUVECs) (H930) were generously supplied by T. Maciag (American Red Cross, Gaithersburg, MD) and were used between passages 9 and 14 to avoid senescence as described (17). HUVECs were chosen because they were of human origin, are

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: CAI, carboxyamidotriazole; FGF2, basic fibroblast growth factor; PLC-γ, phospholipase C-γ; CAM, chicken chorioallantoic membrane; MMP, matrix metalloproteinase; HUVEC, human umbilical vein endothelial cell; DMSO, dimethyl sulfoxide; AIC, aminoimidazolecarboxamide.

primary cultures, and form microvessels under appropriate culture conditions.

Proliferation Assays. Growth inhibition of HUVECs by CAI was measured by crystal violet nuclear stain as described (16). To evaluate the effect of FGF2 on HUVEC growth, cells were plated in low serum medium (1% fetal calf serum) in the presence of FGF2 (10 ng/ml), heparin, and increasing concentrations of CAI. At the indicated time, cells were fixed, stained, and quantitated as described. Quantitative FGF2 immunofluorescence was done to evaluate the effect of CAI on FGF2 cell binding and uptake with HUVECs incubated with CAI (10 μM) or vehicle for 24 h. Cells were then chilled to 4°C, incubated with FGF2 (10 ng/ml) and heparin or control medium, washed, fixed (3.7% paraformaldehyde), and blocked with 50% goat serum. Cells were treated with control antibody or mouse anti-human FGF2 and fluorescein isothiocyanate-labeled goat anti-mouse IgG, and then fluorescence was measured (×400) using the IMAGE1/FLUOR software package (Universal Imaging, West Chester, PA).

Motility and Adhesion Assays. Chemotaxis was assayed by using the modified Boyden chamber as described for tumor cell motility with 2×10^6 cells per ml, 8-μm membrane pore size, and 2-h incubation (16, 18). Laminin (100 μg/ml), fibronectin (30 μg/ml), type IV collagen (100 μg/ml), or thrombospondin (20 μg/ml) was diluted into control medium (M-199 plus 0.1% bovine serum albumin) and used as chemoattractant. HUVECs were incubated with dimethyl sulfoxide (DMSO) control (0.1%) or CAI (0.1–10 μM) for 18 h prior to the motility assay. CAI and DMSO were present throughout the experiment. Five high-power fields per triplicate pellet were counted.

Adhesion of control and CAI-treated HUVECs was tested by using glass slides prepared by placement of 10-μl aliquots of 25 nM fibronectin, laminin, gelatin, or type IV collagen into triplicate wells of multichamber slides. Slides were incubated at 37°C for 2 h, air-dried overnight, and then blocked (50 μM Tris·HCl, pH 7.8, 111 mM NaCl, 5 mM $CaCl_2$/1% bovine serum albumin). HUVECs were pretreated as described above and resuspended at 1×10^6 cells per ml in control medium followed by addition to the coated wells (200 μl) and incubation at 37°C for 90 min. Five high-power fields per triplicate well were counted.

Gelatin Zymography. Gelatin zymography was performed as described (19). HUVECs were seeded in complete medium containing increasing concentrations of CAI (0.1–10 μM) or DMSO vehicle control. After 18 h of culture, the complete medium was removed and the cells were washed once with serum-free M-199 supplemented with bovine insulin (5 μg/ml), human transferrin (5 μg/ml), and sodium selenite (5 ng/ml) (ITS) followed by a further 24-h incubation with 3 ml of fresh ITS-containing M-199 with different concentrations of CAI (0.1–10 μM) or DMSO vehicle control (0.1%). To test the effect of CAI on cytokine-stimulated production of MMPs, the HUVECs were cultured as described above; however, FGF2 at a final concentration of 10 ng/ml was added for the final 24 h of incubation. Conditioned medium from HT1080 tumor cells was used as the standard for identification of the 92-kDa MMP-9 and the 72-kDa MMP-2 and was confirmed by Western immunoblot (antisera were gifts of W. Stetler-Stevenson, Laboratory of Pathology, National Cancer Institute).

Northern Blot Analysis. HUVECs were grown to confluence and then fresh complete medium with test compounds was added to confluent cells for an additional 24-h incubation. FGF2 (10 ng/ml) and/or CAI (10 μM) or DMSO control (0.1%) were added as indicated. Total RNA was isolated and aliquots (7.5 μg) were separated on 1% agarose/formaldehyde gels, blotted, and hybridized with a random-primed 300-bp insert for MMP-2 (gift of W. Stetler-Stevenson). Blots were washed under stringent conditions and then exposed to film. After removal of MMP-2 probe, blots were rehybridized with β-actin probe for assessment of the RNA load.

Growth of HUVECs on Matrigel. Matrigel was used to test the effect of CAI on vascular tube formation as described (20). CAI (1.0, 10, or 20 μM) or DMSO control (0.1%) was added to Matrigel and to the culture medium. HUVECs were incubated on the Matrigel in complete medium for 24 h and then evaluated by phase-contrast microscopy and photographed. Similar results were seen when CAI was present only in the medium.

Chicken Chorioallantoic Membrane (CAM) Assay. The ability of CAI to inhibit angiogenesis *in vivo* was evaluated by the CAM assay (21). On day 4 of development, methylcellulose disks (10 μl of 0.45% methylcellulose) containing DMSO vehicle, hydrocortisone (60 μg), CAI, or AIC (aminoimidazolecarboxamide) inactive control were placed on the yolk sac membrane. The zone around the methylcellulose disk was observed 48 h after disk placement for inhibition of vascular development.

Phosphotyrosine Immunoprecipitation and Immunoblot. HUVECs were grown to confluence, starved with 1% FCS, and preincubated with CAI (10 μM) for 24 h. Cells were exposed to FGF2 (10 ng/ml) for the final 15 min, washed, scraped, and lysed (50 mM Tris·HCl, pH 7.6/300 mM NaCl/10 μg of aprotinin per ml/1 mM phenylmethylsulfonyl fluoride/400 μM sodium orthovanadate/400 μM EDTA/100 mM sodium fluoride/100 mM sodium pyrophosphate/0.5% Triton-X 100) for 1 h on ice. Lysates were centrifuged (4°C, 1200 × *g*, 15 min) and supernatant protein concentration was measured. Lysate was incubated with 10 μg of anti-phosphotyrosine monoclonal antibody 4G10 preconjugated to protein A-Sepharose beads, washed (buffer with 0.1% Triton X-100), eluted with sample buffer, electrophoresed, and transferred (18). Immunoblots were incubated overnight with anti-phosphotyrosine monoclonal antibody 4G10 (1 μg/ml), washed, and visualized with 0.5 μCi of ^{125}I-labeled protein A per ml (1 Ci = 37 GBq).

RESULTS

Dose-Dependent Inhibition of HUVEC Proliferation. The effect of CAI on HUVEC proliferation was examined over a

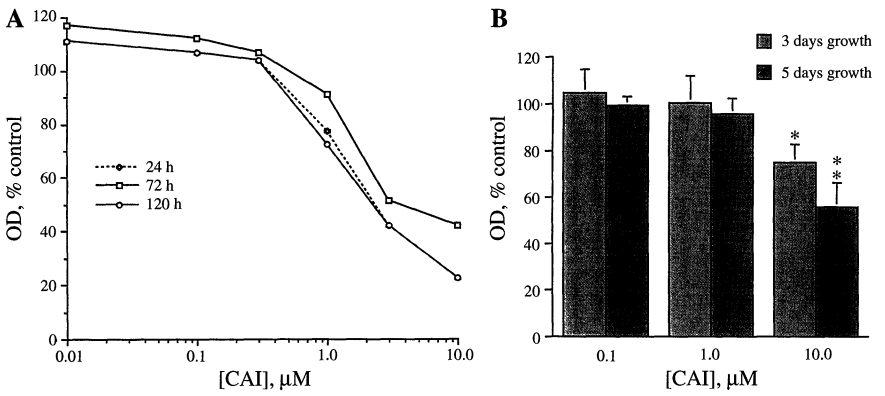

FIG. 1. Effect of CAI on basal and FGF2-stimulated HUVEC growth. Data (mean ± SEM) are expressed as % of control ($n = 3$). (*A*) Serum with endothelial cell growth supplement. HUVECs were grown in 0.1% DMSO vehicle or CAI (0.01–10 μM) as described for 24, 72, and 120 h. (*B*) HUVECs were treated with FGF2 in low serum and assessed after 3 and 5 days. Addition of 10 μM CAI caused a statistically significant inhibition of growth of 25% (*, $P_2 < 0.01$; Student's *t* test) and 45% (**, $P_2 < 0.005$) of control after 3 and 5 days.

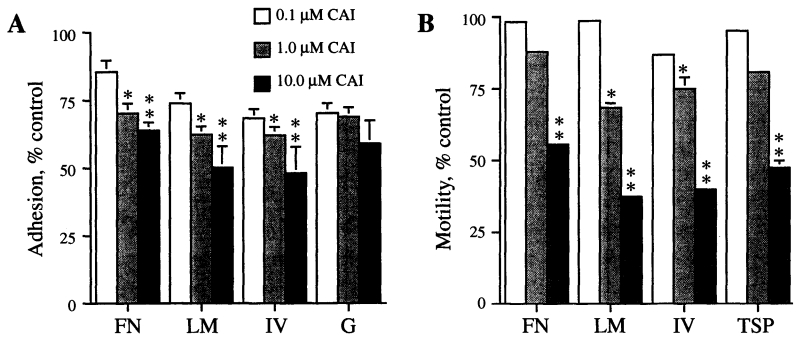

FIG. 2. Effect on adhesion and migration in response to basement membrane components. Data are expressed as % adhesion and motility of control cells and represent mean ± SEM (n = 3). (*A*) Statistically significant inhibition of HUVEC adhesion to fibronectin (FN), laminin (LM) and type IV collagen (IV) (25 nM) at 1 μM CAI (*, $P_2 < 0.043$, 0.04, and 0.027; Student's t test) and 10 μM CAI (**, $P_2 < 0.01$, 0.004, and 0.025) is shown. G, gelatin. (*B*) Motility assays were performed as described. CAI caused a statistically significant dose-dependent inhibition of HUVEC motility toward laminin and type IV collagen at 1 μM (*, $P_2 < 0.009$ and 0.018) and toward all attractants at 10 μM (**, $P_2 < 0.001$). TSP, thrombospondin.

5-day period of culture with increasing concentrations of CAI. CAI had no effect at concentrations up to 0.1 μM but inhibited endothelial cell growth in a dose-dependent manner above 0.5 μM at all time points (Fig. 1*A*). The IC_{50} values at 1, 3, and 5 days of culture were 1.31 ± 0.03 μM, 2.14 ± 0.03 μM, and 2.08 ± 0.01 μM, respectively. DMSO vehicle did not influence endothelial cell proliferation. CAI (10 μM) inhibited HUVEC growth 25% and 45%, respectively, 3 and 5 days after seeding in serum-limited medium with added FGF2 ($P_2 < 0.010$ and 0.005, respectively) (Fig. 1*B*). The effect on FGF2 interaction with HUVECs was determined by quantitative immunofluorescence using antibody to human FGF2 after the cells were exposed to FGF2 at 4°C. The immunofluorescence FGF2 staining patterns observed showed slight cytoplasmic staining and intense nuclear localization as described (22, 23). Treatment with 10 μM CAI for 24 h did not affect FGF2 staining: FGF2 (10 ng/ml), 52 ± 14 relative fluorescence intensity units; CAI (10 μM) + FGF2 (10 ng/ml), 63 ± 19 units, 37 ± 11 antibody only, and 40 ± 12 antibody on CAI-treated cells. These results indicate that CAI can markedly inhibit proliferation of HUVECs in the presence of serum- or FGF2-containing growth conditions.

Inhibition of Adhesion and Stimulated Motility. Basement membrane glycoproteins may be chemotactic to endothelial cells during vascular sprout formation. Adhesion of HUVECs to all basement membrane components was inhibited in a dose-dependent fashion by CAI (0–10 μM; Fig. 2*A*). This was statistically significant for laminin, fibronectin, and collagen type IV at 1.0 μM ($P_2 < 0.04$, 0.043, and 0.027, respectively) and 10.0 μM ($P_2 < 0.004$, 0.011, and 0.025, respectively). Preincubation of HUVECs with CAI did not significantly inhibit adhesion to gelatin, which was chosen as the substratum for filter coating for the motility assays. A reproducible dose-dependent inhibition of HUVEC motility was found after CAI treatment (Fig. 2*B*). A statistically significant inhibition of migration was demonstrated at 1.0 μM CAI for laminin and collagen type IV ($P_2 < 0.009$ and 0.018, respectively) and for all ligands by 10.0 μM CAI ($P_2 < 0.001$). These findings demonstrate selective inhibition of HUVEC adhesion and migration to extracellular matrix components.

Inhibition of HUVEC MMP-2 Gelatinolytic Activity by CAI. We have previously demonstrated that CAI treatment decreases MMP-2 gene expression in cancer cells (15). CAI treatment of HUVECs caused a dose-dependent inhibition of pro- and active MMP-2 (Fig. 3*A*). Densitometric analysis showed that 10.0 μM CAI caused a 50% decrease in MMP-2. No gelatinolytic activity corresponding to MMP-9 was detectable in the conditioned medium of HUVECs. We tested the effect of CAI on the levels of MMPs in HUVECs after incubation with FGF2 (10 ng/ml). CAI (10 μM) reduced FGF2-stimulated synthesis of MMP-2 in HUVECs (90% inhibition; Fig. 3*B*). Northern blot analysis (Fig. 3*C*) shows the inhibition of MMP-2 expression in HUVECs in response to CAI alone or FGF2 plus CAI. These results show that CAI inhibits the production of MMP-2 in HUVECs under native and FGF2-induced conditions and indicates that part or all of this effect may occur at the level of expression.

Inhibition of Vascular Tube Formation on Matrigel. When plated on Matrigel (24), HUVECs formed vessel-like structures from endothelial cell protrusions and had increased cellularity at the central zones (Fig. 4*A*). In contrast, the addition of 10 μM CAI both in Matrigel and in culture medium caused an inhibition of Matrigel-induced network formation (Fig. 4*B*). The tubes were less extensive, thinner, foreshortened, and less cellular when compared with DMSO controls. Treatment with 20 μM CAI resulted in a more striking inhi-

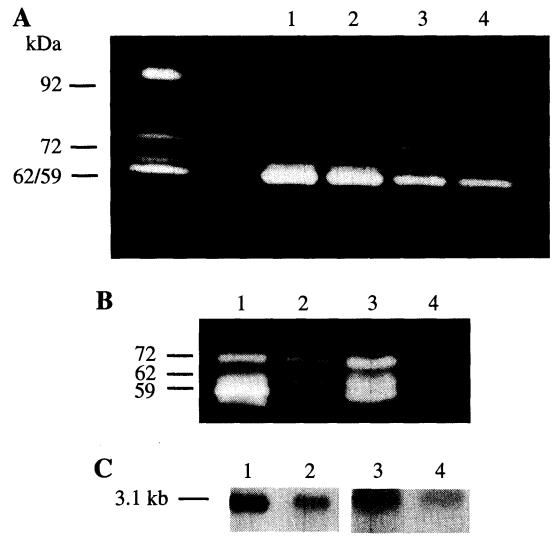

FIG. 3. Effect of CAI on HUVEC gelatinase activity and expression. (*A*) Dose-dependent inhibition of gelatinase activity. Lanes: 1, conditioned medium from DMSO-treated HUVECs; 2–4, conditioned medium from HUVECs treated with 0.1, 1, and 10 μM CAI, respectively. Conditioned medium from HT1080 human fibrosarcoma cells was used as a standard for the MMP-9 (92 kDa) and MMP-2 (72, 62/59 kDa) gelatinases as labeled. (*B*) Inhibition of FGF2-stimulated gelatinase activity. Lanes: 1, DMSO-treated HUVECs; 2, CAI (10 μM); 3, FGF2 (10 ng/ml); 4, FGF2 (10 ng/ml) and CAI (10 μM). (*C*) Northern analysis. mRNA from control (lane 1), CAI (10 μM) (lane 2), FGF2 (10 ng/ml) + CAI (10 μM) (lane 3), or FGF2 (10 ng/ml) (lane 4) was electrophoresed and probed as described for MMP-2 (3.1 kb).

bition of tube formation; similar results were obtained when CAI was omitted from the Matrigel (data not shown).

Inhibition of Angiogenesis *in Vivo*. The CAM assay is an important *in vivo* model of microvessel formation (12, 21). A marked inhibition of angiogenesis (Fig. 5 *B* and *D*) was seen on examination 2 days after CAI-impregnated disks were placed at the advancing edge of the vascular membrane when compared to DMSO vehicle-treated controls (Fig. 5*A*). The angiogenesis inhibitory effect of CAI was dose dependent (Table 1) with inhibition seen in 67% of embryos exposed to 20 μM CAI (Table 1). No effect was produced by AIC, an inactive structural analog of CAI (16). These findings indicate that CAI inhibited angiogenesis *in vivo*.

CAI Inhibits Tyrosine Phosphorylation in Response to FGF2. FGF2 exerts its activity through tyrosine phosphorylation (25). DMSO (Fig. 6, lane 1) or CAI (lane 2) treatment of HUVECs did not alter basal tyrosine phosphorylation. Incubation of HUVECs with CAI for 18 h and with FGF2 for the final 15 min resulted in inhibition of FGF2-stimulated phosphorylation (lanes 3 and 4). The size of the broad affected band is 110–150 kDa, representing the size range for the FGF2 receptor, PLC-γ, and the 110-kDa subunit of phosphatidylinositol 3′-kinase, among other proteins.

DISCUSSION

Invasion is the process through which cells encroach upon surrounding tissue and violate the integrity of that tissue. It is the hallmark of malignancy; however, the invasive process of adhesion, proteolysis, and migration is not limited to cancer. The role of calcium in the regulation of invasion is now emerging (26). It is a key component of cellular adhesion pathways, important in proteolytic degradation of the basement membrane, and is involved in migration in response to motility factors and extracellular matrix components (13, 16, 27). CAI has been used as a tool to confirm the role of calcium-mediated events in invasion (13, 15, 16, 27–29). Angiogenesis is a form of regulated invasion. We now report a role for calcium-mediated signal transduction in angiogenesis as demonstrated by the antiangiogenic efficacy of an inhibitor of calcium-mediated signal transduction, CAI. In concentrations that inhibit calcium influx and calcium-mediated signaling pathways in malignant cells (9, 14, 16), CAI inhibited adhesion, collagenolytic activity, migration, and proliferation of human endothelial cells in culture and capillary outgrowth *in vivo*, as well as tyrosine phosphorylation in response to FGF2.

Acidic and basic FGF (FGF2) are the best characterized and most potent angiogenic factors described (30, 31). Relatively little is known about the mechanisms involved in signal transduction in endothelial cells after their binding to the receptor other than activation of receptor tyrosine kinase activity. This receptor tyrosine kinase can cause autophosphorylation, which represents a very early response to FGF receptor stimulation (25). A role for calcium in the FGF2 signal transduction pathway in endothelial cells has not been elucidated. We have previously shown that CAI can inhibit calcium-mediated tyrosine phosphorylation stimulated by m5 muscarinic receptor activation in m5-transfected CHO cells (9). We now show

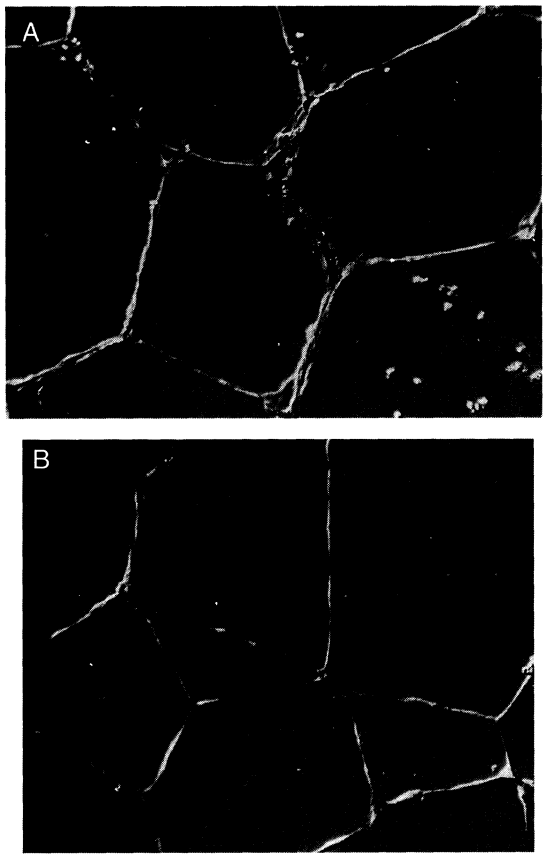

FIG. 4. Influence of CAI treatment on *in vitro* tube formation. HUVECs were plated on Matrigel-coated wells as described. Cultures were photographed after 24 h of incubation. (×80.) (*A*) 0.1% DMSO. (*B*) 10 μM CAI.

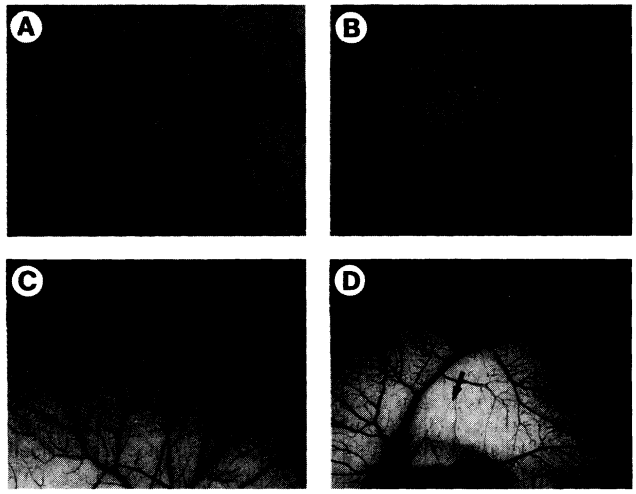

FIG. 5. Inhibition of *in vivo* angiogenesis by CAI. After 4 days of development, a 0.5% methylcellulose disk (arrow) containing CAI (10 or 20 μM), AIC (10 μM, inactive CAI analog), or DMSO (0.05%) was placed at the advancing edge of the vascular membrane of the CAM. After 48 h of exposure to CAI, the membranes were evaluated and photographed. (*A*) DMSO vehicle control. (×30.) (*B*) CAI (20 μM). (×30.) (*C*) AIC. (×7.) (*D*) CAI (20 μM). (×7.)

Table 1. CAI inhibits capillary formation *in vivo*

Agent	Eggs with avascular zones/total	% inhibition*
CAI (10 μM)	19/34	55
CAI (20 μM)	20/30	67
AIC (10 μM)	4/24	16
DMSO (0.05%)	5/42	9
Hydrocortisone (60 μg)	10/18	55

*Percentage inhibition of angiogenesis on yolk sac membrane by CAI. CAI, tested at 10 and 20 μM in 10 μl of 0.45% methylcellulose, caused an inhibition of angiogenesis of 55% and 67%, respectively. AIC, an inactive CAI analogue (16), was not effective when tested on chicken yolk sac membrane.

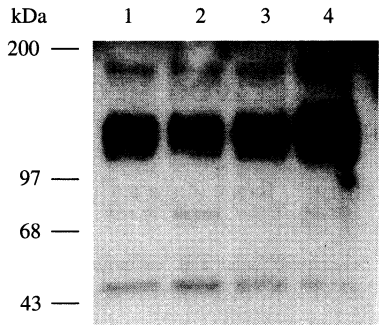

FIG. 6. CAI inhibits phosphotyrosine phosphorylation events in FGF2-stimulated HUVEC cells. Confluent HUVEC cultures were treated with DMSO vehicle control (lane 1), CAI (10 μM) (lane 2), FGF2 (10 ng/ml) with CAI (10 μM) (lane 3), and FGF2 (10 ng/ml) (lane 4) as described. Incubation with both CAI and FGF2 resulted in a marked inhibition of tyrosine phosphorylation of proteins in the range 110–150 kDa. Migration of size standards is indicated on the left.

that CAI inhibited FGF2-mediated tyrosine phosphorylation in stimulated endothelial cells.

The role of tyrosine phosphorylation responsible for the angiogenic properties of FGF2 has been suggested by the work of Montesano and others (30, 32). They observed that treatment of endothelial cells with vanadate, an inhibitor of phosphotyrosine phosphatases, elicited morphologic and biochemical changes similar to that induced by FGF2. These modifications included increases in plasminogen activator levels and formation of tubules resembling blood capillaries. Genistein, an inhibitor of tyrosine phosphorylation (33), has been shown to have antiangiogenic properties (34). Inhibition of tyrosine phosphorylation by genistein was proposed but not demonstrated to be the mechanism by which genistein inhibited angiogenesis.

FGF2 has been linked to calcium-regulated signal transduction pathways. Presta and coworkers (35) studied FGF2 in GM 7373 cells, an immortalized endothelial cell line, and showed that FGF2 acts through two distinct signaling pathways. A protein kinase C-dependent pathway was shown to be involved in endothelial cell mitogenic activity and a calcium-dependent but protein kinase C-independent pathway was shown to trigger the plasminogen activator cascade. An increase in intracellular endothelial cell calcium concentration has been shown to be necessary for the migration of neutrophils through an endothelial cell monolayer (36). A stimulation of arachidonic acid release from endothelial cells in response to the combination of A23187, a calcium ionophore, and FGF2 has also been shown (37). Cytosolic phospholipase A_2 requires an increase in intracellular calcium for activation and subsequent translocation to the membrane and activation (8).

The process of angiogenesis requires the triad of proliferation, local protease activity, and endothelial cell migration. All of these can be stimulated by angiogenesis factors such as FGF2 and may be regulated further through endothelial cell–basement membrane interactions. FGF2-mediated endothelial cell signals offer a target for inhibition of angiogenesis. We have demonstrated a role for calcium mobilization in the regulation of FGF2-induced tyrosine phosphorylation, a key step in activation of the angiogenesis triad, by using an inhibitor of receptor-operated calcium influx. Inhibition of FGF2 signals by CAI caused inhibition of endothelial cell proliferation and proteolysis *in vitro* and *in vivo*. Furthermore, CAI inhibited endothelial cell adhesion and migration in response to basement membrane components to which they are exposed *in vivo* during neoangiogenesis. The antiangiogenic effects of CAI, through inhibition of FGF2-induced tyrosine phosphorylation, may be a further mechanism by which CAI is an effective cancer treatment and prevention agent.

We thank Mr. William Jacobs for his technical support and Drs. Susan Mackem and Marsha Moses for helpful discussion.

1. Folkman, J. & Shing, Y. (1992) *J. Biol. Chem.* **267,** 10931–10934.
2. Folkman, J. (1992) *Semin. Cancer Biol.* **3,** 65–71.
3. Bouck, M. (1990) *Cancer Cells* **2,** 179–185.
4. Folkman, J. & Klagsbrun, M. (1987) *Science* **235,** 442–447.
5. Yardin, Y. & Ulrich, A. (1988) *Annu. Rev. Biochem.* **57,** 443–447.
6. Meldolesi, J., Clementi, E., Fusolato, C., Zacchetti, D. & Pozzan, T. (1991) *Trends Pharmacol. Sci.* **12,** 289–292.
7. Berridge, M. J. & Irvine, R. F. (1989) *Nature (London)* **341,** 197–205.
8. Ling, L., Waratmann, M., Lin, A. Y., Knopf, J. L., Seth, A. & Davis, R. J. (1993) *Cell* **72,** 269–278.
9. Gusovsky, F., Lueders, J. E., Kohn, E. C. & Felder, C. C. (1993) *J. Biol. Chem.* **268,** 7768–7772.
10. Felder, C. C., MacArthur, L., Ma, A. L., Gusovsky, F. & Kohn, E. C. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 1706–1710.
11. Moody, T. W. & Cuttitta, F. (1993) *Life Sci.* **52,** 1161–1173.
12. Takigawa, M., Nishida, Y., Suzuki, F., Kishi, J., Yamashita, K. & Hayakawa, T. (1990) *Biochem. Biophys. Res. Commun.* **171,** 1264–1271.
13. Savarese, D. M. F., Russell, J. T., Fatatis, A. & Liotta, L. A. (1992) *J. Biol. Chem.* **267,** 21928–21935.
14. Felder, C. C., Ma, A. L., Liotta, L. A. & Kohn, E. C. (1991) *J. Pharmacol. Exp. Ther.* **257,** 967–971.
15. Kohn, E. C., Jacobs, W., Kim, Y. S., Alessandro, R., Stetler-Stevenson, W. G. & Liotta, L. A. (1994) *J. Biol. Chem.* **269,** 21505–21511.
16. Kohn, E. C., Felder, C. C., Jacobs, W., Holmes, K. A., Day, A. F., Freer, R. & Liotta, L. A. (1994) *Cancer Res.* **54,** 935–942.
17. Bavisotto, L. M., Schwartz, S. M. & Heimark, R. L. (1990) *J. Cell. Physiol.* **143,** 39–51.
18. Minniti, C. P., Kohn, E. C., Grubb, J. H., Sly, W. S., Oh, Y., Muller, H. L., Rosenfeld, R. G. & Hellman, L. J. (1992) *J. Biol. Chem.* **267,** 9000–9004.
19. Brown, P. D., Levy, A. T., Margulies, I. M. K., Liotta, L. A. & Stetler-Stevenson, W. G. (1990) *Cancer Res.* **50,** 6184–6191.
20. Albini, A., Repetto, L., Carlone, S., Benelli, R., Soria, M., Monaco, L., Gendelman, R., Defilippi, P., Bussolino, F. & Parravicini, C. (1992) *Int. J. Oncol.* **1,** 723–730.
21. Taylor, S. & Folkman, J. (1982) *Nature (London)* **297,** 307–311.
22. Yamamoto, N., Matsutani, S., Yoshitake, Y. & Nishikawa, K. (1991) *Histochemistry* **96,** 479–485.
23. Dell'Rea, P. & Presta, M. (1991) *Exp. Cell Res.* **192,** 505–510.
24. Kubota, Y., Kleinman, H. K., Martin, G. R. & Lawley, T. J. (1988) *J. Cell Biol.* **107,** 1589–1598.
25. Jaye, M., Schlessinger, J. & Dioone, C. A. (1992) *Biochim. Biophys. Acta* **1135,** 185–199.
26. Cole, K. A. & Kohn, E. C. (1994) *Cancer Metastasis Rev.* **13,** 33–41.
27. Kohn, E. C. & Liotta, L. A. (1990) *J. Natl. Cancer Inst.* **82,** 54–60.
28. Kohn, E. C., Liotta, L. A. & Schiffmann, E. (1990) *Biochem. Biophys. Res. Commun.* **166,** 757–764.
29. Kohn, E. C., Sandeen, M. A. & Liotta, L. A. (1992) *Cancer Res.* **52,** 3208–3212.
30. Rifkin, D. B. & Moscatelli, D. (1989) *J. Cell Biol.* **109,** 1–6.
31. Gospodarowicz, D. (1992) *Ann. N.Y. Acad. Sci.* **638,** 1–9.
32. Montesano, R., Pepper, M. S., Belin, D., Vassalli, J.-D. & Orci, L. (1988) *J. Cell. Physiol.* **134,** 460–466.
33. Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H., Watanabe, S., Itoh, N., Shibuya, M. & Fukami, Y. (1987) *J. Cell Biol.* **262,** 5592–5595.
34. Fotsis, T., Pepper, M., Adlercreutz, H., Fleischmann, G., Hase, T., Montesano, R. & Schweigerer, L. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 2690–2694.
35. Presta, M., Maier, J. A. M. & Ragnotti, G. (1989) *J. Cell Biol.* **109,** 1877–1884.
36. Huang, A. J., Manning, J. E., Bandak, T. M., Ratau, M. C., Hanser, K. R. & Silverstein, S. C. (1993) *J. Cell Biol.* **120,** 1371–1380.
37. Fafeur, V., Jiang, Z. P. & Bohlen, P. (1991) *J. Cell. Physiol.* **149,** 277–283.