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Carboxyamido-triazole Inhibits Angiogenesis by Blocking the Calcium-Mediated Nitric-Oxide Synthase-Vascular Endothelial Growth Factor Pathway[1]

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ABSTRACT

The induction of angiogenesis is known to play a critical role in the successful growth, invasion, and metastasis of a tumor. A tumor will not grow beyond a few cubic millimeters without the formation of its own capillary network. Several antiangiogenic agents are under investigation in the clinic setting for the treatment of cancer. Carboxyamido-triazole (CAI), an inhibitor of Ca^{2+}-mediated signal transduction, has been previously shown to inhibit angiogenesis in vitro and in vivo and to down-regulate matrix metalloproteinase-2 in vitro. Diminished levels of intracellular Ca^{2+} result in decreased nitric-oxide synthase (NOS) activity and thereby inhibit the production and release of NO. The antiangiogenic activity of CAI was investigated by assessing microvessel growth from rat aortic segments and in cell culture using human aortic endothelial cells (HAECs). With these models, vascular endothelial growth factor (VEGF) and NOS production and secretion were evaluated. CAI concentrations ranging from 0.25 to 12.0 μg/ml inhibited new microvessel formation in rat aortic cultures and HAEC proliferation in a dose-dependent manner. Additionally, HAECs treated with CAI showed a dose-dependent decrease of NOS expression and a decrease in both VEGF expression and secretion. Rat aortic segments demonstrated decreased VEGF expression in situ on immunostaining. These data suggest that modulation of the NOS-NO-VEGF pathway through Ca^{2+}-mediated signaling by CAI inhibits angiogenesis in vitro.

Angiogenesis, the recruitment and formation of new blood vessels, plays a key role in the growth and survival of developing tumors. It is now well established that unless an intratumoral capillary network is constructed, the tumor will not grow beyond several cubic millimeters (Folkman, 1966; Gimrone et al., 1969). In 1971, Folkman reported that tumor growth is dependent on angiogenesis and suggested inhibition of angiogenesis as a novel approach to the treatment of human cancers (Folkman, 1971). These findings led to the current explosion of research into how angiogenic mechanisms might be manipulated for the treatment of human tumors (Weidner et al., 1991).

Carboxyamido-triazole (CAI) is an inhibitor of signal transduction via nonvoltage-gated Ca^{2+} channels (Hupe et al., 1990). It is proposed that CAI exerts its antiproliferative properties through inhibition of downstream phosphorylation events involving phospholipase-Cγ and inositol triphosphate (Hupe et al., 1990, 1991; Kohn and Liotta, 1990; Gusovsky et al., 1993). CAI inhibits the proliferation and invasive characteristics of several tumor cell lines in vitro, including prostate (Wasilenko et al., 1996), glioblastoma (Lambert et al., 1997), and breast (Jacobs et al., 1997). CAI also demonstrated antiangiogenic activity in the chick chorioallantoic membrane assay, as well as inhibiting the proliferation of human umbilical vein endothelial cells (HUVECs) in vitro (Kohn et al., 1995). It is hypothesized that CAI exerts its antiangiogenic, anti-invasive, and antimetastatic effects by down-regulating key cellular regulatory proteins, including matrix metalloproteinase-2 (Kohn et al., 1994). CAI is under investigation in several phase I studies, as well as phase II studies in glioblastoma, prostate, and ovarian carcinomas (Figg et al., 1995; Berlin et al., 1997; Kohn et al., 1997). It is proposed that antiangiogenic activity of CAI is the mechanism involved in the treatment of solid tumors. In the present study, we investigated one possible pathway to explain the antiangiogenic actions of CAI by using several in vitro models of angiogenesis.

Materials and Methods

Reagents. CAI (NSC 609974) was obtained from the Developmental Therapeutics Program, National Cancer Institute (Rockville,

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ABBREVIATIONS: CAI, carboxyamido-triazole; NO, nitric oxide; NOS, nitric-oxide synthase; DMSO, dimethyl sulfoxide; EBM-II, endothelial cell basal medium; EGM-II, endothelial cell growth medium; VEGF, vascular endothelial growth factor; HUVEC, human umbilical vein endothelial cell; ecNOS, endothelial cell nitric-oxide synthase; HAEC, human aortic endothelial cell.

MD). Doxorubicin was purchased from Sigma Chemical Co. (St. Louis, MO). Suramin was obtained from the Division of Cancer Treatment, National Cancer Institute (Rockville, MD) via Parke-Davis (Ann Arbor, MI). TNP-470 was the gift of Takeda Chemical Industries, Ltd. (Okasaka, Japan). All drugs were prepared as stock solutions in dimethyl sulfoxide (DMSO; Sigma Chemical Co.) and then diluted into culture medium. The final amount of DMSO (vehicle) present in the culture medium was 0.5% (v/v) for all studies performed. The human prostate carcinoma cell line LNCaP was obtained from American Type Culture Collection (Manassas, VA). Human aortic endothelial cells (HAECs), endothelial cell basal medium (EBM-II), and endothelial cell growth medium (EGM-II) were purchased from Clonetics Corporation (San Diego, CA). EGM-II consists of EBM-II after the addition of endothelial growth factors provided as the EGM-II Bulletkit. Matrigel was purchased from Collaborative Biomedical (Bedford, MA). All other cell culture reagents were obtained from Life Technologies (Gaithersburg, MD). Cell lines were grown as directed. The culture of HAECs was limited to seven serial passages, and LNCaP was not used after passage 30. The Quantikine Human VEGF Immunoassay was purchased from R&D Systems (Minneapolis, MN). Protease inhibitors (complete, mini) were purchased from Roche Biochemicals (Indianapolis, IN). Mouse anti-human endothelial cell nitric-oxide synthase (ecNOS, NOS 3) antibody was obtained from Transduction Laboratories (Lexington, KY). The mouse anti-human VEGF antibody (detects the 165-, 189-, and 206-amino acid isoforms) used for immunobloting as well as the immunostaining of rat aortae was purchased from PharMingen (San Diego, CA). Biotinylated goat anti-mouse IgG and the CSPD Western Light chemiluminescence detection kit was obtained from Tropix (Bedford, MA).

Measurement of HAEC Proliferation. HAECs were seeded onto 12-well plates at a density of 5000 cells/well. Cells were allowed to attach for 24 h at 37°C and 5% CO_2. The culture medium was aspirated after 24 h, and fresh culture medium containing the appropriate treatment was added. Culture medium (EGM-II) consisted of EBM-II containing 2% (v/v) FBS, with the addition of the EGM-II Bulletkit. The treatment groups consisted of EGM-II with or without vehicle or EGM-II containing 0.25, 1.0, 4.0, or 12.0 μg/ml CAI. Cell proliferation was assessed daily for 5 days by trypsinization of adherent cells and counting on a Coulter Z1 counter (Coulter Corp., Hialeah, FL). Inhibition of proliferation was assessed by fitting the Hill equation (as follows) to the percent decrease in cell number for each treatment group on day 5:

$$\%\ \mathrm{I} = \frac{\%\ \mathrm{I}_{max} * \mathrm{C}^{s}}{\mathrm{IC}_{50} + \mathrm{C}^{s}}$$

where % I is the cell count represented as percentage of control cell count, % I_{max} is the maximum possible percent inhibition (fixed as 100%), IC_{50} is the concentration of CAI needed to obtain a 50% inhibition, C is the concentration of CAI, and s is the slope factor.

Rat Aortic Ring Culture. Thoracic aortas were carefully excised from juvenile male Sprague-Dawley rats, and the fibroadipose tissue was removed using fine forceps. Under a dissecting microscope, the aorta was dissected into 1-mm-long cross sections and then rinsed eight times with EGM-II. Approximately 200 μl of Matrigel was added to each well of a 12-well plate and allowed to gel at 37°C for 30 min. Each aortic ring was placed into a well containing the set Matrigel and then covered with an additional 300 μl of Matrigel. After the second addition of Matrigel had set, the rings were covered with EGM-II and incubated overnight at 37°C and 5% CO_2. After the overnight incubation, the culture medium was changed to EBM-II with 2% FBS, 0.25 μg/ml amphotericin B, and 10 μg/ml gentamicin (no Bulletkit was added). Each aortic section was exposed to either vehicle or 0.25, 1.0, 4.0, or 12.0 μg/ml CAI. Additional control rings were exposed to 60 ng/ml doxorubicin, 10 μg/ml suramin, or 250 ng/ml TNP-470. All drugs were tested at clinically achievable concentrations. Aortic preparations were cultured for 6 days, with microvessel growth assessed daily. The presence of endothelial cells in new microvessel growths was confirmed by staining the aortic sections for factor VIII and CD34 as previously described (Pinkus et al., 1986; Weidner et al., 1993; Horti et al., 1999). Additionally, rat aortic segments treated with 4.0 μg/ml CAI or vehicle were rinsed thoroughly with PBS, pH 7.4, and frozen at −70°C in O.C.T. compound (Sakura Finetek, Torrance, CA). These rings were cut into 8-μm sections and immunostained for VEGF by Molecular Histology, Inc. (Gaithersburg, MD).

Measurement of VEGF Secretion. HAECs and LNCaP cells were seeded onto 12-well plates at a density of 30,000 cells/well. Cells were allowed to attach for 24 h at 37°C and 5% CO_2. Culture medium for HAECs consisted of EBM-II with 2% FBS, 0.25 μg/ml amphotericin B, and 10 μg/ml gentamicin (no Bulletkit was added); culture medium for the LNCaP cell line consisted of RPMI-1640 containing 10% certified heat-inactivated FBS, 2 mM L-glutamine, and 10 μg/ml gentamicin. Each cell line was treated for 72 h with culture medium alone, culture medium containing vehicle, or culture medium with 0.25, 1.0, 4.0, or 12.0 μg/ml CAI. The conditioned medium was removed for the measurement of vascular endothelial cell growth factor (VEGF) secretion, and the adherent cells were trypsinized and counted. The amount of VEGF secreted into the conditioned culture medium was determined using the Quantikine Human VEGF Immunoassay kit. VEGF secretion was calculated as the amount of VEGF secreted into the conditioned medium per cell per well.

Expression of NOS. HAECs were grown to 80% confluency in 80-cm^2 tissue culture flasks in EGM-II. The cells were split at a ratio of 1:3 into fresh 80-cm^2 flasks and exposed to either EGM-II, EGM-II with vehicle, or EGM-II with CAI at a final concentration of 0.25, 1.0, 4.0, or 12.0 μg/ml. After 72 h, cells were harvested by scraping, and the pellet was washed in PBS, pH 7.4, containing protease inhibitors. Cytosolic extracts and immunoblots were prepared as previously described (Horti, 1999). Blots were developed using mouse anti-human endothelial cell NOS antibody followed by incubation with biotinylated goat anti-mouse IgG. Detection was done using the CSPD Western Light chemiluminescence detection kit.

Expression of VEGF. HAECs were grown to 80% confluency in 80 cm^2 tissue culture flasks in EGM-II. The cells were split at a ratio of 1:3 into fresh 80 cm^2 flasks and exposed to either EGM-II, EGM-II

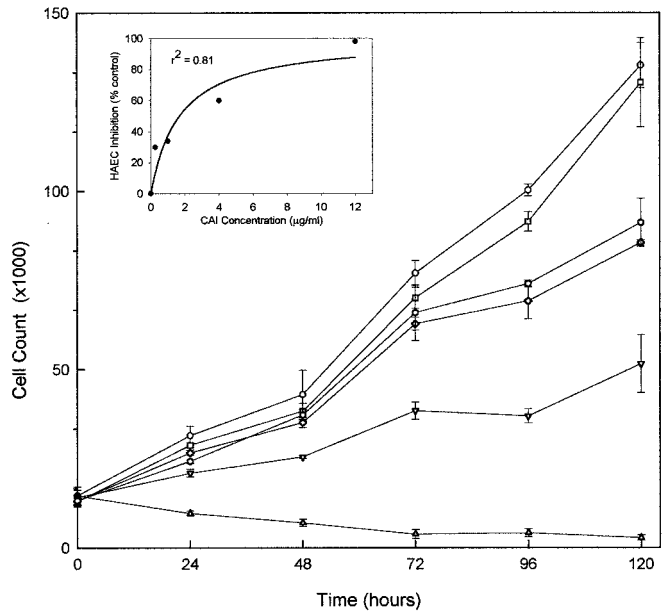

Fig. 1. Cell viability of HAECs plated at 5000 cells/well incubated in EGM-II alone (○) and with vehicle (□) (0.5% DMSO) or 0.25 (○), 1.0 (◇), 4.0 (▽), and 12.0 (△) μg/ml CAI for 5 days. Inset, HAEC percent inhibition by CAI at 120 h. Solid line is the line of best fit using the Hill equation (r^2 = 0.81). IC_{50} = 1.53 μg/ml.

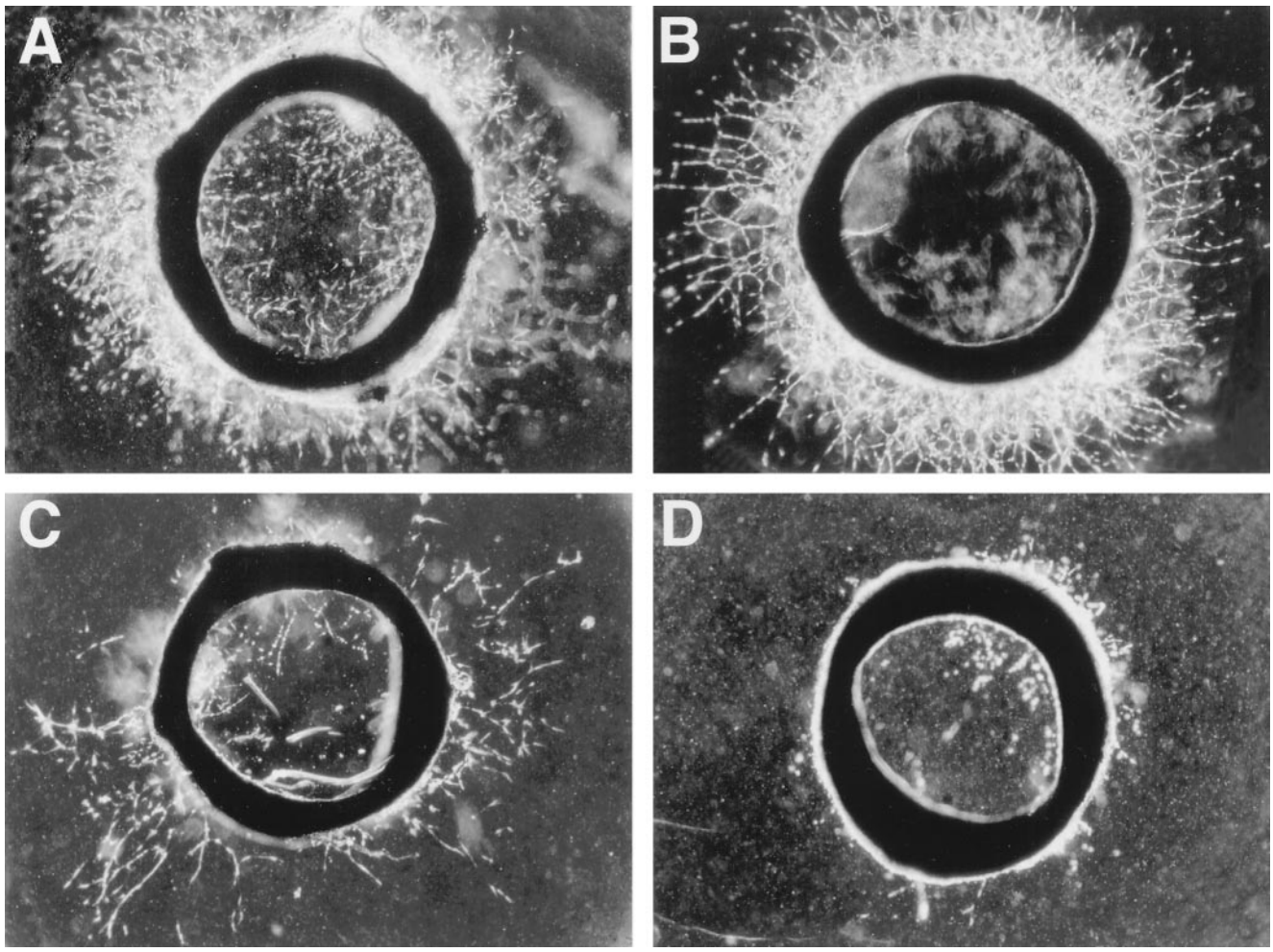

Fig. 2. Rat aortic sections incubated in the presence of EBM-II with CAI at concentrations of 0.25 μg/ml (A), 1.0 μg/ml (B), 4.0 μg/ml (C), and 12.0 μg/ml (D). Photographs are representative rings of duplicate assays. Outgrowths visualized directly by inverted microscopy.

with vehicle, or EGM-II with CAI at a final concentration of 0.25, 1.0, 4.0, or 12.0 μg/ml. After 72 h, cells were harvested by scraping and the pellet washed in PBS, pH 7.4, containing protease inhibitors. Cytosolic extracts and immunoblots were prepared as previously described (Horti, 1999). Blots were developed using mouse antihuman VEGF antibody followed by incubation with biotinylated goat antimouse IgG. Detection was done using the CSPD Western Light chemiluminescence detection kit.

Results

Inhibition of HAEC Proliferation by CAI. As a simplified model of angiogenesis, the effect of CAI on the proliferation of HAECs was assessed. These cells incubated in the presence of CAI exhibited concentration-dependent growth inhibition (Fig. 1). After 5 days of treatment, a modest growth inhibition of 30 and 34% of control was observed at CAI concentrations of 0.25 and 1.0 μg/ml, respectively. Cell growth was inhibited by 60% at 4.0 μg/ml CAI and by more than 98% at 12 μg/ml at day 5. At the 12 μg/ml CAI concentration, the total cell number per well decreased steadily from baseline, indicating a cytotoxic effect as well as growth inhibition. Using the day 5 inhibition determined with the limited number of CAI concentrations tested, the IC_{50} value was calculated to be approximately 1.53 μg/ml based on the estimates obtained by fitting the Hill equation to the data (Fig. 1).

Retardation of Microvessel Outgrowth and Decreased VEGF Expression. Rat aortic rings treated with CAI also demonstrated a concentration-dependent inhibition of microvessel outgrowth in the concentration range studied

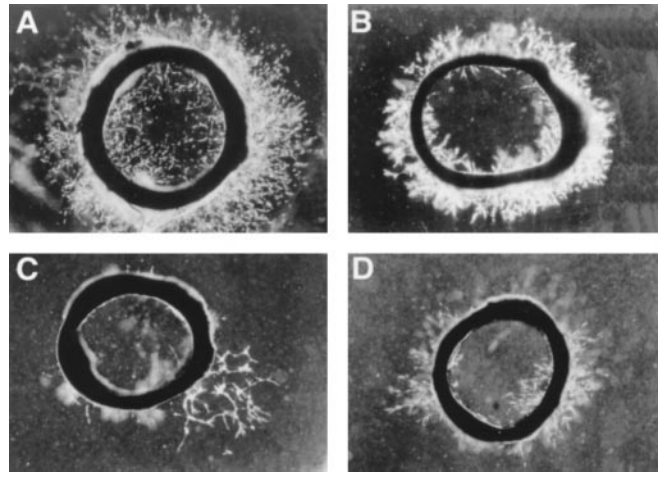

Fig. 3. Rat aortic sections incubated in the presence of EBM-II with vehicle (0.5% DMSO; A), 60 ng/ml doxorubicin (B), 10 μg/ml suramin (C), and 250 ng/ml TNP-470 (D). Photographs are representative rings of duplicate assays. Outgrowths visualized directly by inverted microscopy.

(Fig. 2, A–D). At the lowest concentration, there was no substantial difference in microvessel outgrowth compared with rings treated with culture medium containing the control vehicle (Fig. 3A). The cytotoxic agent doxorubicin (Fig. 3B) had no effect on microvessel outgrowth, indicating that cytoxicity was not responsible for inhibited microvessel outgrowth. In comparison, the known antiangiogenic agents suramin (Fig. 3C) and TNP-470 (Fig. 3D) showed marked inhibition of microvessel outgrowth that was comparable with the rings treated with 12.0 μg/ml CAI (Fig. 2D). Immunostaining of the rings was positive for both factor VIII and CD34 in the microvessels emerging from the aortic ring, suggesting that the sprouts were vascular in nature (data not shown). To determine whether intracellular VEGF expression was affected by CAI treatment, the rat aortic explants were sectioned and stained for VEGF. Figure 4 demonstrates the diminished expression of endothelial VEGF in the rat aortic ring culture in situ.

CAI Down-Regulates VEGF Secretion. To determine whether CAI had any effect on the secretion of VEGF from HAECs, the amount of this growth factor was measured in the conditioned medium of cultured cells. The amount of VEGF secreted per cell showed a concentration-dependent decrease in the presence of CAI (Fig. 5). A 16.1 and 30.5% decrease in VEGF concentration per 1000 cells was noted at the 1.0 and 4.0 μg/ml CAI concentrations, respectively. However, at the 12 μg/ml CAI concentration, an increase of 57.1% in the concentration of VEGF per 1000 cells was observed.

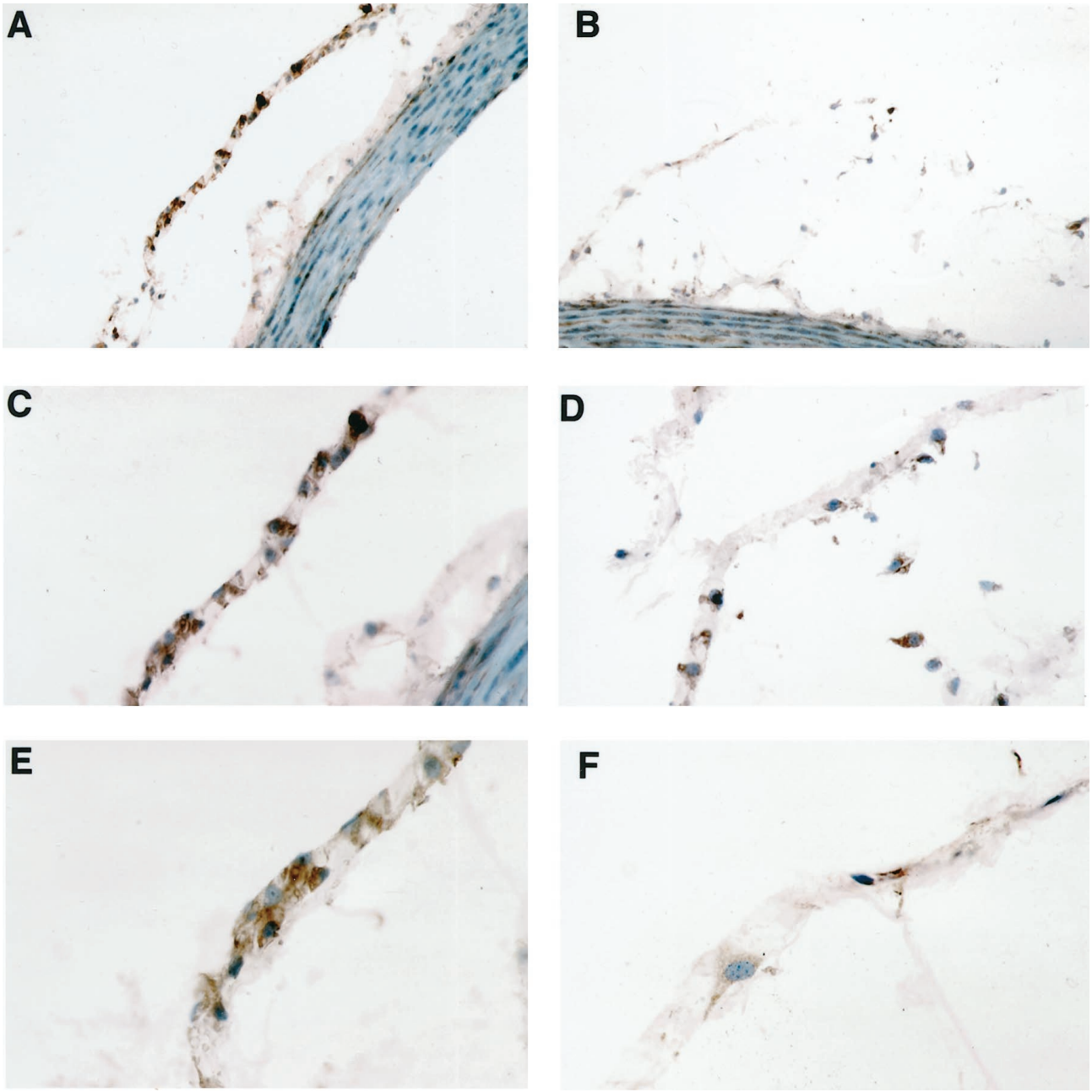

Fig. 4. Rat aortic microvessels stained for VEGF with peroxidase (brown). Aortic sections incubated in the presence of EBM-II with vehicle are shown at 200× (A), 400× (C), and 630× (E) magnification. Aortic sections incubated in the presence of CAI at 4.0 μg/ml shown at 200× (B), 400× (D), and 630× (F) magnification. Aortic wall counterstained with hematoxylin (blue).

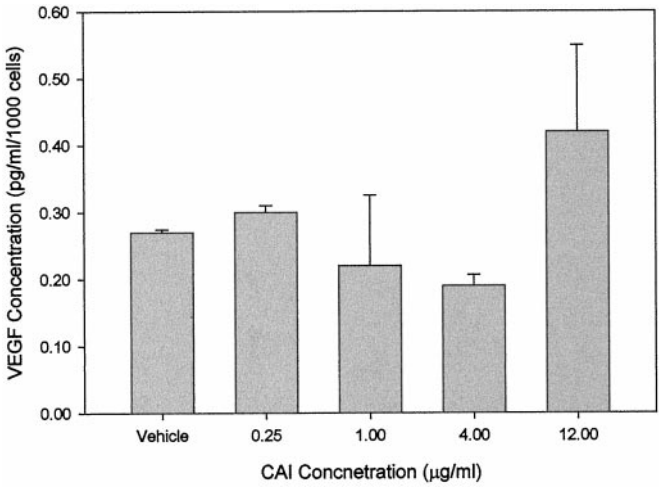

Fig. 5. Concentration of VEGF secreted into the medium per 1000 cells of HAECs incubated in EBM-II, 0.5% DMSO, or 0.25, 1.0, 4.0, or 12.0 μg/ml CAI for 3 days. Data represent duplicate samples.

This increase in VEGF secretion may reflect the cytotoxic effect of CAI noted at this concentration. It is possible that the dying cells released intracellular VEGF into the conditioned medium and thereby increased the secreted VEGF concentration. Alternatively, this increase may represent the induction of a redundant pathway that contributes to the production and secretion of VEGF.

NOS Expression Is Decreased. The same concentrations of CAI that affected HAEC proliferation were used to assess the effect on ecNOS expression. At a concentration of 4.0 μg/ml CAI, intracellular NOS expression in HAECs was clearly diminished (Fig. 6). At this concentration, there was a 72.7% reduction in NOS compared with vehicle control. HAECs treated with 12 μg/ml CAI produced undetectable amounts of NOS. CAI had no detectable effects on NOS production at concentrations of less than 4 μg/ml.

VEGF Expression Is Decreased. VEGF expression was evaluated in both vascular endothelial cells (HAECs) and the prostate tumor cells (LNCaP) by exposing the cell lines to various concentrations of CAI. With the addition of 0.25 μg/ml CAI, the expression of VEGF in LNCaP was unchanged. However, after exposure to 1.0 and 4.0 μg/ml CAI, the intracellular VEGF-2 expression was decreased by 22 and 16%, respectively (Fig. 7). The treatment of HAECs with 0.25, 1.0, and 4.0 μg/ml CAI resulted in a 11, 68, and 50% decrease in VEGF-2 expression, respectively (Fig. 8). However, at 12 μg/ml, a decrease of less than a 29% was observed.

Discussion

Calcium signaling pathways have been implicated in the regulation of several cellular processes, including proliferation and tumorigenic transformation. One major pathway involves G protein-induced production of the cellular signaling molecules inositol triphosphate and diacylglycerol (Berridge and Irvine, 1989). In this pathway, activation of a G protein-associated receptor results in the release of intracellular stores of Ca^{2+} from the endoplasmic reticulum (Kobayashi et al., 1988; Miyazaki, 1988). Other pathways involve the activation/deactivation of membrane channels that results in the uptake or release of intracellular Ca^{2+}. CAI has been identified as an inhibitor of Ca^{2+} influx and is speculated to inhibit both nonvoltage-gated Ca^{2+} channels and components of the G protein-coupled receptor cascade (Hupe et al., 1900, 1991) The antiproliferative properties of CAI have been correlated with the inhibition of the release of Ca^{2+} from intracellular stores (Hupe et al., 1990), whereas the anticancer activity of CAI is thought to result from a down-regulation in transcription of matrix metalloproteinase-2 (Kohn et al., 1994). In addition to its antiproliferative properties, CAI has been observed to have antimetastatic and anti-invasive properties in animal models.

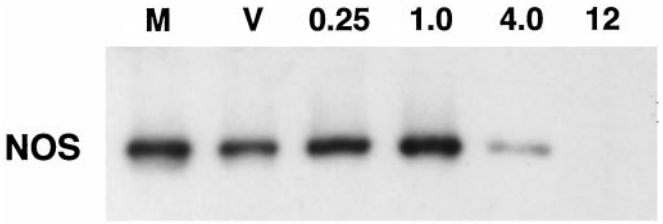

Fig. 6. Western blot for NOS from HAEC lysate after culture for 72 h in EGM-II alone (M), with vehicle (V), and at CAI concentrations of 0.25, 1.0, 4.0, and 12.0 μg/ml.

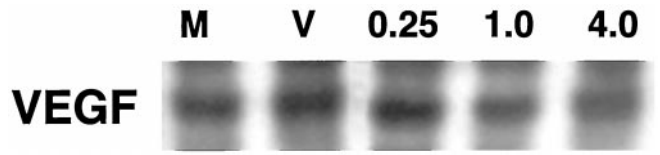

Fig. 7. Western blot for VEGF-2 for LNCaP cell lysate after culture for 72 h with RPMI 1640 culture medium alone (M), with vehicle (V), or at CAI concentrations of 0.25, 1.0, and 4.0 μg/ml.

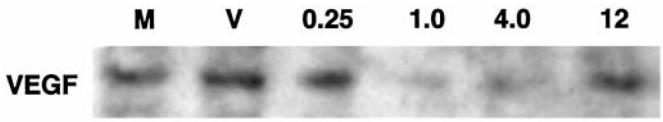

Fig. 8. Western blot for VEGF-2 for HAEC cell lysate after culture for 72 h with EGM-II alone (M), EGM-II with vehicle (V), or at CAI concentrations of 0.25, 1.0, 4.0, and 12.0 μg/ml.

One of the proposed mechanisms for the anticancer activity of CAI is through the inhibition of angiogenesis (Kohn et al., 1995). Previously, CAI has demonstrated antiangiogenic activity in both the chorioallantoic membrane assay and HUVEC proliferation assay (Kohn et al., 1995). This study reported an IC_{50} value of 1.0 μg/ml for HUVEC proliferation. In an effort to establish a possible link between the ability of CAI to inhibit Ca^{2+} influx and its antiangiogenic properties, we undertook a series of investigations.

There are several isoforms of NOS, including the Ca^{2+}-dependent ecNOS and cytokine-induced inducible NOS. Several groups have demonstrated that transient increases in intracellular Ca^{2+} concentrations can induce the production of ecNOS at both the mRNA and protein level (Park et al., 1996; Sunyer et al., 1997; Weikert et al., 1997). This Ca^{2+}-induced increase in ecNOS expression results in elevated levels of NO.

Recent studies have reported an association between NO and the angiogenesis-promoting factor VEGF. Several studies have observed an up-regulation of VEGF after induction by NO (Morbidelli et al., 1996; Frank et al., 1998); furthermore, VEGF has been found to induce NOS and thereby increase NO (Kroll and Waltenberger, 1998; Trachtman et al., 1998). These findings illustrate one possible relationship

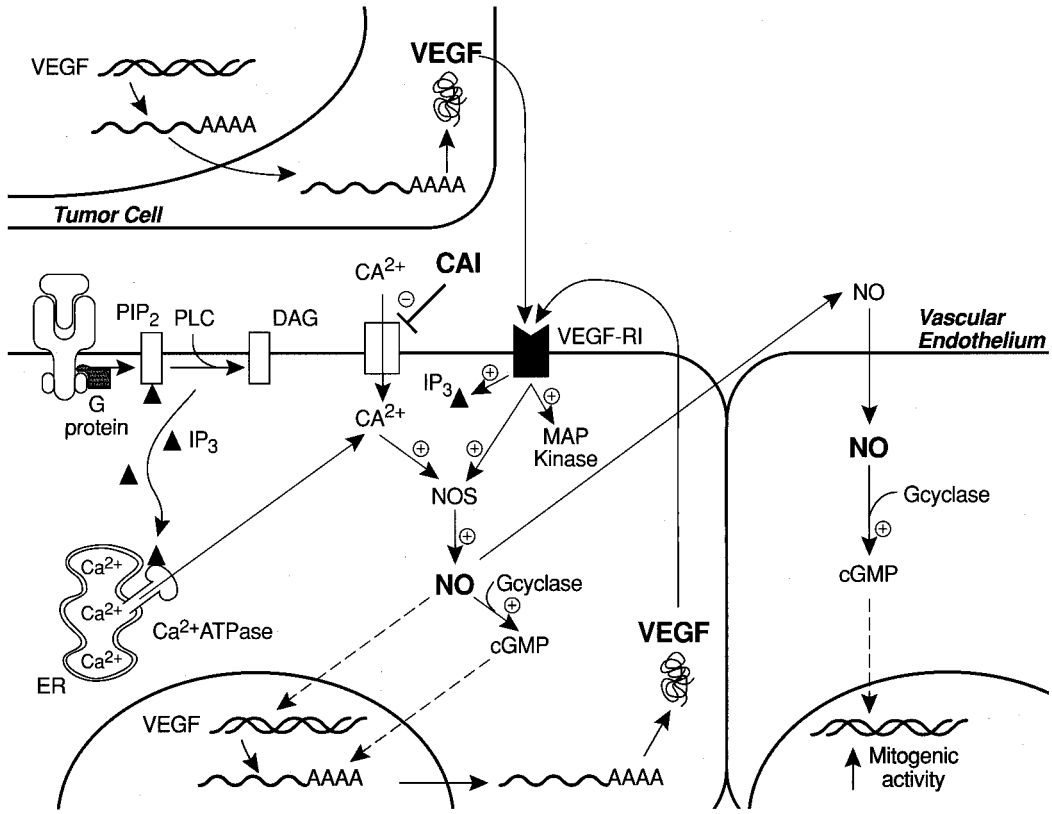

Fig. 9. Diagrammatic representation of the proposed inhibition of the Ca^{2+}-NOS-NO-VEGF pathway and angiogenesis by CAI. VEGF-RI, vascular endothelial growth factor receptor 1; IP3, inositol 1,4,5-trisphosphate, PLC, phospholipase C; DAG, 1,2-diacylglycerol; PiP_2, phosphatidylinositol-4,5-bisphosphate; G cyclase, guanylate cyclase.

among Ca^{2+}, VEGF, NOS, and NO and the regulation of angiogenesis (Fig. 9).

We have demonstrated that CAI inhibits the proliferation of HAECs and retards microvessel outgrowth in a rat aortic ring explant model. Both of these effects occurred in a concentration-dependent manner. The inhibition of HAEC proliferation was similar to that reported by Kohn et al. (1995) for HUVECs, with IC_{50} values of 1.53 and 1.0 μg/ml, respectively. Treatment with CAI led to diminished VEGF secretion into the HAEC conditioned culture medium, as well as decreased expression of cytoplasmic VEGF in the endothelial cell sprouts arising from the rat aortic rings. The intracellular levels of NOS were also substantially decreased after the treatment of HAECs with 4.0 to12.0 μg/ml CAI. These findings suggest that a possible mechanism for the antiangiogenic properties of CAI involve the regulation of a Ca^{2+}-NOS-NO-VEGF signaling pathway (Fig. 9).

It has been suggested that within a tumor, induction of neovascularization results from a rapid transient influx of Ca^{2+}. The increased intracellular Ca^{2+} induces the production of NOS, which in turn yields an increased NO concentration in the local tumor environment. Subsequently, NO stimulates VEGF production and secretion. VEGF exerts its potent induction of neovascularization via its mitogenic and microvascular hyperpermeability effects on the surrounding vascular endothelium. Alternatively, VEGF may indirectly promote angiogenesis by stimulating the production of NO through its feedback induction of NOS. In this proposed model, CAI blocks the influx of intracellular Ca^{2+}, leading to the decreased expression of NOS; subsequently, NO production decreases and leads to a down-regulation of the NOS-NO-VEGF regulatory loop. Thus, the speculated end result of treatment with CAI is the inhibition of angiogenesis within the tumor.

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