ELSEVIER

European Journal of Pharmacology 398 (2000) 19–29

ejp

www.elsevier.nl/locate/ejphar

Dissociation between the Ca^{2+} signal and tube formation induced by vascular endothelial growth factor in bovine aortic endothelial cells

Junya Kawasaki [a], Katsuya Hirano [a], Mayumi Hirano [a], Junji Nishimura [a], Akio Nakatsuka [b], Masatoshi Fujishima [c], Hideo Kanaide [a,*]

[a] *Department of Molecular Cardiology, Research Institute of Angiocardiology, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan*

[b] *Department of Cancer Therapy and Research, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan*

[c] *Department of Medicine and Clinical Science, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan*

Received 15 March 2000; received in revised form 4 April 2000; accepted 7 April 2000

Abstract

The correlation between the intracellular Ca^{2+} signal and the tube formation in collagen gels induced by vascular endothelial cell growth factor (VEGF) was investigated using cultured bovine aortic endothelial cells. The VEGF-induced sustained elevation of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) was similarly inhibited by 10 μM 1-{β-[3-(4-methoxyphenyl)propyl]-4-methoxyphenethyl}-1*H*-imidazole hydrochloride (SKF 96365) and 10 μM troglitazone. However, 10 μM diltiazem had no effect. The basal tube formation obtained with 1% serum was augmented twofold by 100 ng/ml VEGF. SKF 96365 (0.1–10 μM) inhibited the VEGF-induced and basal tube formation, while 10 μM troglitazone or 10 μM diltiazem had no effect. The proliferation of endothelial cells was markedly inhibited by SKF 96365 but only slightly by troglitazone and diltiazem. The inhibition of tube formation by three Ca^{2+} entry blockers thus correlated with the inhibition of cell proliferation. The $[Ca^{2+}]_i$ elevation is thus not a prerequisite for VEGF to induce tube formation.

Keywords: Endothelial cell; Angiogenesis; Ca^{2+} influx; Proliferation

1. Introduction

Angiogenesis is defined as the development of new capillaries from existing vessels. It plays an important role not only in a number of physiological processes, such as embryonic development, ovulation and wound healing, but also in various pathological conditions such as solid tumor growth, inflammation, tissue ischemia and diabetic retinopathy (Folkman and Klagsburn, 1987). Angiogenesis can be accomplished through a complex series of steps including the degradation of the basement membrane by cellular protease, the penetration and migration of endothelial cells into the extracellular matrix, the proliferation of endothelial cells and the formation of vascular networks (Beck and D'Amore, 1997; Folkman and Klagsburn, 1987).

Several experimental methods have been developed to assess angiogenesis in vivo and in vitro. The models of in vivo angiogenesis include the micropocket technique, which permits capillary growth toward an angiogenic substance implanted on the rabbit, mouse or rat cornea, or on the chick embryo chorioallantoic membrane (Folkman and Klagsburn, 1987). A method to observe the transformation of endothelial cells into capillary-like tube structures in a three-dimensional collagen gel culture system has been widely and successfully used as a model of in vitro angiogenesis (Davis and Camarillo, 1996; Madri and Williams, 1983; Montesano et al., 1983; Okamura et al., 1992; Satake et al., 1998; Xin et al., 1999). The final tube formation observed in the three-dimensional collagen gel culture system is considered to depend on the three steps required for angiogenesis: degradation of the collagen matrix, migration and proliferation.

Vascular endothelial growth factor (VEGF) is one of the most potent angiogenic factors. VEGF exerts its effects

* Corresponding author. Tel.: +81-92-642-5548; fax: +81-92-642-5552.

E-mail address: kanaide@molcar.med.kyushu-u.ac.jp (H. Kanaide).

0014-2999/00/$ - see front matter
PII: S0014-2999(00)00296-X

by binding to its specific receptors Flt-1 and KDR/Flk-1 in endothelial cells. Flt-1 is considered to be a critical survival factor involved in endothelial cell morphogenesis, while Flk-1/KDR functions as a transducer to signal endothelial cell proliferation and differentiation (Ferrara and Davis-Smyth, 1997; Fong et al., 1995; Ilan et al., 1998). VEGF was shown to accelerate endothelial proliferation and angiogenesis both in vivo and in vitro (Hanahan, 1997), while also inducing endothelium-dependent vasorelaxation (Ku et al., 1993). VEGF is also reported to upregulate the expression of endothelial constitutive NO synthase (NOS) mRNA and to stimulate NO production (Hood et al., 1998). VEGF increases cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_i$) through both Ca^{2+} influx and Ca^{2+} release from inositol trisphosphate-sensitive stores in endothelial cells (Brock et al., 1991). The intracellular Ca^{2+} signal may thus play a key role in the VEGF-mediated biological effects. In fact, carboxy-amidotriazole, an inhibitor of non-voltage-operated Ca^{2+} channels, was shown to suppress tube formation in Matrigel and the development of new capillaries induced by basic fibroblast growth factor on the rabbit cornea (Kohn et al., 1995). The Ca^{2+} influx was thus suggested to be a prerequisite for angiogenesis. So far, however, there has been no report demonstrating the relationship between Ca^{2+} signaling in endothelial cells and angiogenesis.

In the present study, the correlation between the Ca^{2+} signal and angiogenesis in vascular endothelial cells was investigated. The three-dimensional culture system in collagen gel was utilized, and in vitro angiogenesis was evaluated by examining capillary-like tube formation. The change in $[Ca^{2+}]_i$ in vascular endothelial cells was examined using microfluorometry (Hirano et al., 1993). We have previously shown that an anti-diabetic drug troglitazone ((±)-5-[4-(6-hydroxy-2,5,7,8-tetramethylchroman-2-ylmethoxy)benzyl]-2,4-thiazolidinedione) inhibits not only voltage-operated Ca^{2+} channels, but also other Ca^{2+} entry pathways in porcine arterial smooth muscle cells (Kawasaki et al., 1998), and the thapsigargin-induced Ca^{2+} influx in endothelial cells (Kawasaki et al., 1999). We used 1-{β-[3-(4-methoxyphenyl)propyl]-4-methoxyphenethyl}-1*H*-imidazole hydrochloride (SKF 96365) (Merritt et al., 1990) and troglitazone (Bressler and Johnson, 1997) to inhibit the Ca^{2+} influx in endothelial cells to clarify the role of the Ca^{2+} influx and the resultant elevation of $[Ca^{2+}]_i$ in VEGF-induced angiogenesis.

2. Materials and methods

2.1. Culture of bovine aortic endothelial cells

Bovine aortic endothelial cells without any contamination of fibroblasts were obtained as previously described (Hirano et al., 1993), with minor modifications. In brief, the bovine aortas were obtained at a local slaughterhouse. The endothelial lining was scraped off with a scalpel blade, collected in growth medium by centrifugation at 1000 rpm for 5 min, and then mechanically dispersed into smaller clumps by pipetting. The dispersed cells were then plated in 48-well plastic plates (Nalge Nunc International, Denmark) in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum, 100 unit/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml gentamycin. The wells that showed an overriding growth of needle-shaped fibroblasts were discarded, and the cells without any contamination of fibroblasts were subcultured and expanded for experimental use. The endothelial cells thus obtained showed a monolayer with a typical cobblestone appearance at confluence and with an active uptake of fluorescent dye-labeled acetylated low-density lipoprotein (data not shown). In all experiments, the cells obtained from passages 5 through 9 were used.

2.2. Fura-2 fluorometry of bovine aortic endothelial cells

For the $[Ca^{2+}]_i$ measurement, bovine aortic endothelial cells were cultured until confluence on sterilized round glass coverslips (diameter 25 mm; Matsunami, Osaka, Japan) placed in 35-mm culture dishes (Nalge Nunc International). The cells were incubated in DMEM containing 5 μM fura-2/AM (an acetoxymethyl ester form of fura-2), 1 mM probenecid (*p*-[dipropylsulfamoyl]benzoic acid) and 10% fetal bovine serum for 1 h at 37°C. After fura-2 loading, cells were washed in *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulphonic acid] (HEPES)-buffered saline (HBS). The fluorometry of the bovine aortic endothelial cells was performed as described (Hirano et al., 1993). The change in the ratio of fluorescence intensity (500 nm) at 340 nm to that at 380 nm excitation (F340/F380) was recorded as an indicator of $[Ca^{2+}]_i$ by using an inverted fluorescent microscope (TMD-56, Nikon, Tokyo, Japan), equipped with a fluorometer, CAM 220 (JASCO, Tokyo, Japan). All measurements were performed at 25°C to prevent sequestration of the fluorescent dye (Kawasaki et al., 1999). The fluorescence ratio is expressed as a percentage, with the values at rest and at a peak response to 10 μM ATP being considered 0% and 100%, respectively. Repetitive stimulation with 10 μM ATP at 15-min intervals reproducibly induced a similar increase in $[Ca^{2+}]_i$ (Aoki et al., 1994). All data were collected using a computerized data acquisition system (MacLab; Analog Digital Instruments, Australia).

2.3. Assay for tube formation of bovine aortic endothelial cells in three-dimensional collagen gel

Angiogenesis was evaluated by observing the tube formation of bovine aortic endothelial cells according to the reported methods (Satake et al., 1998) with minor modifications. Briefly, the 0.5 ml/well collagen gel (basal gel) was first prepared in 12-well plastic plates (Nalge Nunc

International). The collagen gel consisted of 7 vol of 3 mg/ml type I collagen (Nitta Gelatin, Osaka, Japan; final concentration of collagen was 2.1 mg/ml), 2 vol of a 5 × concentrated DMEM and 1 vol of a buffer containing 0.05 N NaOH, 2.2% $NaHCO_3$ and 200 mM HEPES. After the gel solidified, each well was equilibrated with an equal volume (0.5 ml) of 2% serum-containing medium for 24 h. On the next day, 0.5 ml of the collagen gel with the same composition, but also containing bovine aortic endothelial cells (2×10^6 cells/ml), was overlaid onto the basal gel (day 0). After the gel solidified, 2% serum-containing medium (0.5 ml/well) was added, and cells were incubated for 5 days. The tube formation observed with no additional stimulation (control condition) was thus considered to be obtained under stimulation with 1% serum. When the effects of the drugs on tube formation were examined, they were added to the top medium on day 0. After 5 days of culture, tube formation was evaluated by measuring the total length of the tubes (mm) per 0.06 mm^3 (0.26 mm × 0.23 mm field area × 1 mm depth) with a Video micrometer VM-30 (Olympus, Tokyo, Japan), on a phase contrast microscopic image (magnification ×200) obtained by a Color CCD camera CS900 (Olympus). In all experiments, the total length of the tubes was measured in three different fields for each well and the mean value was regarded as the value of each well.

2.4. Cell proliferation and viability

The effects of the Ca^{2+} entry blockers on cell proliferation and viability were investigated by the use of the following three methods: counting cell number, a MTT [3-(4,5-dimethylthiazol-2-yl-2,5-diphenyl) tetrazolium bromide] assay and the incorporation of bromodoxyuridine (BrdU). To determine the effect on cell number, the endothelial cells were cultured in 35-mm dishes (961 mm^2) at a density of 30,000 cells/2 ml medium. After 3 days of culture, the cell number increased to $2.3 \pm 0.2 \times 10^5$ cells/dish, and the medium was changed to a 1% serum-containing medium in the absence and presence of either 100 ng/ml VEGF alone or VEGF with SKF 96365, troglitazone or diltiazem. After 8 days of culture, the cells attached to the dishes were detached after treating them with trypsin, and the total number of cells was counted on a hemocytometer in triplicate. Cell viability was evaluated by the Trypan blue exclusion test.

A MTT assay was performed as described earlier (Gralinski et al., 1998; Nisoli et al., 1998). Cells were plated in 48-well plates at a density of 3200 cells/well. After 3 days of culture in the normal growth medium, cells were exposed to 1% serum-containing media and treated with drugs either with and without 100 ng/ml VEGF for 5 days. Cells were then incubated with 1 mg/ml MTT for 4 h, and a blue formazan product was subsequently extracted in 100 μl isopropanol containing 0.04 N HCl. The extract was transferred to a 96-well plate and its absorbance was determined on a microplate reader EAR 340AT (SLT-Labinstrumets, Austria). The data were obtained by subtracting the absorbance at a reference wavelength of 620 nm (A_{620}) from that at a test wavelength of 570 nm (A_{570}) (Nisoli et al., 1998).

The effect of the Ca^{2+} entry blockers on DNA synthesis was evaluated by the incorporation of BrdU. Cells were prepared on a 2×2 cm^2 square coverglass in 35-mm dishes as described for the cell count experiment, and the BrdU incorporation during 1.5 h labeling was determined by immunofluorescent staining (Boehringer Mannheim, Tokyo, Japan) after 24 h treatment with the Ca^{2+} entry blockers. The fluorescence image was observed under a fluorescence microscope, and the number of the BrdU-containing cells as a fraction of the total number of cells was determined by counting more than 350 cells, as previously described (Hirano et al., 1999).

2.5. Drugs and solutions

The composition of HBS was as follows (mM): 133.9 NaCl, 5.9 KCl, 1.2 $MgCl_2$, 1.25 $CaCl_2$,11.5 D-glucose, 10 HEPES (containing 4.6 Na^+), pH 7.4 at 25°C. Ca^{2+}-free HBS was prepared by adding 2 mM ethylenglycol-bis (β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) instead of $CaCl_2$. Human recombinant VEGF (disulfide-linked homodimer of 165 amino acid VEGF) was purchased from Genzyme (Cambridge, MA, USA). Fura-2/AM and EGTA were purchased from Dojindo (Kumamoto, Japan). Troglitazone was donated by Sankyo (Tokyo, Japan). SKF 96365 was purchased from Calbiochem (La Jolla, CA, USA). ATP was purchased from Boehringer Mannheim. Diltiazem, probenecid and MTT were purchased from Sigma (St. Louis, MO, USA).

2.6. Data analysis

The values are expressed as the means ± standard error of the mean (S.E.M.). Student's *t*-test was used to determine significant differences between the two groups, and an analysis of variance was used to determine the concentration-dependent effect of VEGF and SKF 96365. *P* values of less than 0.05 were considered to be statistically significant.

3. Results

3.1. Effect of SKF 96365, troglitazone and diltiazem on the VEGF-induced $[Ca^{2+}]_i$ elevation

Fig. 1a shows a representative recording of the $[Ca^{2+}]_i$ increase induced by 100 ng/ml VEGF in bovine aortic endothelial cells. At about 2 min after the application of VEGF, the $[Ca^{2+}]_i$ reached a peak level (70.1 ± 4.1% of the peak $[Ca^{2+}]_i$ increase induced by 10 μM ATP, $n = 6$),

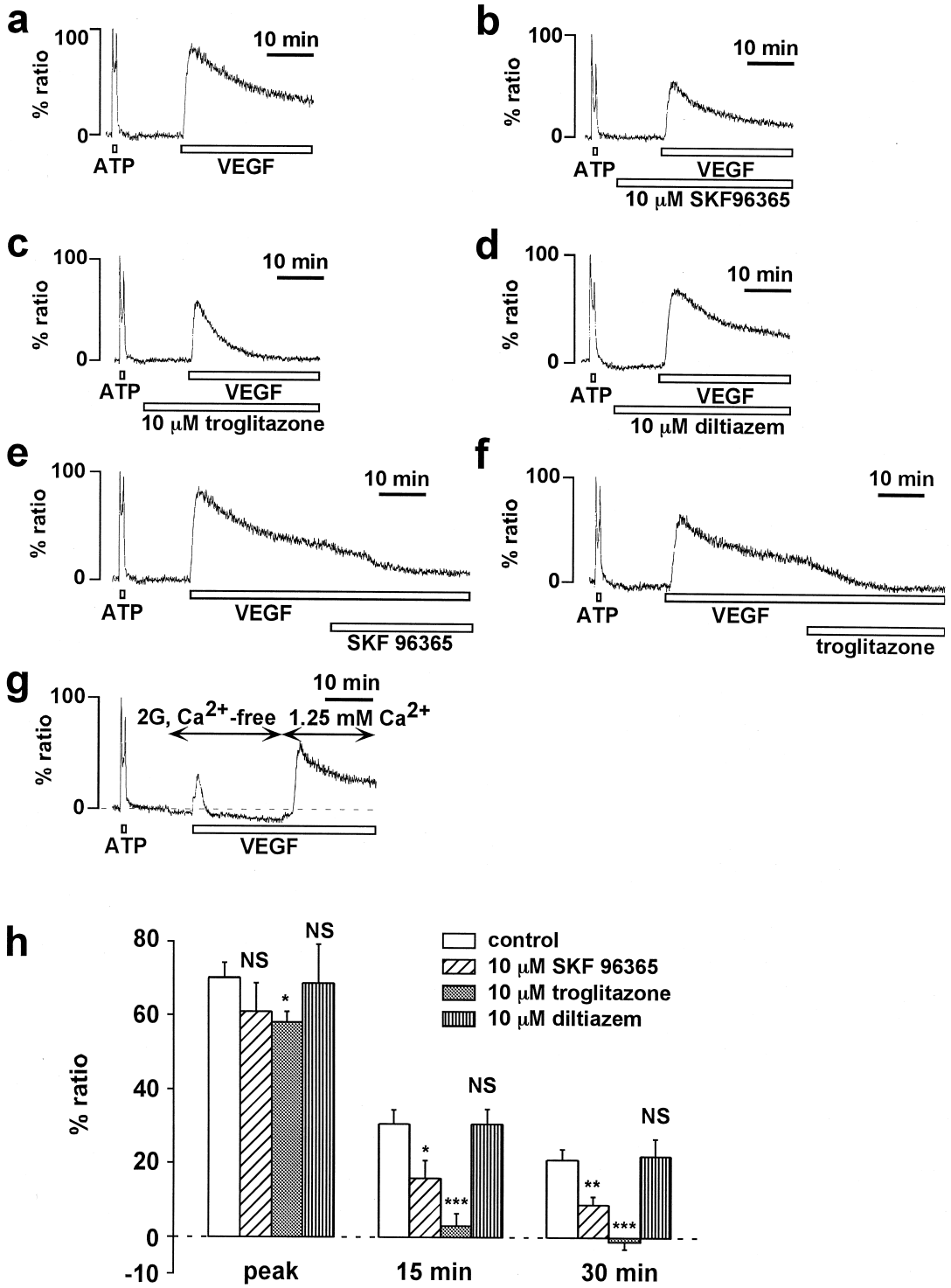

Fig. 1. Effects of SKF 96365, troglitazone and diltiazem on the $[Ca^{2+}]_i$ increase induced by 100 ng/ml VEGF in bovine aortic endothelial cells. (a–d) Representative recordings of 100 ng/ml VEGF-induced $[Ca^{2+}]_i$ transient in normal PSS in the absence (a) and presence of 10 μM SKF 96365 (b), 10 μM troglitazone (c), and 10 μM diltiazem (d). SKF 96365, troglitazone and diltiazem were applied 10 min before the application of VEGF. (e, f) Representative recordings showing the effect of application of 10 μM SKF 96365 (e) and 10 μM troglitazone (f) during the declining phase of the 100 ng/ml VEGF-induced $[Ca^{2+}]_i$ increase. SKF 96365 and troglitazone were applied 30 min after stimulation with VEGF. (g) A representative recording of 100 ng/ml VEGF-induced $[Ca^{2+}]_i$ transient in the Ca^{2+}-free media containing 2 mM EGTA. Extracellular Ca^{2+} (1.25 mM) was replaced at 20 min after simulation with VEGF. (h) Summary of the effect of SKF 96365, troglitazone and diltiazem on the $[Ca^{2+}]_i$ increase induced by VEGF as shown in (a–d). The top of each column represents the level of $[Ca^{2+}]_i$ (% ratio) at the peak $[Ca^{2+}]_i$ increase, and at 15 and 30 min after the application of VEGF, obtained in the absence (open columns) and presence of 10 μM SKF 96365 (hatched column), 10 μM troglitazone (shaded columns), and 10 μM diltiazem (vertically striped columns). In all experiments, the level of the % ratio obtained at the peak response to 10 μM ATP in normal PSS was considered to be 100%. Data are the means ± S.E.M. ($n = 6$). $^{***}P < 0.005$; $^{**}P < 0.01$; $^{*}P < 0.05$; NS, not significantly different compared to the values without pretreatment.

and remained at a sustained level with a slow decline. The $[Ca^{2+}]_i$ levels were 30.7 ± 3.6% and 20.9 ± 2.9% ($n = 6$) at 15 and 30 min, respectively. In Ca^{2+}-free solution containing 2 mM EGTA, 100 ng/ml VEGF induced only a transient $[Ca^{2+}]_i$ elevation (Fig. 1g). The $[Ca^{2+}]_i$ rapidly reached its peak (27.0 ± 3.7%, $n = 6$) at about 2 min, and subsequently decreased to the pre-stimulation level within 5 min (Fig. 1g). However, the subsequent replacement of the extracellular Ca^{2+} (1.25 mM) caused a rapid increase in $[Ca^{2+}]_i$, followed by a declining phase as observed in the control (Fig. 1a). As a result, the sustained increase in $[Ca^{2+}]_i$ observed in the presence of extracellular Ca^{2+} was considered to depend on the influx of extracellular Ca^{2+}.

Troglitazone, SKF 96365 or diltiazem was applied 10 min prior to and during the stimulation with VEGF (Fig. 1b–d). These three reagents showed no significant effect on the resting level of $[Ca^{2+}]_i$. Both troglitazone and SKF 96365 inhibited the sustained increase in $[Ca^{2+}]_i$ induced by VEGF, while only troglitazone significantly inhibited the peak increase (Fig. 1h). Diltiazem had no effect on either the peak or the declining phase of the $[Ca^{2+}]_i$ increase induced by VEGF (Fig. 1d and h). In the presence of 10 μM SKF 96365, 10 μM troglitazone and 10 μM diltiazem, the peak levels of $[Ca^{2+}]_i$ increase were 61.1 ± 7.7%, 58.3 ± 2.9% and 68.7 ± 10.5%, respectively ($n = 6$) (Fig. 1h). The $[Ca^{2+}]_i$ levels during the sustained phase observed in the presence of SKF 96365, troglitazone and diltiazem were 15.8 ± 4.9%, 3.3 ± 3.2% and 30.6 ± 4.1% at 15 min, and 9.0 ± 2.1%, −1.2 ± 1.9% and 21.8 ± 4.8% at 30 min after the application of VEGF, respectively (Fig. 1h). As shown in Fig. 1e and f, we also confirmed that SKF 96365 and troglitazone inhibited the influx compo-

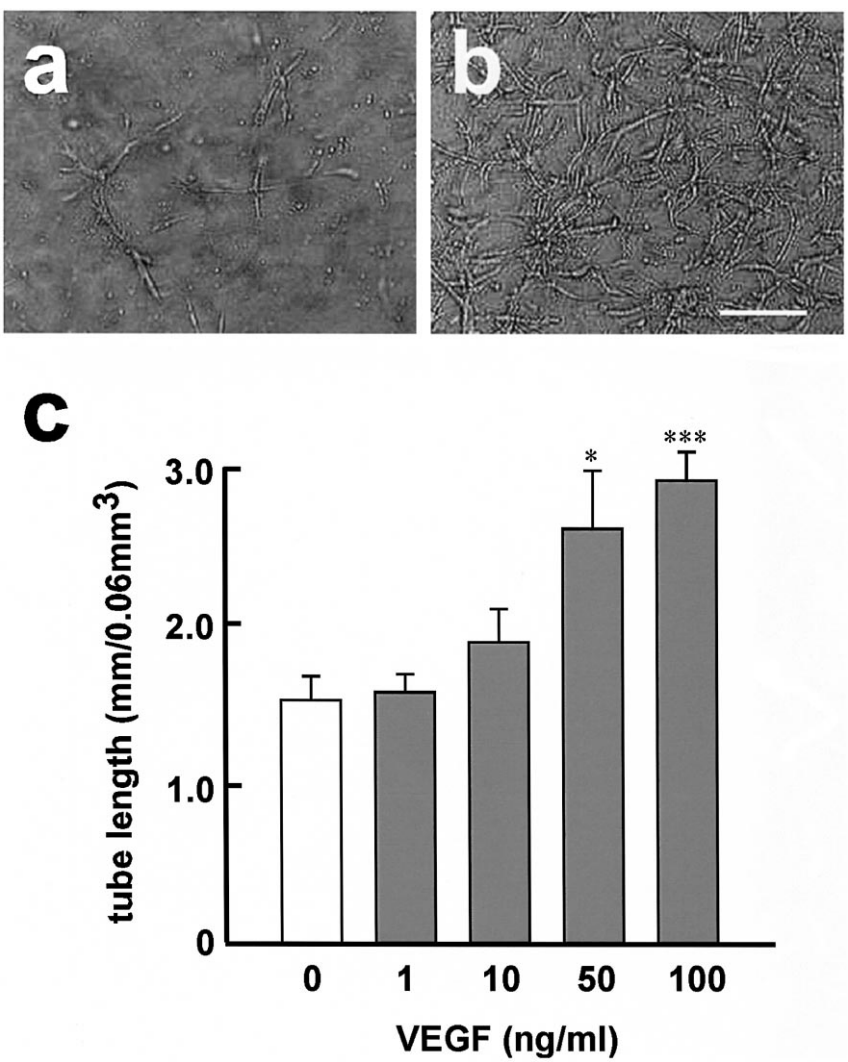

Fig. 2. Effects of VEGF on the tube formation of bovine aortic endothelial cells in three-dimensional collagen gel culture. (a, b) Representative pictures showing the tube formation of bovine aortic endothelial cells after 5 days of culture in the 1% serum-containing collagen gels matrix in the absence (a, basal tube formation) and presence of 100 ng/ml VEGF (b). Scale bar = 200 μm. (c) Concentration-dependent effect of VEGF on the tube formation of bovine aortic endothelial cells. Each column represents the total length of the developed tube (mm) per 0.06 mm^3 collagen gel. Data are the means ± S.E.M. ($n = 6$). $^{***}P < 0.005$; $^{**}P < 0.01$; $^{*}P < 0.05$ compared to the values obtained without VEGF.

nent of the VEGF-induced $[Ca^{2+}]_i$ increase when they were applied during the declining phase of the VEGF-induced $[Ca^{2+}]_i$ increase. After both pretreatment and post-treatment, troglitazone almost completely inhibited the declining phase of the $[Ca^{2+}]_i$ increase, while SKF 96365 showed a decreased inhibitory effect.

3.2. Effect of SKF 96365, troglitazone and diltiazem on tube formation

Fig. 2 shows the effect of VEGF on the tube formation of endothelial cells in the collagen gel. One percent serum induced the transformation of endothelial cells into capil-

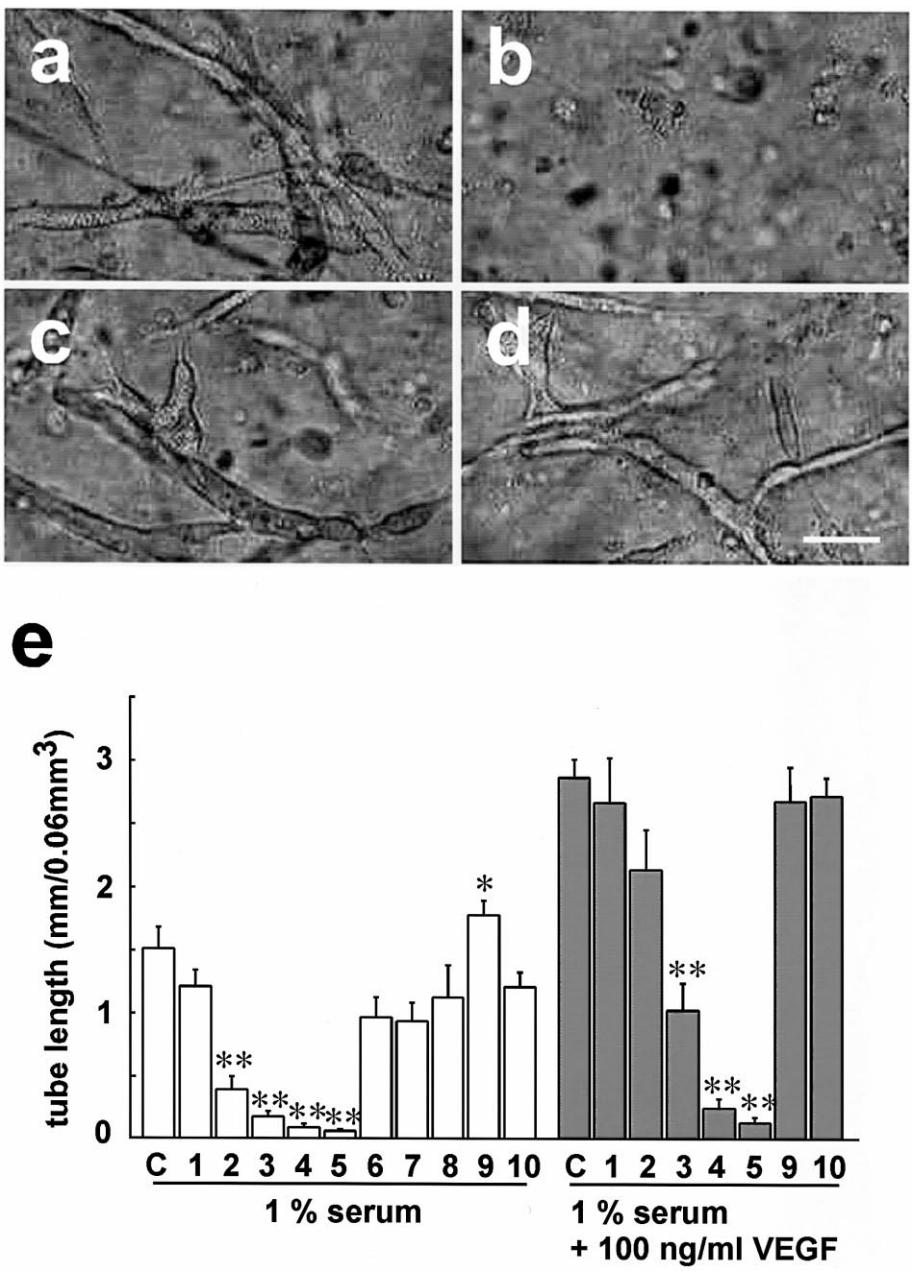

Fig. 3. Effects of SKF 96365, troglitazone and diltiazem on the VEGF-induced tube formation of bovine aortic endothelial cells in collagen gels. (a–d) Representative pictures showing the tube formation of bovine aortic endothelial cells after 5 days of culture in collagen gels with 1% serum-containing medium induced by 100 ng/ml VEGF in the absence (a, control) and presence of either 10 μM SKF 96365 (b), 10 μM troglitazone (c) or 10 μM diltiazem (d). Scale bar = 50 μm. (e) Summary of the effect of SKF 96365, troglitazone and diltiazem on the tube formation with (shaded columns) and without (open columns) 100 ng/ml VEGF. Each column represents the total length of the developed tubes (mm) per 0.06 mm^3 collagen gel. Columns C, no Ca^{2+} entry blockers; columns 1, 2, 3, 4 and 5, 0.1, 1, 3, 6 and 10 μM SKF 96365, respectively; columns 6, 7, 8 and 9, 0.1, 1, 3, 10 μM troglitazone, respectively; column 10, 10 μM diltiazem. The data are the means ± S.E.M. ($n = 5–13$). $^{**}P < 0.01$; $^{*}P < 0.05$.

lary-like tube structures (Fig. 2a; a basal tube formation). The total length of the tubes was 1.52 ± 0.16 mm/0.06 mm^3 of collagen gel ($n = 6$). Addition of VEGF (100 ng/ml) increased tube formation by twofold (2.88 ± 0.18 mm/0.06 mm^3; $n = 6$) compared to the basal tube formation (Fig. 2b and c). The augmentation of tube formation by VEGF was concentration dependent (1–100 ng/ml; Fig. 2c).

Fig. 3a–d represents microscopic pictures showing the effects of SKF 96365, troglitazone and diltiazem on the tube formation obtained in the presence of 100 ng/ml VEGF. Only SKF 96365 completely inhibited both the basal and VEGF-augmented tube formation. The majority of cells remained rounded in the collagen gels and were devoid of any capillary-like structure in the presence of SKF 96365 (Fig. 3b). Fig. 3e shows the concentration-dependent effect of SKF 96365 in both the presence and absence of VEGF (Fig. 3e). The inhibitory effect of SKF 96365 was observed at concentrations between 0.1 and 10 μM in both the presence (EC_{50}; 2.28 ± 0.31 μM) and absence (EC_{50}; 0.38 ± 0.019 μM) of VEGF. At 10 μM SKF 96365, tube formation was almost completely inhibited in both the presence and absence of VEGF (Fig. 3e). It thus appeared that the inhibitory effect of SKF 96365 on VEGF-induced tube formation closely correlated with that on the $[Ca^{2+}]_i$ elevation induced by VEGF. However,

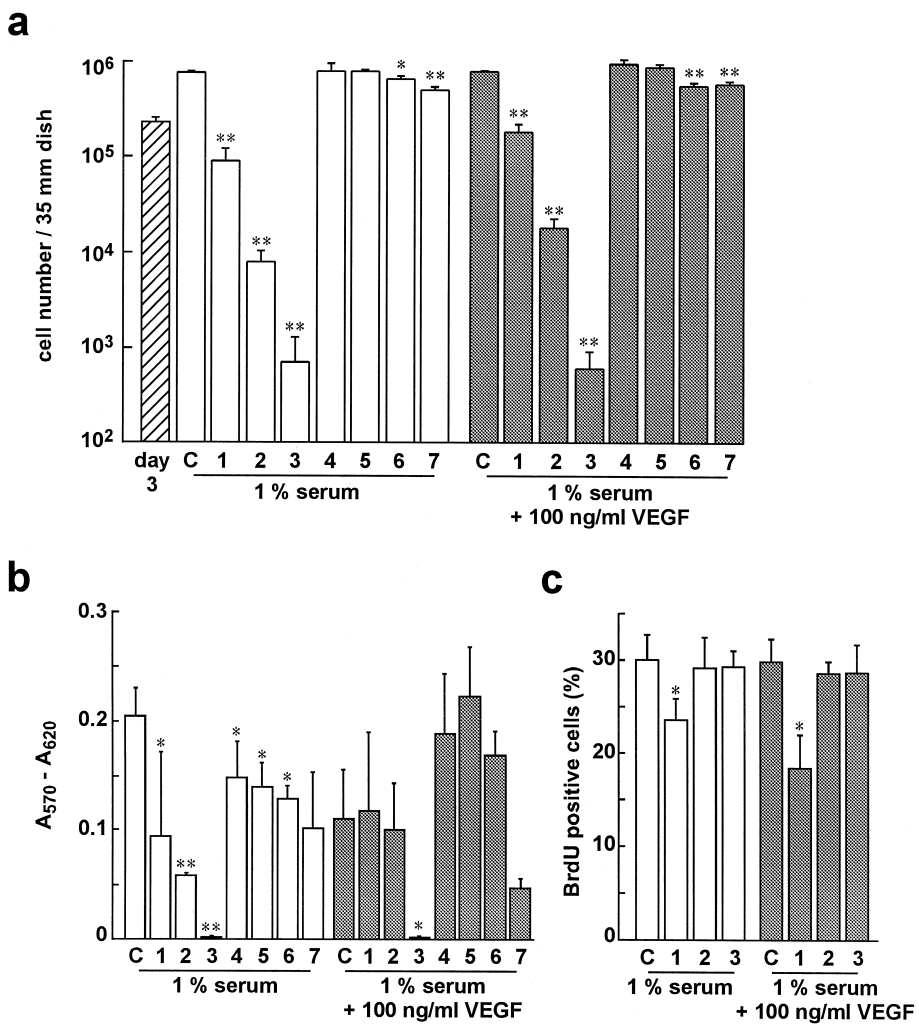

Fig. 4. Effects of SKF 96365, troglitazone and diltiazem on cell proliferation. (a) The effect of Ca^{2+} entry blockers on the increase in cell numbers. Endothelial cells were cultured in 10% serum-containing growth media for 3 days, and then in 1% serum-containing medium for an additional 5 days (at day 8) without (open columns) and with (shaded columns) 100 ng/ml VEGF, and in the absence (column C) and in the presence of 0.1 (column 1), 1 (column 2), 10 μM SKF 96365 (column 3), 0.1 (column 4), 1 (column 5), 10 μM troglitazone (column 6), and 10 μM diltiazem (column 7). Hatched column; the cell number after 3 days of culture in the 10% serum-containing growth media. The data are the means ± S.E.M. ($n = 3$). $^{*}P < 0.05$; $^{**}P < 0.01$. (b) The effect of Ca^{2+} entry blockers on cell proliferation as assessed in a MTT assay. The cells were cultured in the normal growth media for 3 days, and then treated for 5 days with the Ca^{2+} entry blockers in 1% serum-containing media with and without 100 ng/ml VEGF. The numbering of columns is the same as in panel a. The data are the means ± S.E.M. ($n = 3$). $^{*}P < 0.05$; $^{**}P < 0.01$. (c) The effect of the Ca^{2+} entry blockers on BrdU incorporation. BrdU incorporation was assessed after 24 h treatment with the Ca^{2+} entry blockers without (open columns) and with (shaded columns) 100 ng/ml VEGF. Column C, no Ca^{2+} entry blocker; column 1, 10 μM SKF 96365; column 2, 10 μM troglitazone; column 3, 10 μM diltiazem. The data are the means ± S.E.M. ($n = 3$). $^{*}P < 0.05$.

troglitazone had no significant effect on VEGF-induced tube formation even at the concentration of 10 μM (2.68 ± 0.27 mm/0.06 mm^3; $n = 5$), although it completely inhibited the VEGF-induced $[Ca^{2+}]_i$ elevation (Fig. 1). The capillary-like structure obtained with troglitazone appeared to be similar to that observed in its absence (Fig. 3a and c). Interestingly, 10 μM troglitazone slightly but significantly enhanced the basal tube formation to 1.91 ± 0.13 mm/0.06 mm^3 ($n = 5$) in the absence of VEGF. Diltiazem, which did not inhibit the VEGF-induced $[Ca^{2+}]_i$ elevation (Fig. 1), had no effect on either the basal tube formation or that observed in the presence of VEGF (Fig. 3d and e).

We also examined the effect of removing external Ca^{2+} on tube formation. To obtain nominally Ca^{2+}-free conditions, we included 2 mM EGTA in the collagen gel. Under this condition, the tube formation was almost completely inhibited even in the presence of VEGF (0.29 ± 0.05 mm/0.06 mm^3; $n = 6$). However, cell viability was also lost in about 80% cells, as determined by a Trypan blue exclusion test. Thus, the apparent inhibition of tube formation was due to the cytotoxic effect of removing external Ca^{2+} on the endothelial cells.

3.3. Cell proliferation and viability

The effects of SKF 96365, troglitazone and diltiazem on the proliferation of endothelial cells were examined by cell counting (Fig. 4a). Since tube formation was evaluated after 5 days of culture in the 1% serum-containing media, the effect of Ca^{2+} entry blockers on cell numbers was evaluated after 5 days of treatment (Fig. 4a). During the 5 days of culture in 1% serum, cells increased from 2.3 ± 0.2 × 10^5 cells/dish to 7.7 ± 0.4 × 10^5 cells/dish (Fig. 4a), and reached confluence. The addition of 100 ng/ml VEGF caused no additional increase in cell number (7.9 ± 0.2 × 10^5 cells). The addition of SKF 96365 decreased the cell number in a concentration-dependent manner in both the presence and absence of VEGF (Fig. 4a). Troglitazone had no effect on cell numbers at concentrations of 0.1 and 1 μM either with or without VEGF, while it slightly, but significantly, decreased cell numbers at 10 μM. Diltiazem slightly, but significantly, decreased cell numbers in both the presence and absence of VEGF. The Trypan blue exclusion test revealed that more than 90% of the cells attached to the dished remained viable under all experimental conditions. As a result, the inhibitory effects of the Ca^{2+} entry blockers on cell proliferation appeared to correlate with those on tube formation.

The potent inhibitory effect of SKF 96365 on cell proliferation was confirmed in two other assays (Fig. 4b and c). In a MTT assay (Fig. 4b), SKF 96365 markedly inhibited cell proliferation in both the presence and absence of VEGF. Troglitazone slightly, but significantly, inhibited cell proliferation in the absence of VEGF, while it had no inhibitory effect on the cell proliferation seen with VEGF. Diltiazem slightly inhibited cell proliferation, but its effect was not statistically significant ($P > 0.05$). The incorporation of BrdU was examined to determine the effect of the Ca^{2+} entry blockers on DNA synthesis (Fig. 4c). After 24 h exposure to the 1% serum-containing medium, 30.0 ± 2.7% (without VEGF, $n = 3$) and 29.9 ± 2.3% (with VEGF, $n = 3$) of cells incorporated BrdU during the 1.5-h labeling period. SKF 96365 significantly inhibited the BrdU incorporation (23.6 ± 2.3% without VEGF; 18.4 ± 3.6% with VEGF). However, troglitazone or diltiazem had no effect on BrdU incorporation in either the presence or absence of VEGF. Collectively, SKF 96365 had a potent inhibitory effect on cell proliferation, as assessed by cell counting, a MTT assay and BrdU incorporation, while troglitazone and diltiazem had a negligible effect on cell proliferation.

4. Discussion

By using three different Ca^{2+} entry blockers, we examined the role of Ca^{2+} influx in the angiogenesis induced by VEGF in bovine aortic endothelial cells. Angiogenesis was evaluated in vitro by observing tube formation in a three-dimensional culture system. A voltage-operated Ca^{2+} channel blocker, diltiazem, had no effect on either the $[Ca^{2+}]_i$ elevation or the tube formation induced by VEGF. This finding is consistent with both our previous observations (Kawasaki et al., 1999) and the electrophysiological studies demonstrating no activity of voltage-operated Ca^{2+} channels in either freshly isolated (Busse et al., 1988) or cultured endothelial cells (Colden-Stanfield et al., 1987). In contrast, SKF 96365 inhibited the extracellular Ca^{2+}-dependent elevation, namely, Ca^{2+} influx and tube formation induced by VEGF. This observation is consistent with a report showing the inhibitory effect of carboxy-amidotriazole, an inhibitor of non-voltage-operated Ca^{2+} channels on the tube formation of human umbilical vein endothelial cells (Kohn et al., 1995). The concentrations of SKF 96365 required to inhibit tube formation (0.1–10 μM) closely correlated with those required to inhibit thapsigargin-induced Ca^{2+} influx in endothelial cells (Kawasaki et al., 1999). These observations with SKF 96365 and carboxy-amidotriazole suggested a possible correlation between the inhibition of Ca^{2+} influx and the inhibition of tube formation.

Troglitazone, an oral anti-diabetic drug, had no effect on tube formation while it potently inhibited the Ca^{2+} influx induced by VEGF. We previously reported that troglitazone inhibited both the Ca^{2+} influx induced by thapsigargin in porcine endothelial cells in culture and in situ and that induced by endothelin and bradykinin in in situ endothelial cells (Kawasaki et al., 1999). An important finding was that troglitazone inhibited Ca^{2+} influx in endothelial cells more potently than did SKF 96365. We also showed that troglitazone inhibited voltage-operated

Ca^{2+} channels in smooth muscles (Kawasaki et al., 1998). We suggested that troglitazone was a non-selective Ca^{2+} entry blocker similar to SKF 96365. Despite the similarity between SKF 96365 and troglitazone in inhibiting Ca^{2+} influx, troglitazone did not inhibit tube formation, while SKF 96365 did inhibit it. This observation suggested that the inhibition of Ca^{2+} influx by SKF 96365 did not correlate with the inhibition of tube formation. This consideration is supported by the observation that SKF 96365 inhibited not only VEGF-stimulated tube formation, but also basal tube formation. As a result, the inhibition of tube formation by SKF 96365 was considered to be due to some effect other than the inhibition of Ca^{2+} influx. Moreover, Ca^{2+} influx was not suggested to be a prerequisite for tube formation.

The present study also showed that SKF 96365 markedly inhibited the proliferation of endothelial cells, while troglitazone and diltiazem only slightly inhibited it. As a result, the inhibitory effects of these reagents on proliferation correlated well with those on tube formation. Therefore, the inhibition of cell proliferation by SKF 96365 was suggested to be linked to the inhibition of tube formation. These findings also indicated that $[Ca^{2+}]_i$ elevation is not linked to cell proliferation. In addition, carboxyamidotriazole has also been reported to inhibit the proliferation of human umbilical vein endothelial cells in response to serum or basic fibroblast growth factor (Kohn et al., 1995). The reported inhibitory effect of carboxyamidotriazole on in vitro tube formation and in vivo angiogenesis is thus considered to be related to the inhibition of proliferation and not to the inhibition of Ca^{2+} influx. It is thus concluded that the inhibition of tube formation by SKF 96365 may be due to the inhibition of cell proliferation.

It is generally accepted that angiogenesis requires cell migration and proliferation of endothelial cells (Beck and D'Amore, 1997). VEGF is a potent angiogenic and mitogenic factor for vascular endothelial cells (Neufeld et al., 1999). There are two types of VEGF receptor, Flt-1 and KDR/Flk-1 (Neufeld et al., 1999). Both of them belong to subtype 5 of receptor tyrosine kinase (Terman and Dougher-Vermazen, 1996), and they are very similar to the platelet-derived growth factor (PDGF) receptor. They consist of seven immunogobulin-like repeats in the ligand-binding domain, a single transmembrane domain and two tyrosine kinase domains split by a kinase insert domain. The different roles of the two receptors are still controversial. KDR/Flk-1 was shown to induce both cell migration and proliferation, while Flt-1 induced cell migration but not cell proliferation (Neufeld et al., 1999). Flt-1 is thus considered to act as a survival factor involved in endothelial cell morphogenesis, while Flk-1/KDR functions as a transducer to signal endothelial cell proliferation and differentiation (Ferrara and Davis-Smyth, 1997; Fong et al., 1995; Ilan et al., 1998). Although the intracellular signaling induced by VEGF receptors is not fully understood, some of the signals induced by VEGF are similar to those induced by PDGF (Neufeld et al., 1999; Terman and Dougher-Vermazen, 1996). In smooth muscle cells in primary culture, the $[Ca^{2+}]_i$ elevation induced by PDGF was shown to be unrelated to the mitogenic activity of PDGF (Kobayashi et al., 1994). This finding is consistent with reports on PDGF receptor mutants (Escobedo and Williams, 1988; Williams, 1989). Three different mutants of tyrosine kinase-deficient receptor failed to induce $[Ca^{2+}]_i$ elevation and DNA synthesis (Escobedo et al., 1988), while a kinase insert-deficient receptor induced $[Ca^{2+}]_i$ elevation, but not DNA synthesis (Escobedo and Williams, 1988). It was thus suggested that $[Ca^{2+}]_i$ elevation is not required for the mitogenic activity induced by PDGF. VEGF was also shown to induce $[Ca^{2+}]_i$ elevation in endothelial cells (Brock et al., 1991). However, the role of the Ca^{2+} signal in the cell proliferation induced by VEGF has not been established. In the present study, the potent inhibition of VEGF-induced Ca^{2+} influx by troglitazone was not associated with the inhibition of cell proliferation. SKF 96365 had no significant effect on the initial transient elevation of $[Ca^{2+}]_i$ induced by VEGF while inhibiting cell proliferation, suggesting that the transient elevation of $[Ca^{2+}]_i$ is not a prerequisite for cell proliferation. Collectively, the present study suggests a dissociation between the transient Ca^{2+} signal and the mitogenic activity of VEGF, as in the case of PDGF.

In our study, VEGF increased $[Ca^{2+}]_i$ due to the Ca^{2+} influx as well as Ca^{2+} release. This observation was also consistent with previous reports (Brock et al., 1991; Papapetropoulos et al., 1997). The mechanism of the activation of Ca^{2+} influx by VEGF has not yet been clearly elucidated. However, the present study clearly demonstrated that the VEGF-induced Ca^{2+} influx was inhibited by both SKF 96365 and troglitazone. At 10 μM, troglitazone completely inhibited the Ca^{2+} elevation, while SKF 96365 only partially inhibited it. These observations are similar to those of our previous study, in which we reported that troglitazone inhibited the Ca^{2+} influx induced by the depletion of the intracellular Ca^{2+} stores by thapsigargin more potently than did SKF 96365 (Kawasaki et al., 1999). The similarity of the sensitivity to the Ca^{2+} entry blockers suggests that the VEGF-activated Ca^{2+} influx pathway is similar to that activated by thapsigargin. It is possible that VEGF induces the depletion of Ca^{2+} stores due to Ca^{2+} release, since VEGF was shown to activate both phospholipase C-γ and inositol trisphosphate production (Brock et al., 1991).

Troglitazone is one of the anti-diabetic thiazolidinediones. Thiazolidinedione has been recently shown as a high affinity ligand for peroxisome proliferator-activated receptor-γ (PPAR-γ) (Lehmann et al., 1995). The activation of PPAR-γ by 15-deoxy-δ12,14-prostaglandin J2 or other thiazolidinediones, such as BRL49653 and ciglitizone, was shown to markedly suppress the tube formation of human umbilical vein endothelial cells in three-dimen-

sional collagen gels (Xin et al., 1999). This inhibition of tube formation was shown to correlate with the inhibition of cell proliferation, as evaluated by DNA synthesis (Xin et al., 1999). The observed correlation between the inhibition of tube formation and the inhibition of cell proliferation is consistent with our finding of the inhibitory effect of SKF 96365 on the proliferation and tube formation of bovine aortic endothelial cells. However, the above report is apparently inconsistent with our observation that troglitazone slightly but significantly accelerated tube formation in the 1% serum medium but had no effect on VEGF-stimulated tube formation. The mechanism for the slight enhancement of basal tube formation by troglitazone remains to be elucidated; however, the absence of an inhibitory effect of troglitazone on tube formation may be due to differences in the level of expression of PPAR-γ between human umbilical vein endothelial cells and bovine aortic endothelial cells. The expression of PPAR-γ in endothelial cells was first reported in human umbilical vein endothelial cells (Xin et al., 1999); however, the expression of PPAR-γ in bovine aortic endothelial cells has yet to be determined. Troglitazone was reported to have no direct effect on the proliferation of mouse aortic endothelial cells, while it significantly inhibited basic fibroblast growth factor-induced proliferation (Gralinski et al., 1998). Troglitazone was also shown to inhibit the proliferation of vascular smooth muscle cells (Marx et al., 1998; Yasunari et al., 1997). Our observations of the slight inhibition of the proliferation of bovine aortic endothelial cells by troglitazone may be consistent with these reports.

We herein showed the dissociation between the inhibition of Ca^{2+} influx and the inhibition of tube formation in bovine aortic endothelial cells, based on the different effects of SKF 96365 and troglitazone. While both SKF 96365 and troglitazone inhibited VEGF-induced Ca^{2+} influx, only SKF 96365 inhibited tube formation. Moreover, SKF 96365 not only inhibited VEGF-stimulated tube formation but also basal tube formation induced in the presence of 1% serum. These observations suggested that Ca^{2+} influx is not a prerequisite for the angiogenesis of endothelial cells. An effect of SKF 96365 other than inhibition of Ca^{2+} influx maybe related to the inhibition of tube formation. The inhibition of cell proliferation is therefore considered to be one of the major mechanisms involved in the inhibition of tube formation.

Acknowledgements

We thank Mr. Brian Quinn for comments and help with the manuscript. This study was supported in part by Grants-in-Aid for Scientific Research (Nos. 10557072, 11838013, 11670687), for the Encouragement of Young Scientists (No. 10770308) from the Ministry of Education, Science, Sports and Culture, Japan, by the Research Grant for Cardiovascular Diseases (11C-1) from the Ministry of Health and Welfare, Japan, and by grants from the Vehicle Racing Commemorative Foundation, the Foundation for the Promotion of Clinical Medicine and the Suzuken Memorial Foundation.

References

Aoki, H., Kobayashi, S., Nishimura, J., Kanaide, H., 1994. Sensitivity of G-protein involved in endothelin-1-induced Ca^{2+} influx to pertussis toxin in porcine endothelial cells in situ. Br. J. Pharmacol. 111, 989–996.

Beck, L.Jr., D'Amore, P.A., 1997. Vascular development: cellular and molecular regulation. FASEB J. 11, 365–373.

Bressler, R., Johnson, D.G., 1997. Pharmacological regulation of blood glucose levels in non-insulin-dependent diabetes mellitus. Arch. Intern. Med. 157, 836–848.

Brock, T.A., Dvorak, H.F., Senger, D.R., 1991. Tumor-secreted vascular permeability factor increases cytosolic Ca^{2+} and von Willebrand factor release in human endothelial cells. Am. J. Pathol. 138, 213–221.

Busse, R., Fichtner, H., Luckhoff, A., Kohlhardt, M., 1988. Hyperpolarization and increased free calcium in acetylcholine-stimulated endothelial cells. Am. J. Physiol. 255, H965–H969.

Colden-Stanfield, M., Schilling, W.P., Ritchie, A.K., Eskin, S.G., Navarro, L.T., Kunze, D.L., 1987. Bradykinin-induced increases in cytosolic calcium and ionic currents in cultured bovine aortic endothelial cells. Circ. Res. 61, 632–640.

Davis, G.E., Camarillo, C.W., 1996. An α2 β1 integrin-dependent pinocytic mechanism involving intracellular vacuole formation and coalescence regulates capillary lumen and tube formation in three-dimensional collagen matrix. Exp. Cell Res. 224, 39–51.

Escobedo, J.A., Williams, L.T., 1988. A PDGF receptor domain essential for mitogenesis but not for many other responses to PDGF. Nature 335, 85–87.

Escobedo, J.A., Barr, P.J., Williams, L.T., 1988. Role of tyrosine kinase and membrane-spanning domains in signal transduction by the platelet-derived growth factor receptor. Mol. Cell. Biol. 8, 5126–5131.

Ferrara, N., Davis-Smyth, T., 1997. The biology of vascular endothelial growth factor. Endocr. Rev. 18, 4–25.

Folkman, J., Klagsburn, M., 1987. Angiogenic factors. Science 235, 442–447.

Fong, G.H., Rossant, J., Gertsenstein, M., Breitman, M.L., 1995. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature 376, 66–70.

Gralinski, M.R., Rowse, P.E., Breider, M.A., 1998. Effects of troglitazone and pioglitazone on cytokine-mediated endothelial cell proliferation in vitro. J. Cardiovasc. Pharmacol. 31, 909–913.

Hanahan, D., 1997. Signaling vascular morphogenesis and maintenance. Science 277, 48–50.

Hirano, K., Hirano, M., Kanaide, H., 1993. Enhancement by captopril of bradykinin-induced calcium transients in cultured endothelial cells of the bovine aorta. Eur. J. Pharmacol. 244, 133–137.

Hirano, M., Niiro, N., Hirano, K., Nishimura, J., Hartshorne, D.J., Kanaide, H., 1999. Expression, subcellular localization, and cloning of the 130-kDa regulatory subunit of myosin phosphatase in porcine aortic endothelial cells. Biochem. Biophys. Res. Commun. 254, 490–496.

Hood, J.D., Meininger, C.J., Ziche, M., Granger, H.J., 1998. VEGF upregulates ecNOS message, protein, and NO production in human endothelial cells. Am. J. Physiol. 274, H1054–H1058.

Ilan, N., Mahooti, S., Madri, J.A., 1998. Distinct signal transduction pathways are utilized during the tube formation and survival phases of in vitro angiogenesis. J. Cell Sci. 111, 3621–3631.

Kawasaki, J., Hirano, K., Nishimura, J., Fujishima, M., Kanaide, H., 1998. Mechanisms of vasorelaxation induced by troglitazone, a novel

antidiabetic drug, in the porcine coronary artery. Circulation 98, 2446–2452.

Kawasaki, J., Hirano, K., Hirano, M., Nishimura, J., Fujishima, M., Kanaide, H., 1999. Troglitazone inhibits capacitative Ca^{2+} entry in endothelial cell. Eur. J. Pharmacol. 373, 111–120.

Kobayashi, S., Nishimura, J., Kanaide, H., 1994. Cytosolic Ca^{2+} transients are not required for platelet-derived growth factor to induce cell cycle progression of vascular smooth muscle cells in primary culture. Actions of tyrosine kinase. J. Biol. Chem. 269, 9011–9018.

Kohn, E.C., Alessandro, R., Spoonster, J., Wersto, R.P., Liotta, L.A., 1995. Angiogenesis: role of calcium-mediated signal transduction. Proc. Natl. Acad. Sci. U. S. A. 92, 1307–1311.

Ku, D.D., Zaleski, J.K., Liu, S., Brock, T.A., 1993. Vascular endothelial growth factor induces EDRF-dependent relaxation in coronary arteries. Am. J. Physiol. 265, H586–H592.

Lehmann, J.M., Moore, L.B., Smith-Oliver, T.A., Wilkison, W.O., Willson, T.M., Kliewer, S.A., 1995. An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor γ (PPARγ). J. Biol. Chem. 270, 12953–12956.

Madri, J.A., Williams, S.K., 1983. Capillary endothelial cell cultures: phenotypic modulation by matrix components. J. Cell Biol. 97, 153–165.

Marx, N., Schonbeck, U., Lazar, M.A., Libby, P., Plutzky, J., 1998. Peroxisome proliferator-activated receptor γ activators inhibit gene expression and migration in human vascular smooth muscle cells. Circ. Res. 83, 1097–1103.

Merritt, J.E., Armstrong, W.P., Benham, C.D., Hallam, T.J., Jacob, R., Jaxa-Chamiec, A., Leigh, B.K., McCarthy, S.A., Moores, K.E., Rink, T.J., 1990. SK & F 96365, a novel inhibitor of receptor-mediated calcium entry. Biochem. J. 271, 515–522.

Montesano, R., Orci, L., Vassalli, P., 1983. In vitro rapid organization of endothelial cells into capillary-like networks is promoted by collagen matrices. J. Cell Biol. 97, 1648–1652.

Neufeld, G., Cohen, T., Gengrinovitch, S., Poltorak, Z., 1999. Vascular endothelial growth factor (VEGF) and its receptors. FASEB J. 13, 9–22.

Nisoli, E., Clementi, E., Tonello, C., Sciorati, C., Briscini, L., Carruba, M.O., 1998. Effects of nitric oxide on proliferation and differentiation of rat brown adipocytes in primary cultures. Br. J. Pharmacol. 125, 888–894.

Okamura, K., Morimoto, A., Hamanaka, R., Ono, M., Kohno, K., Uchida, Y., Kuwano, M., 1992. A model system for tumor angiogenesis: involvement of transforming growth factor-α in tube formation of human microvascular endothelial cells induced by esophageal cancer cells. Biochem. Biophys. Res. Commun. 186, 1471–1479.

Papapetropoulos, A., Garcia-Cardena, G., Madri, J.A., Sessa, W.C., 1997. Nitric oxide production contributes to the angiogenic properties of vascular endothelial growth factor in human endothelial cells. J. Clin. Invest. 100, 3131–3139.

Satake, S., Kuzuya, M., Ramos, M.A., Kanda, S., Iguchi, A., 1998. Angiogenic stimuli are essential for survival of vascular endothelial cells in three-dimensional collagen lattice. Biochem. Biophys. Res. Commun. 244, 642–646.

Terman, B.I., Dougher-Vermazen, M., 1996. Biological properties of VEGF/VPF receptors. Cancer Metastasis Rev. 15, 159–163.

Williams, L.T., 1989. Signal transduction by the platelet-derived growth factor receptor. Science 243, 1564–1570.

Xin, X., Yang, S., Kowalski, J., Gerritsen, M.E., 1999. Peroxisome proliferator-activated receptor γ ligands are potent inhibitors of angiogenesis in vitro and in vivo. J. Biol. Chem. 274, 9116–9121.

Yasunari, K., Kohno, M., Kano, H., Yokokawa, K., Minami, M., Yoshikawa, J., 1997. Mechanisms of action of troglitazone in the prevention of high glucose-induced migration and proliferation of cultured coronary smooth muscle cells. Circ. Res. 81, 953–962.