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Thapsigargin inhibits angiogenesis in the rat isolated aorta: studies on the role of intracellular calcium pools

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Abstract

Objective: Since the role of Ca^{2+} in angiogenesis is not fully understood, we investigated the effect of thapsigargin (TG: depletes intracellular Ca^{2+} pools) and other Ca^{2+} modulators [ionomycin, calcium ionophore A23187 and dibutylhydroquinone (DBHQ)] on in vitro angiogenesis by rat aortic rings. **Methods:** Aortae from Sprague–Dawley rats were cut into 2-mm rings, embedded in a fibrin clot and cultured for 15 days in serum-free medium containing drugs and the microvessels counted. Rings were also pre-treated with TG and Ca^{2+} modulators for 1 h prior to embedding and culture. Viability was examined by the measurement of lactic acid dehydrogenase release. Rings were also treated with hydrocortisone and lavendustin A (a tyrosine kinase inhibitor), as positive controls. The effect of TG on the proliferation and migration of human umbilical artery endothelial cells (HUVECs) was studied in parallel. **Results:** TG significantly inhibited microvessel formation and HUVEC proliferation and migration in a dose-dependent manner, all at <10 nmol/l, without affecting viability. In contrast, ionomycin, A23187 and DBHQ were cytotoxic at inhibitory concentrations. Continual exposure to hydrocortisone and lavendustin A also inhibited angiogenesis without affecting viability. **Conclusion:** Since low concentrations of TG deplete intracellular Ca^{2+} stores, it is concluded that these pools play a central role in mediating angiogenesis. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Angiogenesis; Calcium (cellular)

1. Introduction

Angiogenesis plays a pivotal role in tumourigenesis [1,2]. In turn, the inhibition of angiogenesis has become an important therapeutic strategy in oncology [1,2]. Angiogenesis involves the proliferation and migration of endothelial cells (ECs) which form tubes [1]. Vascular smooth muscle cells (VSMCs) later migrate and associate with these tubes to form the microvessel proper [1,2]. It follows that the inhibition of any of these aforementioned processes would inhibit angiogenesis and therefore constitute therapeutic targets.

Of the factors that control angiogenesis, Ca^{2+} plays a key role, but the precise mechanisms involved are not fully

elucidated [3]. It was recently demonstrated, however, that low concentrations (<10 nmol/l) of thapsigargin (a Ca^{2+} ATPase inhibitor) inhibits the proliferation and migration of human vascular smooth muscle cells (VSMCs) [4,5]. This effect was mediated by the depletion of intracellular Ca^{2+} pools and not by an elevation of cytosolic Ca^{2+} [6]. Furthermore, pre-exposure of human saphenous vein segments to <10 nmol/l thapsigargin, followed by washing, prevented the proliferation of VSMCs for 14 days in culture after treatment [7]. Notably, thapsigargin was not cytotoxic at the concentrations that inhibited migration or proliferation of VSMCs [7]. In contrast, other Ca^{2+} modulators including dibutylhydroquinone (DBHQ), Ca^{2+} ionophore A23187 (A23187) and ionomycin did not exert a similar effect at non-toxic concentrations [4]. It was concluded that intracellular Ca^{2+} pools are crucial for both the proliferation and migration of VSMCs [4–7].

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In order to investigate further the role of intracellular Ca^{2+} pools in mediating angiogenesis, the effect of prolonged and short-term exposure to thapsigargin on microvessel formation and growth in rat isolated aorta was investigated using a modified method of Nicosia and Ottinetti [8]. DBHQ, A23187 and ionomycin were also investigated. As positive controls, two drugs which had previously been shown to inhibit angiogenesis; hydrocortisone [8] and lavendustin A [9,10] were also studied. The effect of thapsigargin on the proliferation and migration of human umbilical endothelial cells (HUVECs) was studied in parallel, since these processes are central to angiogenesis [1,2].

2. Methods

2.1. Materials

Dulbecco's modified eagle medium with L-glutamine sodium pyruvate and 5 mM HEPES, Medium 199 and phosphate-buffered saline (PBS) were purchased from Gibco Life Technologies (Paisley, Renfrew, UK) and EBM[®] (endothelial basal medium) from Clonetics, normal human cell system distributed by TCS Biologicals (Buckingham, UK). Lectin 1 isolectin B4 antibody was purchased from R&D Systems (Oxford, UK). Aluminium ammonium sulphate, ϵ -aminocaproic acid, buffer components, calcium ionophore A23187, citric acid, chloral hydrate, collagen type IV, collagenase, dibutylhydroquinone (DBHQ), dimethylsulfoxide (DMSO) fibrinogen, forskolin, haematoxylin, hydrocortisone, ionomycin, isobutylmethylxanthine, laminin, perchloric acid, sodium iodate and thapsigargin, were purchased from Sigma (Poole, Dorset, UK). Lavendustin A was purchased from Calbiochem-Novabiochem (Nottingham, UK). Lactic acid dehydrogenase assay kits were purchased from Boehringer Mannheim (Mannheim, Germany). [¹²⁵I]-radioimmunoassay kits for the measurement of cAMP were purchased from Amersham (Aylesbury, Bucks, UK).

Thapsigargin, ionomycin, A23187, DBHQ, lavendustin A and hydrocortisone were all initially dissolved in DMSO and diluted subsequently in medium for addition to cells or tissues. Controls contained equivalent amounts of DMSO.

2.2. Angiogenesis assay

Male Sprague–Dawley rats (250 g) were killed by decapitation, their thoracic aortae rapidly excised and placed in Dulbecco's modified eagle medium (DMEM) supplemented with 100 U/ml penicillin and 100 U/ml streptomycin and 0.25 μ g/ml amphotericin. Under sterile conditions, adventitia was removed and the aortae cut into 2-mm rings and rinsed in DMEM. The rings were then transferred to 12-well culture plates containing fibrinogen

solution (50 U/ml). Thrombin (3 mg/ml) which promotes the conversion of fibrinogen to fibrin, was added to each well, thereby embedding and immobilising the rings. The entire fibrin-ring complex was then covered with serum-free DMEM containing ϵ -aminocaproic acid (300 μ g/ml), a protease inhibitor which prevents the dissolution of the fibrin gel by secretion of proteases from the aortic ring. The culture plates were then placed in a 95% O_2 –5% CO_2 humidified incubator for 15 days and microvessels counted daily under a microscope (Fig. 1). Tissues were incubated with thapsigargin, calcium ionophore A23187, ionomycin or DBHQ over the entire 15 days of culture.

As positive controls, aortic rings were also treated with 100 nmol/l hydrocortisone and 100 nmol/l lavendustin A, since these drugs at these concentrations had previously been shown to inhibit angiogenesis in this model [8–10]. In other experiments, rings were pre-incubated with thapsigargin and other drugs for 1 h, washed three times in DMEM and subsequently cultured for 15 days in fibrin clots and angiogenesis measured as above.

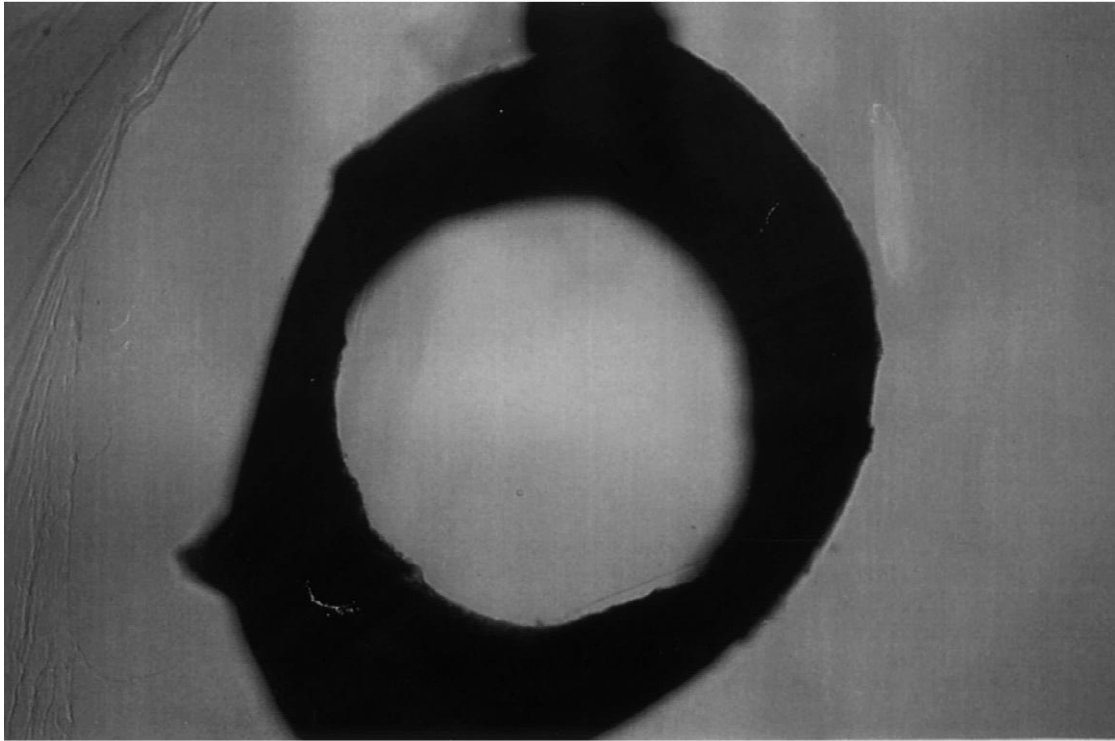
2.3. Endothelial cell cultures

The method of Jaffe et al. [11] was used to prepare HUVECs for culture. Fresh umbilical cords were obtained from the delivery ward and cannulated. They were then flushed with warm PBS to remove blood and filled with a 2% collagenase solution for 15 min at room temperature. The cell suspension was then removed, neutralised and washed with DMEM containing 20% FCS and then the cells placed in culture with M199 culture medium and 20% FCS and cultured in a 5% CO_2 humidified incubator. When confluent, the cells were trypsinized and seeded into 96-well culture plates and then grown to confluence in the same medium. Cells stained positive for lectin and von Willebrand's factor.

2.4. Thymidine incorporation

Confluent HUVECs at third passage were subcultured into 96-well plates and then rendered quiescent by changing the medium to 0.4% FCS in M199 and incubated for 48 h in a 5% CO_2 humidified incubator. The cells were then washed with FCS-free medium prior to drug treatment and thymidine incorporation experiments. Thapsigargin, ionomycin, DBHQ or A23187 in quadruplicate for each drug dose were added to the cells over a range of concentrations. [³H]-thymidine (1 μ Ci) was then added to each well and the plates incubated for a further 48 h in a CO_2 humidified incubator. After incubation, the medium was discarded and the cells washed three times with phosphate buffered saline. To each well was added ice-cold 7.5% trichloroacetic acid and this was discarded after incubation for 1 h at 4°C. A 200- μ l volume of 1 mol/l NaOH was then added to each well and the plates then left at room temperature overnight. The dissolved DNA

A



B

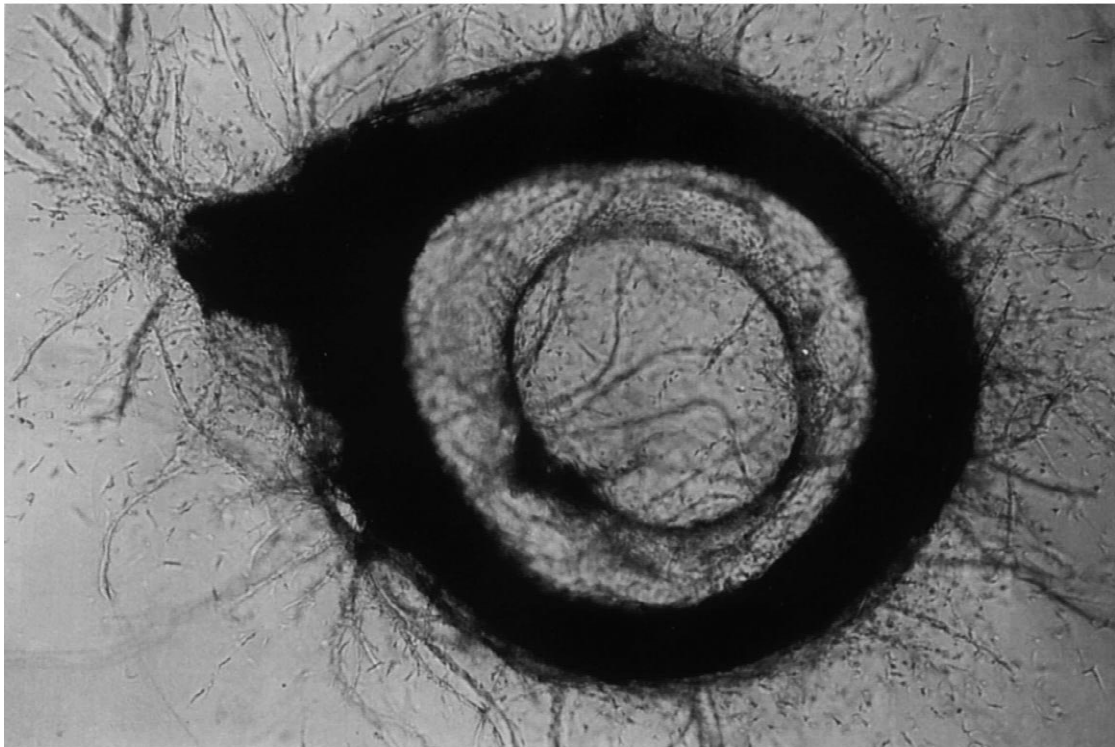


Fig. 1. Photomicrographs of rat aorta embedded in fibrin at (A) day zero and (B) 15 days after culture showing the degree of microvessel formation over this time course.

was then pipetted into liquid scintillation vials and which well rinsed with hydrochloric acid which was then pipetted into equivalent vials. Liquid scintillation fluid was added and the vials counted for radioactivity. For cell counts, culture medium was discarded and the cells washed with calcium and magnesium free phosphate buffered saline. Cells were then treated with trypsin and the suspended cells counted using a haemocytometer.

2.5. Tests for tissue and HUVEC viability

In order to check for tissue and cell viability and drug cytotoxicity, several methods were used. First for histology, following culture for 15 days, fibrin clots containing aortae were removed and placed in Carsen's fixative for 24 h, after which fixed gels were washed and placed in PBS containing 2% sucrose at 4°C until processing. Gels were embedded in wax and 4- μ m sections prepared and mounted on slides which were then treated with haematoxylin and eosin stain. In parallel experiments, after 15 days of culture, rings were removed from the fibrin matrix and assessed for viability using lactic acid dehydrogenase (LDH) release using commercial kits (Boehringer-Mannheim) and cAMP formation [4,12].

For tissue LDH, rings were incubated in DMEM at 37°C for 12 h and supernatants and tissues stored in protein coated tubes in liquid nitrogen until assay. Tissue was homogenised in assay buffer on ice and solubilized with 1% Triton X-100 for 1 h at room temperature. HUVECs were seeded into 24-well plates and cultured for 48 h to reach semi-confluence and rendered quiescent as described above. The HUVECs were treated with thapsigargin or other drugs as for thymidine incorporation and incubated for a further 48 h. Supernatants were carefully removed and stored as above and the monolayer of cells solubilized with 1% Triton X-100 for 1 h at room temperature. These supernatants and solubilized cells were then processed for measurement of LDH levels according to protocols supplied by Boehringer Mannheim [4]. LDH release was expressed as a percentage of LDH in the supernatant relative to total LDH (LDH in supernatants+LDH in cells) [4].

For cAMP, rings were placed in DMEM containing 250 μ mol/l isobutylmethylxanthine (IBMX) and incubated with 1 μ mol/l forskolin (stimulates the formation of cyclic adenosine-3'5'-monophosphate (cAMP) [12]. Reactions were stopped with the addition of 1 mol/l perchloric acid and cyclic nucleotides extracted and measured using radioimmunoassays [12]. The rationale for this approach is that since cAMP formation is dependent upon ATP stores and tissue/cell ATP levels are an index of tissue viability, cAMP is, ipso facto, an index of viability [12].

2.6. Chemotaxis

Chemotaxis experiments were carried out using a modi-

fied method of Kohn et al. [13]. DMEM and 1% BSA was pipetted into one group of wells of the chemotaxis chamber, to act as a control and to monitor spontaneous chemotaxis. DMEM containing a mixture of laminin (100 μ g/ml) and collagen type IV (100 μ g/ml) were pipetted into the rest of the wells and a Nucleopore polyvinyl propylene-free chemotaxis filter was placed over the wells. The top plate was pushed down against the bottom plate and held in place with thumb nuts. The cultured HUVECs were washed with PBS containing 0.1% BSA, without calcium and magnesium ions and sodium bicarbonate, and then with wash buffer (0.1% BSA and 0.1% acetic acid in PBS) at 4°C for 2–3 min. The cells were then washed in PBS containing 0.02% EDTA to give a monosuspension, an aliquot being taken to count. The cell count was then adjusted by addition of PBS to give a final cell density of $1-1.2 \times 10^5$ /ml ($=5-6 \times 10^4$ /well). To each well cell suspensions were added containing different amounts of thapsigargin. In other experiments, cells were pre-incubated with thapsigargin for 1 h prior to the onset of migration assay. The chambers were then incubated for 4 h at 37°C in a humidified O₂-CO₂ (95:5%) incubator. The chamber was inverted and the filter removed. Wetting the lower side in PBS and wiping removed unigrated cells. The cells were then fixed by placing the filter in methanol for 2–3 min and then stained in haematoxylin (haematoxylin, 4 g, sodium iodate, 0.8 g, aluminium ammonium sulphate, 200 g, citric acid, 4 g, chloral hydrate, 200 g) for 1 h. The filter was then washed in tap water and placed on a microscope slide, and allowed to dry. A counting template was placed over the filter and the cells in each well were counted manually using a microscope.

2.7. Data analysis and statistics

Data was analysed using ANOVA for multiple comparisons. Paired analysis between two groups was performed using paired Student's *t*-test where ANOVA indicated significance for the multiple comparison. Statistical significance was accepted when $P < 0.05$.

3. Results

3.1. Continuous exposure

The profile of the rate and extent of microvessel growth was sigmoidal over 15 days (Fig. 1). Continual exposure to thapsigargin over 15 days inhibited microvessel formation in a dose-dependent manner (IC₅₀; 2 nmol/l; Fig. 2). This treatment had no obvious deleterious effect on the histological appearance of the tissue and had no effect on LDH release (Table 1) or forskolin-stimulated cAMP formation (index of ATP levels; Table 2). Continual exposure to the other Ca²⁺ modulators, A23187, ionomycin and DBHQ also inhibited angiogenesis at

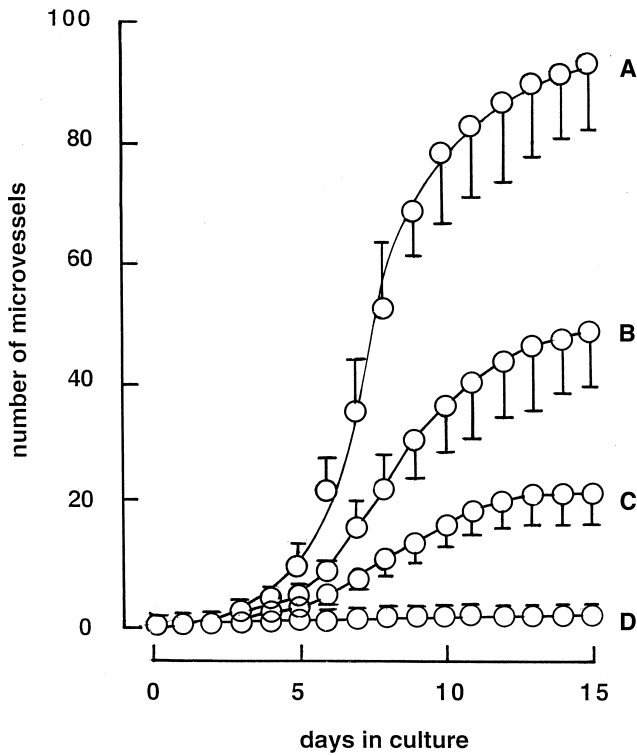


Fig. 2. Effect of continuous exposure to different concentrations of thapsigargin: zero control (A), 1 nmol/l (B), 10 nmol/l (C), 100 nmol/l (D) on microvessel formation in rat aortic rings cultured for 15 days in fibrin. Each point=mean±S.E.M., n=6.

higher concentrations than thapsigargin (Fig. 3) but at these concentrations and greater they also markedly reduced LDH release and cAMP formation indicating a cytotoxic effect (Tables 1 and 2). The inhibition of angiogenesis could therefore be ascribed to cytotoxic effects rather than a true physiological response.

Continual exposure of thapsigargin to HUVECs over 48 h inhibited thymidine incorporation relative to cell numbers (Fig. 4) and migration (Table 3) in a dose-dependent manner and almost identical to that of angiogenesis. At concentrations that inhibited proliferation and migration, thapsigargin had no effect on LDH levels but at higher concentrations elicited a significant release of LDH (Table 4). Continual exposure to the other Ca²⁺ modulators, A23187, ionomycin and DBHQ also inhibited proliferation but at concentrations greater than 100 nmol/l caused a significant release of LDH indicating that the drugs elicited a cytotoxic effect which would explain the inhibitory effects of these drugs on proliferation (Table 4). These data also consolidate the conclusions based on LDH and cAMP levels in the angiogenesis assay above. Continual exposure to hydrocortisone and lavendustin A, both at 100 nmol/l, also inhibited microvessel formation (Fig. 5) without affecting viability (Tables 1 and 2), consolidating the validity of the system, since previous studies have shown that these two drugs are inhibitors of angiogenesis [8–10].

Table 1
Effect of continual exposure of different drugs on LDH release (% increase) by rat aortic rings after culture for 15 days in fibrin gels^a

	-log Drug concentration (mol/l)					
	0	9	8	7	6	5
Thapsigargin	0±0	2±0.1	1±0.1	3±0.2	12±5*	40±6*
A23187	0±0	2±0.1	2±0.1	6±0.3	32±3*	88±15*
DBHQ	0±0	1±0.1	2±0.2	2±0.4	33±5*	75±8*
Ionomycin	0±0	3±0.2	3±0.4	4±0.5	40±5*	64±6*
Lavendustin A	0±0	2±0.2	4±0.3	4±0.8	38±4*	38±7*
Hydrocortisone	0±0	1±0.2	3±0.2	3±0.2	2±2	49±8*

^a Each value represents the mean±S.E.M., n=6. *, P<0.05.

Table 2
Effect of continual exposure of different drugs on forskolin (1 µmol/l)-stimulated cAMP concentrations (pmol/mg tissue/min) in rat aortic rings cultured for 15 days in fibrin gels^a

	-log Drug concentration (mol/l)					
	0	9	8	7	6	5
Thapsigargin	5.4±0.6	5.5±0.6	5.5±0.5	5.6±0.4	0.4±0.03*	0.2±0.01*
A23187	5.6±0.4	5.8±0.5	5.3±0.7	3.6±0.5	0.2±0.02*	0.08±0.03*
DBHQ	5.3±0.5	5.5±0.4	5.4±0.8	4.6±0.4	0.4±0.03*	0.07±0.01*
Ionomycin	5.4±0.7	5.7±0.8	5.4±0.8	3.8±0.4	0.4±0.02*	0.2±0.03*
Lavendustin A	5.6±0.3	5.6±0.4	5.5±0.7	3.9±0.5	0.4±0.05*	0.2±0.03*
Hydrocortisone	5.4±0.4	5.5±0.9	5.4±0.6	4.6±0.3	4.4±0.50	3.2±0.03*

^a Each value represents the mean±S.E.M., n=6. *, P<0.05.

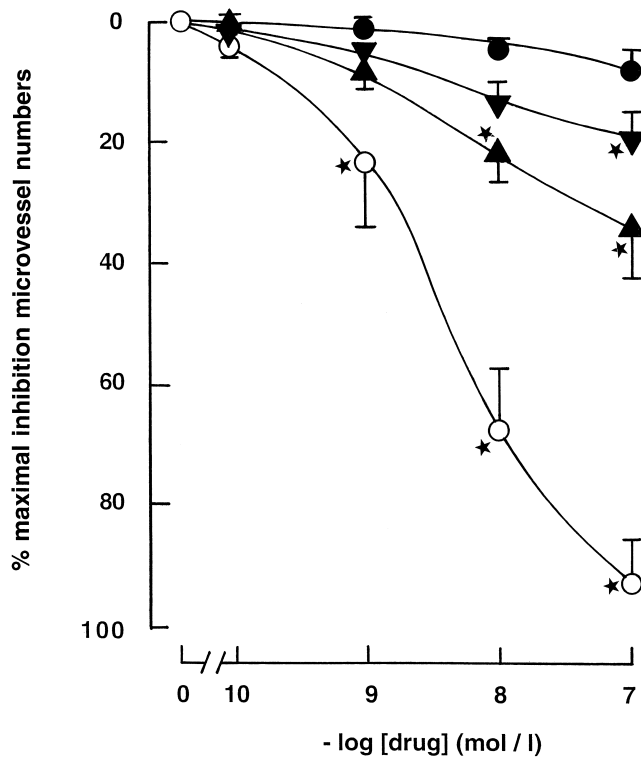


Fig. 3. Angiogenesis: effect of continual exposure to thapsigargin (○), dibutyrylhydroquinone (▲), calcium ionophore A23187 (▼) and ionomycin (●) on microvessel formation in rat aortic rings cultured for 15 days in fibrin. Each point=mean±S.E.M., $n=6$. ★, $P<0.05$.

3.2. One hour pre-exposure experiments

Following pre-exposure for 1 h followed by washing, thapsigargin inhibited microvessel formation in rat aortae over 15 days in a dose dependent manner, although less potent than with continuous exposure (Figs. 6 and 7). Pre-exposure to the other Ca^{2+} modulators, A23187, ionomycin and DBHQ had no effect on angiogenesis (Fig. 7). Pre-exposure to lavendustin A or hydrocortisone (both at 100 nmol/l) for 1 h had no significant effect on microvessel formation over 15 days of culture (Fig. 5).

Pre-exposure of HUVECs to thapsigargin inhibited both the proliferation and migration of HUVECs whereas at non-cytotoxic concentrations, ionomycin, A23187 and DBHQ had no effect on the proliferation or migration of HUVECs (data not shown).

4. Discussion

The present study demonstrates that continual or pre-exposure to thapsigargin inhibited angiogenesis in rat aortae at concentrations that were not cytotoxic. Similarly, both continual or pre-exposure to thapsigargin inhibited the proliferation and migration of cultured endothelial cells. Since angiogenesis involves tube formation from resident endothelial cells [1,2] it is firstly suggested that thapsigar-

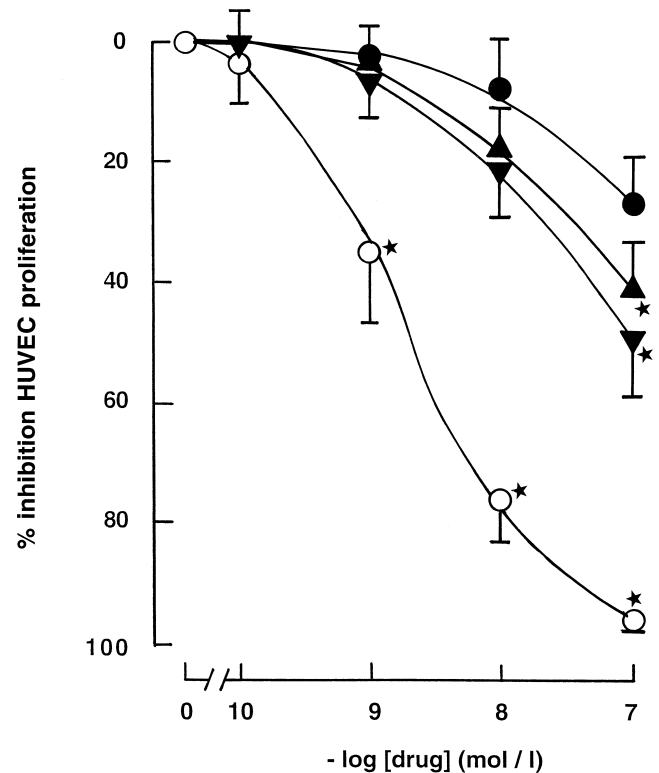


Fig. 4. Proliferation of HUVECs. Effect of continual exposure to thapsigargin (○), dibutyrylhydroquinone (▲), calcium ionophore A23187 (▼) and ionomycin (●) on thymidine incorporation relative to cell numbers in HUVECs cultured for 48 h. Each point=mean±S.E.M., $n=6$. ★, $P<0.05$.

Table 3

Effect of thapsigargin on the migration of HUVECS (% inhibition control)^a

	-log Thapsigargin (mol/l)				
	0	9	8	7	6
Continual	0±0	10±2*	30±3*	48±15*	69±26*
Pre-exposure	0±0	5±2*	10±3*	28±15*	39±26*

^a Each value represents the mean±S.E.M., $n=6$. ★, $P<0.05$.

gin is acting upon endothelial cells in this rat aortic model. In contrast to thapsigargin, the other Ca^{2+} modulators investigated exerted a similar effect at much higher concentrations than thapsigargin but this was ascribable to direct cytotoxic effects. Similar toxic effects of these drugs

Table 4

Effect of continual exposure of different drugs on LDH release (% increase) by HUVECs cultured for 48 h^a

	-log Drug concentration (mol/l)					
	0	9	8	7	6	5
Thapsigargin	0±0	1±0.1	1±0.1	3±0.2	15±5*	60±6*
A23187	0±0	1±0.1	3±0.1	3±0.3	32±3*	88±15*
DBHQ	0±0	1±0.1	4±0.2	2±0.4	43±5*	69±7*
Ionomycin	0±0	3±0.2	3±0.4	4±0.3	45±2*	74±5*

^a Each value represents the mean±S.E.M., $n=6$. ★, $P<0.05$.

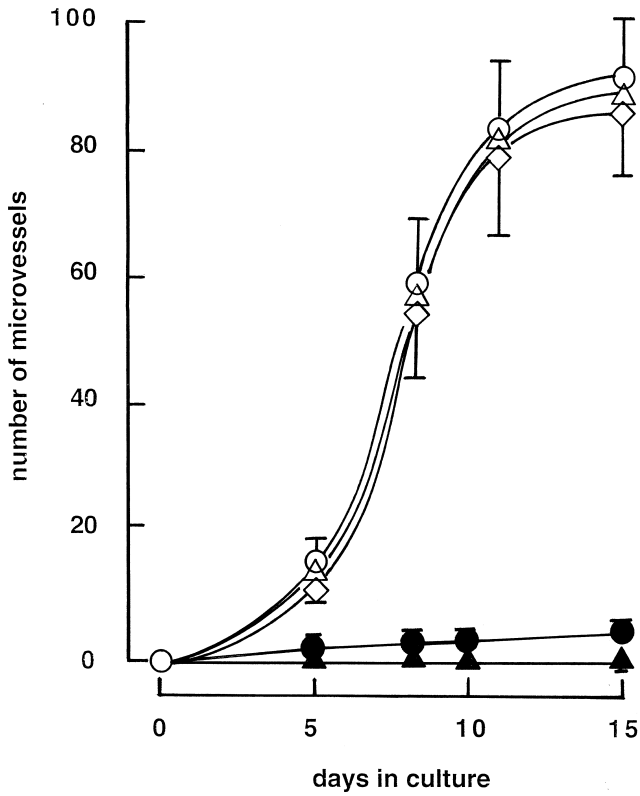


Fig. 5. Effect of continuous and pre-exposure to hydrocortisone and lavendustin A on microvessel formation in cultured rat aortae. Continuous exposure for 15 days: 100 nmol/l lavendustin A (▲), 100 nmol/l hydrocortisone (●). Rings were also pre-exposed to drugs for 1 h followed by 15 days in culture: control (○), 100 nmol/l lavendustin A (△), 100 nmol/l hydrocortisone (◇). Each point=mean±S.E.M., n=6.

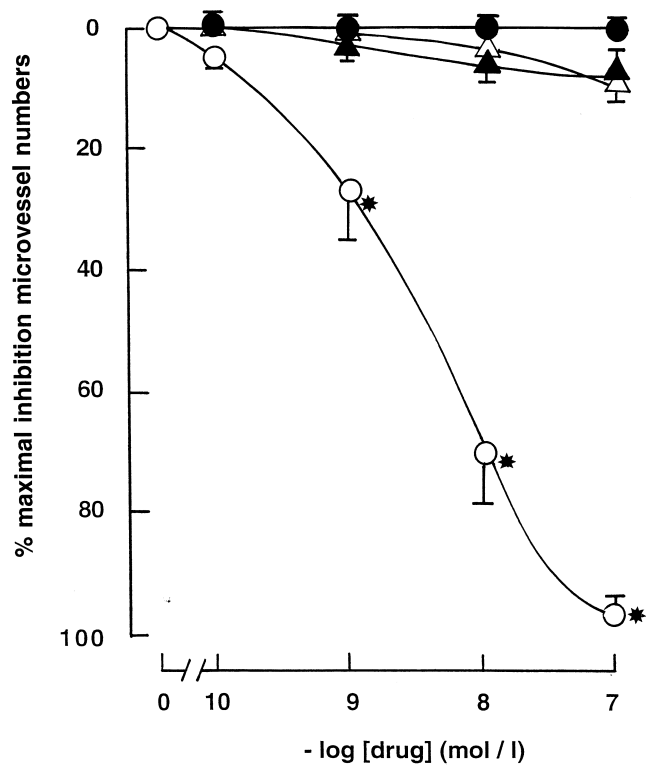


Fig. 7. Effect of pre-exposure to thapsigargin (○), dibutylhydroquinone (▲), calcium ionophore A23187 (△) and ionomycin (●) on microvessel formation in rat aortic rings cultured for 15 days in fibrin. Each point=mean±S.E.M., n=6. ★, P<0.05.

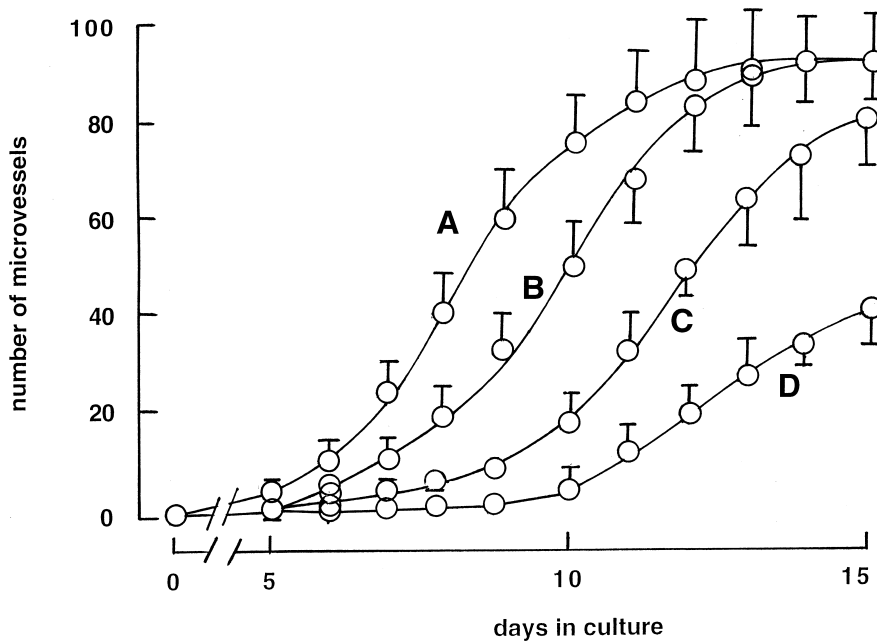


Fig. 6. Effect of pre-exposure to different concentrations of thapsigargin for 1 h on microvessel formation in rat aortic rings cultured for 15 days in fibrin: zero control (A), 1 nmol/l (B), 10 nmol/l (C), 100 nmol/l (D). Each point=mean±S.E.M., n=6.

on VSMCs have been previously reported [4]. In particular, although DBHQ has the same site of action as thapsigargin (inhibition of Ca^{2+} ATPase) and elevates intracellular Ca^{2+} [14], this drug is clearly toxic to cells in the long term [4]. Caution should therefore be exercised in interpreting data from prolonged culture models, since drug-induced cytotoxicity may give rise to spurious conclusions.

The observation that 1 h pre-exposure to thapsigargin but not lavendustin A and hydrocortisone, still resulted in the inhibition of angiogenesis 15 days later consolidates that thapsigargin binds avidly to its target, Ca^{2+} ATPase of the sarcoplasmic reticulum. Thus, thapsigargin possesses three distinct properties: (1) extreme potency at low concentrations, (2) long term action following brief exposure and (3) relative lack of cytotoxicity at effective concentrations. These properties have also been demonstrated in a human saphenous vein organ culture model. Pre-exposure to 10 nmol/l thapsigargin for 1 h, followed by washing and culture for 14 days, resulted in a suppression of VSMC proliferation and migration and neointima formation [7]. It was concluded that thapsigargin inhibits both the migration and proliferation of VSMCs in this whole tissue model. Furthermore, there were no changes in apoptosis or cell death that may have accounted for these observations [7]. Taken together, these above observations indicate that thapsigargin enters tissues rapidly and remains associated with its site of action (i.e. Ca^{2+} ATPase) for an extended period of time or at least during the initial phase of microvessel formation.

Mechanistically, thapsigargin is a highly selective inhibitor of Ca^{2+} ATPase which sequesters Ca^{2+} into IP_3 mobilisable intracellular stores (the endoplasmic reticulum) in many cells including VSMCs [14,15]. As a consequence, thapsigargin tends to increase cytosolic Ca^{2+} concentration, and then on sustained exposure, to deplete intracellular hormone-sensitive Ca^{2+} pools [14,15]. However, at 10 nmol/l, the concentration at which angiogenesis was completely inhibited in this study, thapsigargin had no effect on cytosolic Ca^{2+} levels in cultured VSMCs, as assessed with FURA-2 loading methods [5]. In contrast, exposure of VSMCs to 10 nmol/l thapsigargin completely inhibited ionomycin-stimulated Ca^{2+} release, demonstrating that at these concentrations, thapsigargin depletes intracellular Ca^{2+} pools without influencing cytosolic Ca^{2+} concentrations [5]. Since thapsigargin also inhibited proliferation at identical concentrations, it was concluded that the release of Ca^{2+} from these pools are obligatory for VSMC replication [5]. In the present study, since thapsigargin at 10 nmol/l also completely inhibited angiogenesis as well as HUVEC proliferation, it is reasonable to suggest that intracellular Ca^{2+} pools are obligatory for endothelial migration and replication and therefore microvessel formation. The lack of effect of ionomycin and A23187 on angiogenesis also consolidates that the effect of thapsigargin is not mediated through an increase in cytosolic Ca^{2+} per se.

With regard to intracellular mechanisms underlying the effect of thapsigargin, there are many candidates for Ca^{2+} -sensitive systems that mediate cellular proliferation and migration. These include phospholipase C- γ , protein kinase C, MAP kinase (ERK1 and ERK2), Ca^{2+} calmodulin kinase and Ca^{2+} sensitive cyclins [16]. In this context, we have demonstrated that thapsigargin has little effect on MAP kinase activation but prevents its translocation to the nucleus which influences cyclin D1 activity [17]. MAPK is axiomatic in the progression of cell division as well as of migration and motility of cells [16]. Ca^{2+} also controls the formation of nitric oxide (NO) an important positive modulator of endothelial cell proliferation and angiogenesis [16,18,19]. Since we have demonstrated that thapsigargin inhibits the activation of eNOS (calcium-calmodulin dependent), it is tempting to postulate that thapsigargin prevents NO-mediated angiogenesis through a similar mechanism. Although the complete analysis of these systems are beyond the scope of the present study, we are currently investigating the sites of action of thapsigargin.

In conclusion, it is proposed that thapsigargin inhibits angiogenesis probably through depletion of intracellular Ca^{2+} pools in endothelial cells, thereby blocking their proliferation and migration. These Ca^{2+} pools constitute a potential therapeutic target for diseases associated with angiogenesis, in particular cancer. Although it is unlikely that thapsigargin itself could be used systemically, its properties render it amenable to local application. For example, prior to implantation as vein grafts, pre-exposure of saphenous veins to thapsigargin inhibits neointima formation in vivo [20]. Since, neointima formation involves the migration and proliferation of VSMCs, we concluded that the targeted administration of thapsigargin may be a therapeutically valid approach to the treatment of vein graft failure. With regard to the prevention of tumour growth, the local administration of thapsigargin may be achieved by using pluronic gels. Apart from inhibiting angiogenesis, such a strategy may also directly inhibit the proliferation of malignant cells.

Acknowledgements

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