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Selective inhibition of agonist-induced but not shear stress-dependent release of endothelial autacoids by thapsigargin

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1 The effects of the Ca^{2+}-ATPase inhibitor, thapsigargin, on the shear stress-dependent and on the agonist-stimulated release of endothelium-derived relaxing factor, i.e. nitric oxide (NO), and prostacyclin (PGI_2) were studied in bovine and human cultured endothelial cells as well as in endothelium-intact arterial segments of the rabbit.

2 Preincubation with thapsigargin (1 μM for 10 min) had no effect on the shear stress-dependent release of NO from bovine aortic endothelial cells grown on beads, but abolished the release of NO induced by ADP, bradykinin, ionomycin or poly-L-lysine. Similarly, thapsigargin completely abrogated the agonist-stimulated PGI_2 release from these cells, but had no effect on the shear stress-dependent release of PGI_2.

3 The acetylcholine-induced release of NO from the luminally perfused thoracic aorta and femoral artery of the rabbit was suppressed by pretreatment with thapsigargin (1 μM). In contrast, thapsigargin did not affect the shear stress-dependent release of NO from the femoral artery.

4 Administration of thapsigargin to these vascular preparations or to cultured endothelial cells alone produced a substantial release of both NO and PGI_2. This release declined towards previous values after washout of thapsigargin.

5 In human and bovine cultured endothelial cells, thapsigargin (1–1000 nM) caused a dose-dependent sustained rise in $[Ca^{2+}]_i$, an effect that was abolished in the absence of extracellular Ca^{2+}. Stimulation of these cells with bradykinin, histamine, ADP or ionomycin after previous exposure to thapsigargin (30–1000 nM) no longer caused an increase in $[Ca^{2+}]_i$.

6 These findings demonstrate that by emptying intracellular Ca^{2+} stores, thapsigargin selectively blocks the agonist-stimulated release of both NO and PGI_2, suggesting a principal difference in the control by $[Ca^{2+}]_i$ of the release of these endothelial autacoids caused by shear stress or receptor-dependent and independent agonists.

Keywords: Vascular endothelium; nitric oxide; prostacyclin; endothelium-derived relaxing factor; thapsigargin; calcium signalling; shear stress

Introduction

In both conduit and resistance-sized arteries, even after deduction of neurogenic, metabolic as well as hormonal influences, a sizeable component of vascular tone persists which is commonly referred to as basal tone. This component of vascular tone is modulated and presumably controlled by two physical forces, the distending transmural pressure and fluid shear stress, resulting from the movement of viscous blood along the endothelial surface. While an increase in transmural pressure is balanced by viscoelastic forces of the media and adventitia, the fluid shear stress only affects the endothelium. An increase in fluid shear stress is thought to be responsible for the phenomenon of flow-induced endothelium-dependent dilatation (Davies, 1989), which is crucial for the maintenance of an adequate oxygen supply to the tissue(s).

Flow-induced endothelium-dependent dilatations were first recognized in conduit arteries (Smiesko *et al.*, 1985; Pohl *et al.*, 1986a; Melkumyants *et al.*, 1987), and since then have also been documented in resistance-sized arteries (Griffith *et al.*, 1987; Koller & Kaley, 1990a). These endothelium-dependent dilatations are generally considered to be mediated by an enhanced release of endothelium-derived relaxing factor (EDRF; for reference see Hutcheson & Griffith, 1991), the active principle of which has been identified as nitric oxide (NO; Palmer *et al.*, 1987), and additionally, in some vascular regions, by prostacylin (PGI_2; Pohl *et al.*, 1986b; Koller & Kaley, 1990b). However, little is known about the precise mechanism coupling the increase in blood flow to the enhanced formation and release of these autacoids.

It is now well established that increases in $[Ca^{2+}]_i$ of vascular endothelial cells produced by physiological agonists such as ATP, bradykinin or histamine cause the release of EDRF (from here on referred to as NO) and PGI_2 (for references see Newby & Henderson, 1990). This effect occurs via activation of specific cell surface receptors and subsequent stimulation of the phosphoinositol pathway, and can be maintained by an influx of extracellular Ca^{2+}, presumably through non-selective cation channels (Olesen *et al.*, 1988; Hallam *et al.*, 1989).

Removal of extracellular Ca^{2+} inhibits the formation of NO, but not that of PGI_2, stimulated by both shear stress and receptor-dependent and independent agonists (Griffith *et al.*, 1986; Lückhoff *et al.*, 1988; White & Martin, 1989; Hecker *et al.*, 1992). Whereas the inhibition of the agonist-stimulated release of NO usually progresses with subsequent challenges by the agonist until a full blockade is reached, the inhibition of the shear stress-dependent component is more immediate but often incomplete (Hecker *et al.*, 1992). Moreover, conflicting data have been reported on intracellular Ca^{2+} levels in cultured endothelial cells subjected to increased fluid flow (Ando *et al.*, 1988; Dull & Davies, 1991; Mo *et al.*, 1991; Geiger *et al.*, 1992; Schwartz *et al.*, 1992; Shen *et al.*, 1992). Thus, a definite role of $[Ca^{2+}]_i$ in the shear stress-induced release of NO remains to be established.

Sulfhydryl reagents, such as N-ethylmaleimide and 2,2'-dithiodipyridine, inhibit the agonist-stimulated but not the shear stress-induced formation of both NO and PGI_2 by bovine cultured aortic endothelial cells, presumably by interfering with the mobilization of $[Ca^{2+}]_i$ (Hecker *et al.*, 1992). We have now examined this potential regulatory difference further by using thapsigargin, the specific endoplasmic reti-

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culum Ca^{2+}-ATPase inhibitor, and have extended our studies to both native and cultured endothelial cells from different species. Thapsigargin, a non-phorbol ester-type tumour promoter, acts by irreversibly inhibiting the uptake of Ca^{2+} into inositol-1,4,5-trisphosphate ($InsP_3$)-sensitive and insensitive Ca^{2+} stores, thus preventing their refilling during or after hormonal stimulation (Thastrup *et al.*, 1990; Dolor *et al.*, 1992). Here, we demonstrate that thapsigargin selectively inhibits the agonist-stimulated but not the shear stress-dependent release of NO and PGI_2 from cultured as well as that of NO from native endothelial cells, suggesting than an $InsP_3$-mediated mobilization of intracellular $[Ca^{2+}]_i$ is responsible for the agonist-stimulated release of NO and PGI_2, but does not play a role in the shear stress-dependent release of these autacoids from the vascular endothelium.

Methods

Cell culture

Human umbilical vein endothelial cells were isolated from umbilical cords and grown to confluence on coverslips in M-199 culture medium (Gibco BRL, Eggenstein, Germany) as described previously (Busse & Lamontagne, 1991). Bovine aortic endothelial cells were isolated and grown to confluence in Dulbecco's modified Eagle's medium (Flow Laboratories, Irvine, UK) on Cytodex-3 microcarrier beads (Pharmacia-LKB, Milton Keynes, UK) or in T75 culture flasks as described by de Nucci *et al.* (1988).

Measurement of intracellular Ca^{2+}

Confluent human endothelial cells on coverslips or bovine endothelial cells in suspension were loaded with the fluorescence Ca^{2+} indicator dyes Indo-1/AM or Fura-2/AM, respectively, and changes in $[Ca^{2+}]_i$ were monitored as described previously (Busse & Lamontagne, 1991; Hecker *et al.*, 1992).

Determination of NO release from cultured endothelial cells by bioassay

Approximately 6×10^7 bovine aortic endothelial cells on beads were packed into a jacketed chromatography column and superfused at 5 ml × min^{-1} with warmed (37°C), oxygenated (95% O_2/5% CO_2) Krebs solution (composition mM: Na^+ 143.0, K^+ 5.9, Cl^- 127.7, Ca^{2+} 2.5, Mg^{2+} 1.2, HPO_4^{2-} 1.2, HCO_3^- 25.0, glucose 5.6), pH 7.4. The effluent from the column superfused a rabbit aortic ring with the endothelium removed which was equilibrated for 60 min under a resting tension of 2 g. Thereafter, the detector tissue was constricted further to 3–4 g of tension with 10 nM U46619 (11α,9α-epoxymethano-5*Z*,13*E*-prostaglandin $F_{2\alpha}$). Changes in tension were measured with a force displacement transducer (type 351 from Hugo Sachs Elektronik, March, Germany). Glyceryl trinitrate (GTN; 45 or 90 pmol) was given as a bolus over the detector tissue (o.t.) to calibrate its relaxant responses. Superoxide dismutase (10 u ml^{-1}) was infused through the column of endothelial cells (t.c.) to detect flow- and agonist-induced NO release. The agonists were first given o.t. to assess their effect on the bioassay preparation and then t.c. to stimulate NO release. In separate experiments, the cells were stimulated before and after a 10 min infusion t.c. of thapsigargin (1 μM), haemoglobin (10 μM) or N^G-nitro-L-arginine methyl ester (100 μM). The same bioassay technique was also applied to monitor the release of NO from the luminally perfused rabbit aorta.

Determination of PGI_2 release

The effluent from the endothelial cell column was collected after passing over the detector ring and analyzed by a specific radioimmunoassay for 6-keto-$PGF_{1\alpha}$ (Salmon, 1978), the stable hydrolysis product of PGI_2, and values are expressed as ng 6-keto-$PGF_{1\alpha}$ min^{-1}.

Preparation of arterial segments and measurement of NO release

Endothelium-intact segments of rabbit femoral arteries (8–12 mm in length) were cleaned of adventitial connective tissue, cannulated at both ends and mounted in a heated organ bath (37°C). The lumen of the segments and the organ bath were perfused separately (organ bath: 30 ml h^{-1}; lumen: 10.4 ml h^{-1}) with oxygenated (organ bath: 95% O_2/5% CO_2, Po_2 > 300 mmHg; lumen: 20% O_2/75% N_2/5% CO_2, Po_2 = 140 mmHg) Tyrode solution (composition mM: Na^+ 144.3, K^+ 4.0, Cl^- 138.6, Ca^{2+} 1.7, Mg^{2+} 1.0, HPO_4^{2-} 0.4, HCO_3^- 11.9, glucose 10.0), pH 7.4. The fluid shear stress at the luminal surface of the endothelium was increased by reducing the vessel diameter with endothelin-1 (60 nM) while maintaining the same luminal flow rate. Activation of soluble guanylyl cyclase was used to assay luminally released NO. The effluent from the perfused segments was collected for 15 s into a reaction mixture containing [α-^{32}P]-GTP and purified guanylyl cyclase. Labelled cyclic GMP formed was determined and enzyme activity (expressed as nmol mg^{-1} min^{-1}) calculated as described previously (Jackson *et al.*, 1991).

The same technique was also applied to determine the release of NO from human umbilical vein endothelial cells on coverslips superfused in a circular perfusion chamber (Bachofer) with warmed (37°C), HEPES-buffered (10 mM) Tyrode solution. Shear stress was enhanced by increasing the flow rate from 5.2 ml h^{-1} to 20.8 ml h^{-1}.

Materials

Indo-1/AM was purchased from Boehringer (Mannheim, Germany), Fura-2/AM and ionomycin were from Calbiochem Novabiochem Ltd (Nottingham, UK), and endothelin-1 from Peninsula Laboratories (Belmont, U.S.A.), respectively. ADP, bradykinin, haemoglobin, 6-keto-prostaglandin $F_{1\alpha}$ antiserum, N^G-nitro-L-arginine methyl ester, poly-L-lysine (degree of polymerization 254), superoxide dismutase, and thapsigargin were obtained from Sigma Chemical Co (Poole, U.K.). 6-Keto-prostaglandin $F_{1\alpha}$ was purchased from Cascade Biochem Ltd (Reading, U.K.), glyceryl trinitrate was from Lipha Pharmaceuticals Ltd (West Drayton, U.K.), and U46619 was a generous gift from Dr J. Pike (Upjohn Co, Kalamazoo, U.S.A.). [^{3}H]-6-keto-prostaglandin $F_{1\alpha}$ was obtained from Amersham International (Amersham, U.K.) and [α-^{32}P]-GTP from Amersham Buchler (Braunschweig, Germany).

Statistical analysis

Unless indicated otherwise, all data in the figures and text are expressed as mean ± s.e.mean. Statistical evaluation was performed by Student's *t* test or one-way analysis of variance (ANOVA; followed by a Bonferroni *t* test), where appropriate, with a P value < 0.05 considered statistically significant.

Results

NO release from cultured endothelial cells

Infusion of superoxide dismutase (SOD; 10 u ml^{-1}) through the column of bovine aortic endothelial cells on beads (t.c.) caused an average relaxation of 17 ± 3% ($n = 6$; Figures 1 and 2), which was abolished by infusions of the NO scavenger haemoglobin (10 μM; $n = 3$) as well as the NO biosynthesis inhibitor N^G-nitro-L-arginine methyl ester (Moore *et al.*, 1989; 100 μM; $n = 3$), demonstrating the involvement of NO. This relaxant response was defined as shear stress-

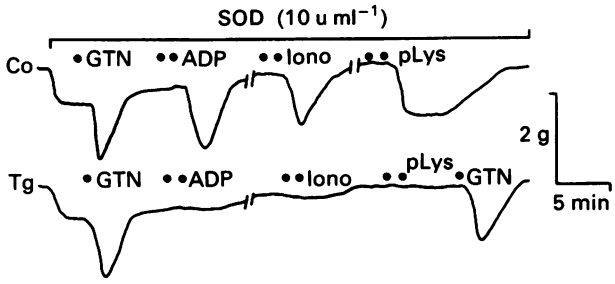

Figure 1 Effects of thapsigargin on shear stress-dependent and agonist-stimulated NO release from bovine aortic endothelial cells. The figure depicts a typical bioassay trace from 6 individual experiments with different batches of cultured endothelial cells. Shown are the relaxant responses of the detector tissue to infusion of superoxide dismutase (SOD; 10 u ml^{-1}) through the column of endothelial cells (t.c.) and bolus injections of ADP (9 nmol), ionomycin (Iono; 60 pmol) or poly-L-lysine (pLys; 550 pmol) over the tissue (o.t.; one circle) or t.c. (two circles) before (Co; top trace) and after (Tg; bottom trace) a 10 min infusion t.c. of thapsigargin (1 μM) followed by a 5 min washout period. Glyceryl trinitrate (GTN; 90 pmol) was given as a bolus injection o.t. to monitor changes in the sensitivity of the detector tissue. Note the lack of effect of thapsigargin on the relaxant response to infusion of SOD, i.e. shear stress-dependent NO release.

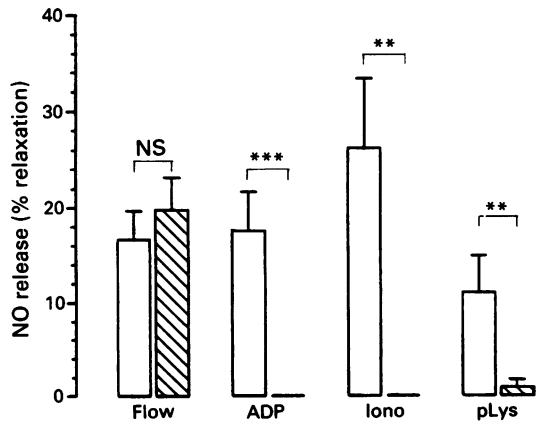

Figure 2 Selective inhibition of agonist-stimulated NO release from cultured bovine aortic endothelial cells by thapsigargin. Shown are the relaxant responses of the detector tissue (expressed as % relaxation of induced tone) to infusion t.c. of superoxide dismutase (SOD, 10 u ml^{-1}; Flow) and bolus injections t.c. of ADP (9 nmol), ionomycin (Iono; 60 pmol) or poly-L-lysine (pLys; 550 pmol) before (open columns) and after (hatched columns) a 10 min infusion t.c. of thapsigargin (1 μM) followed by a 5 min washout period ($n = 6$; $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control; NS, not significant).

induced release of NO, for bovine aortic endothelial cells on beads produce only small amounts of NO under basal conditions (not shown), but at flow rates of 0.3–3 ml min^{-1} show a linear correlation between the amount of NO being released and the rate of perfusion (Buga *et al.*, 1991). The relatively high flow rate of 5 ml min^{-1} used in this study can thus be considered to produce an almost maximum shear stress-dependent release of NO from these cells. Moreover, the observed relaxations were largely attributable to the release of NO from the endothelial cell column, as PGI_2 does not relax the rabbit aorta (Moncada *et al.*, 1976). Therefore and because of the simultaneous determination of PGI_2 release, a cyclo-oxygenase inhibitor was not included in the bioassay.

The shear stress-induced relaxation of the detector tissue was further enhanced (additional 10–25% relaxation; $n = 5$) by bolus injections t.c. of ADP (9 nmol), ionomycin (60 pmol) or poly-L-lysine (550 pmol; Figures 1 and 2). Another receptor-dependent agonist, bradykinin (19 pmol), also caused an additional 14 ± 3% relaxation ($n = 3$). All of these relaxant responses were abolished by infusions t.c. of haemoglobin or N^G-nitro-L-arginine methyl ester ($n = 3$).

Infusions t.c. of thapsigargin (1 μM for 10 min) caused a sustained release of NO (additional 30 ± 8% relaxation; $n = 5$) which declined after the infusion was stopped (not shown). After removal of the thapsigargin infusion and a 5 min washout period, the shear stress-dependent release of NO remained unaffected, whereas the release of NO induced by all four agonists was completely abolished (Figures 1 and 2; $P < 0.01$ for bradykinin; $n = 3$). This also occurred when the thapsigargin infusion was continued throughout the experiment ($n = 3$) or when the Ca^{2+}-ATPase inhibitor was infused for 5 min followed by a 10 min washout period ($n = 3$). Control responses of the bioassay tissue to glyceryl trinitrate were not affected by treatment of the endothelial cell column with thapsigargin (from 37 ± 4 to 30 ± 5% relaxation; $n = 5$).

Thapsigargin administration (infusion at 1 μM for 5 min followed by a 15 min washout period) also inhibited the bradykinin-induced (30 nM) but not the shear stress-dependent release of NO from superfused human endothelial cells grown on coverslips (79 ± 5% inhibition; $n = 3$).

NO release from endothelium-intact arterial segments

In perfused femoral artery segments from the rabbit, infusion of endothelin-1 (60 nM) elicited a decrease in vessel diameter from 1651 ± 53 mm to 1156 ± 53 mm ($n = 3$; $P < 0.01$) accompanied by a distinct release of NO (Figure 3). As the segments were perfused at a constant flow rate (10.4 ml h^{-1}), the endothelin-induced decrease in vessel diameter caused an increase in shear stress at the luminal surface of the endothelium (from 0.12 to 0.55 dyn cm^{-2}, Busse *et al.*, 1991). Acetylcholine (1 μM) also significantly augmented the flow-induced release of NO (Figure 3), an effect that was strongly inhibited by pretreatment of the arterial segments with thapsigargin (infusion at 1 μM for 5 min followed by a 15 min washout period). In contrast, administration of thapsigargin did not affect the decrease in vessel diameter elicited by endothelin-1 (from 1680 ± 79 mm to 1176 ± 67 mm; $n = 3$) or the shear stress-dependent release of NO (Figure 3).

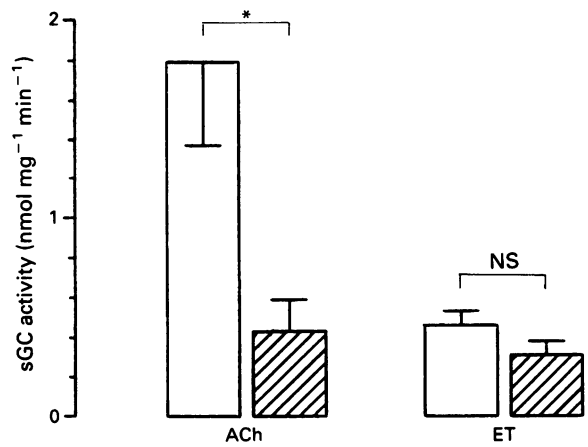

Figure 3 Effects of thapsigargin on the release of NO from isolated, luminally perfused rabbit femoral artery segments. The figure shows the net release of NO from the arterial segments (expressed as increase in specific guanylyl cyclase activity over the level induced by perfusion of the segments alone) elicited by infusions of acetylcholine (ACh; 1 μM) or endothelin-1 (ET; 60 nM) before (open columns) and after (hatched columns) a 5 min infusion of thapsigargin (1 μM) followed by a 15 min washout period ($n \geq 3$; $^{*}P < 0.05$ vs. control; NS, not significant).

Thapsigarin also suppressed the acetylcholine-stimulated release of NO from the luminally perfused, endothelium-intact rabbit aorta. Bolus injections of acetylcholine (55 nmol) stimulated the release of NO, as detected by relaxation of the endothelium-free rabbit aortic ring (15 ± 1% relaxation; $n = 3$), and this effect was abolished by pretreatment of the aorta with thapsigargin (infusion at 1 μM for 5 min followed by a 10 min washout period; $P < 0.01$). Thapsigargin alone elicited a relaxant response similar to that of acetylcholine which declined upon removal of the infusion ($n = 3$).

Moreover, thapsigargin blunted the relaxant response to acetylcholine in femoral artery segments preconstricted with noradrenaline (62% inhibition; not shown), and caused both a significant endothelium-dependent relaxation (88% increase in vessel diameter; not shown) and a substantial release of NO when administered alone (increase in soluble guanylate cyclase activity over basal levels: 1.43 ± 0.10 nmol mg^{-1} min^{-1}; $n = 3$). Both vessel diameter (from 88 to 33% increase) and NO release declined towards previous levels 15 min after washout of the Ca^{2+}-ATPase inhibitor ($n = 3$).

Release of PGI_2 from cultured endothelial cells

As determined by radioimmunoassay for 6-keto-$PGF_{1\alpha}$, the column of bovine aortic endothelial cells released 1.04 ± 0.27 ng PGI_2 min^{-1} ($n = 5$). Bolus injections of ADP, ionomycin, poly-L-lysine or bradykinin all increased the release of PGI_2 by 3 to 14 fold (Figure 4). Pretreatment of the cells with thapsigargin (1 μM for 10 min followed by a 5 min washout period) had no effect on the shear stress-dependent PGI_2 release, but abolished the release of PGI_2 induced by all four agonists (Figure 4; bradykinin: from 13.9 ± 5.5 to 1.09 ± 0.16 ng min^{-1}; $P < 0.05$; $n = 3$). Administration of thapsigargin alone caused an increase in PGI_2 release to 5.04 ± 1.99 ng min^{-1} which declined towards basal levels after removal of the thapsigargin infusion and subsequent superfusion of the cells with thapsigargin-free buffer (Figure 4).

Changes in $[Ca^{2+}]_i$ in cultured endothelial cells

Human endothelial cells grown on coverslips were loaded with Indo-1/AM. Addition of thapsigargin caused a sustained rise in $[Ca^{2+}]_i$ (Figures 5 and 6). This effect was dose-dependent with a maximum increase in $[Ca^{2+}]_i$ at approximately 100 nM thapsigargin, and a threshold concentration of 1–10 nM thapsigargin ($n = 8$). With high concentrations of thapsigargin (100–300 nM), a maximum increase in $[Ca^{2+}]_i$ was attained after 2–3 min, and sustained for more than 10 min. Replacement of the thapsigargin-containing solution by thapsigargin-free buffer had no significant effect on the elevated level of $[Ca^{2+}]_i$ (not shown), but removal of extracellular Ca^{2+} rapidly brought the thapsigargin-elevated $[Ca^{2+}]_i$ signal back to resting values (Figure 6). Stimulation of the cells with bradykinin (3 nM; $n = 4$) or histamine (1 μM; $n = 3$), after previous exposure to thapsigargin (30–300 nM for 10 min), did not result in a further increase in $[Ca^{2+}]_i$ (Figure 6).

In Fura-2/AM-loaded bovine endothelial cells in suspension, thapsigargin (1 μM) produced a rapid increase in $[Ca^{2+}]_i$ from 70–100 nM to 800–1200 nM which decreased slowly to approximately 500 nM over 15 min ($n = 4$). In the absence of extracellular Ca^{2+}, thapsigargin (1 μM) elicited only a small transient increase in $[Ca^{2+}]_i$ ($n = 4$). Addition of ADP (1 μM) or ionomycin (30 nM) to the cell suspension after 10 min exposure to thapsigargin (1 μM) did not produce any further increase in $[Ca^{2+}]_i$ ($n = 4$). This also occurred when the cells were exposed to thapsigargin for 20–60 min followed by a 5 min washout period before the measurement of $[Ca^{2+}]_i$. Resting intracellular Ca^{2+} levels in cells pretreated with thapsigargin, however, were significantly elevated (200–400 nM; $n = 4$) compared to control cells.

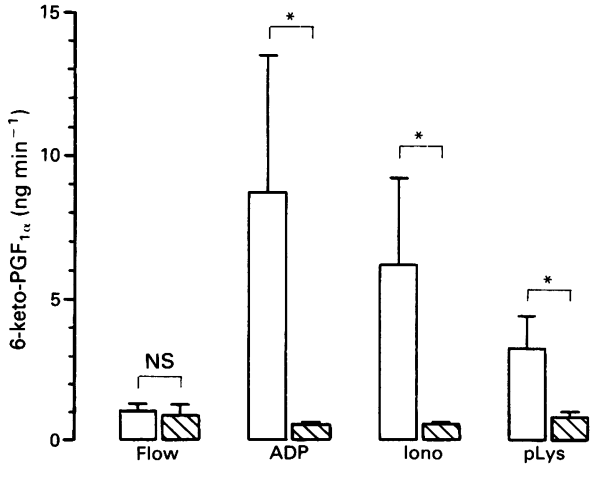

Figure 4 Selective inhibition of agonist-stimulated prostacyclin (PGI_2) release from cultured bovine aortic endothelial cells by thapsigargin. The figure depicts the release of PGI_2 (expressed as ng 6-keto-$PGF_{1\alpha}$ min^{-1}) in the absence (Flow) or presence of bolus injections t.c. of ADP (9 nmol), ionomycin (Iono; 60 pmol) or poly-L-lysine (pLys; 550 pmol) before (open columns) and after (hatched columns) a 10 min infusion t.c. of thapsigargin (1 μM) followed by a 5 min washout period ($n = 5$; $^*P < 0.05$ vs. control; NS, not significant).

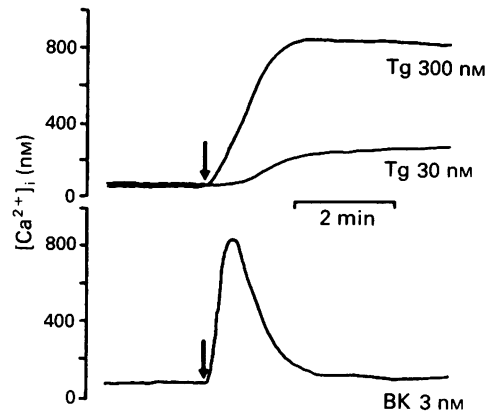

Figure 5 Changes in $[Ca^{2+}]_i$ in human cultured umbilical vein endothelial cells elicited by thapsigargin. The figure shows 2 typical traces obtained with 30 and 300 nM thapsigargin (Tg; top panel) as well as a control response to bradykinin (BK; 3 nM; bottom panel) from 8 individual experiments with different batches of endothelial cells. Arrows indicate the addition of thapsigargin or bradykinin.

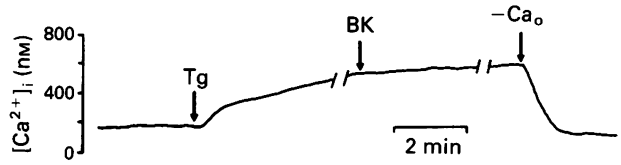

Figure 6 Inhibition of the bradykinin-induced mobilization of $[Ca^{2+}]_i$ by thapsigargin and effect of removal of extracellular Ca^{2+}. Shown is a typical trace from 8 individual experiments with different batches of human endothelial cells. Arrows indicate the addition of thapsigargin (Tg; 30 nM), bradykinin (BK; 3 nM) or Ca^{2+}-free buffer ($-Ca_o$).

Discussion

The data of the present investigation support the conclusion that a principle regulatory difference exists between the shear

stress- and agonist-induced release of both NO and PGI_2 from cultured and native endothelial cells which can be unmasked by the Ca^{2+}-ATPase inhibitor, thapsigargin. By emptying intracellular Ca^{2+} stores, including the $InsP_3$-sensitive Ca^{2+} pool, thapsigargin abolished the agonist-stimulated but did not affect the shear stress-induced release of these autacoids. This effect of the Ca^{2+}-ATPase inhibitor confirms previous findings with 2,2'-dithiodipyridine and N-ethylmaleimide, two sulphydryl reagents which, presumably by a mechanism closely related to that of thapsigargin, selectively inhibit the agonist-stimulated, but not the flow-dependent release of NO and PGI_2 from cultured bovine aortic endothelial cells (Hecker *et al.*, 1992).

Moreover, the sustained elevation of $[Ca^{2+}]_i$ in the presence of thapsigargin caused a substantial release of NO and PGI_2 from both cultured and native endothelial cells. This effect was very similar to that of thimerosal (Hecker *et al.*, 1992), a sulphydryl reagent which also seems to interfere with the uptake of Ca^{2+} by the endoplasmic reticulum (Hecker *et al.*, 1989). The molecular mechanism by which thapsigargin and thimerosal discharge intracellular Ca^{2+} pools, however, seems to be different, for in human platelets thimerosal inhibits the endoplasmic reticulum Ca^{2+}-ATPase by oxidizing one or several sulphydryl groups of this Ca^{2+} pump which are essential for its catalytic activity (Hecker *et al.*, 1989).

The agonist-stimulated release of NO and PGI_2 from endothelial cells is initiated by an increase in $[Ca^{2+}]_i$ via the phosphoinositol pathway (for references see Newby & Henderson, 1990), and is sustained by an influx of extracellular Ca^{2+}, presumably through non-selective cation channels (Olesen *et al.*, 1988; Hallam *et al.*, 1989). Although the regulation of Ca^{2+} entry into endothelial cells is still poorly understood, the most plausible hypothesis is the capacitance model proposed by Putney (1990). Therein, emptying of the $InsP_3$-sensitive intracellular Ca^{2+} store(s) initiates an influx of extracellular Ca^{2+} which is terminated upon refilling of the pool. Indeed, this process seems to be operating in endothelial cells (Dolor *et al.*, 1992), even though it is not clear whether extracellular Ca^{2+} refills endothelial Ca^{2+} store(s) directly (Buchan & Martin, 1991) or indirectly via entry into the cytoplasm and subsequent uptake by the endoplasmic reticulum (Lückhoff & Busse, 1990; Dolor *et al.*, 1992). The sensing mechanism for the filling state of these stores is also still a matter of investigation. However, it seems likely that extracellular Ca^{2+} enters the endothelial cell through non-selective cation channels (Olesen *et al.*, 1988; Hallam *et al.*, 1989), the activation of which is triggered both by an elevation in cytoplasmic Ca^{2+} and formation of inositol-1,3,4,5-tetrakisphosphate (Lückhoff & Clapham, 1992).

Thapsigargin depletes intracellular Ca^{2+} stores, and thus greatly reduces (Dolor *et al.*, 1992) or abolishes (this study) the subsequent mobilization of $[Ca^{2+}]_i$ induced by receptor-dependent agonists such as ADP, bradykinin or histamine in human and bovine endothelial cells. In contrast to the rapid transient rise in $[Ca^{2+}]_i$ initiated by these agonists, thapsigargin stimulated a slowly developing, prolonged elevation of $[Ca^{2+}]_i$ that required the presence of extracellular Ca^{2+}, and was clearly concentration-dependent. This increase in $[Ca^{2+}]_i$ attained maximum levels 2–3 min after the addition of thapsigargin (see also Dolor *et al.*, 1992), and $[Ca^{2+}]_i$ remained significantly elevated above resting levels for periods of up to 60 min, even when the thapsigargin-containing solution was replaced by thapsigargin-free buffer. Thus, as in many other cell types, thapsigargin appears to be a fast acting, irreversible inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase also in endothelial cells.

Interestingly, thapsigargin also suppressed the release of NO and PGI_2 induced by the receptor-independent agonists ionomycin and poly-L-lysine, both of which seem to activate a transmembrane Ca^{2+} influx into endothelial cells (Hecker *et al.*, 1992). The complete inhibition by thapsigargin of both the ionomycin-induced rise in $[Ca^{2+}]_i$ and the release of NO and PGI_2 indicates that the ionophore at low nanomolar concentrations releases Ca^{2+} primarily from intracellular Ca^{2+} stores which can be refilled by extracellular Ca^{2+} but were continuously depleted by thapsigargin.

Although the precise mechanism of action of poly-L-lysine has yet to be established, it seems likely that the polycation partially neutralizes the negatively charged endothelial cell membrane (Thomas *et al.*, 1989), thereby possibly modulating the activity of Ca^{2+}-permeable cation channels. One explanation for the inhibitory effect of the Ca^{2+}-ATPase inhibitor may be, therefore, that poly-L-lysine activates the same set of Ca^{2+}-permeable cation channels that are required for the refilling of endothelial Ca^{2+} stores and were already discharged by thapsigargin.

In contrast to the agonist-stimulated release of NO and PGI_2, pretreatment of both cultured and native endothelial cells with thapsigargin did not affect the shear stress-induced release of these autacoids. Thus, a mobilization of $[Ca^{2+}]_i$ from $InsP_3$-sensitive Ca^{2+} store(s) does not seem to be involved in this response. Indirect support for this conclusion comes from findings that removal of extracellular Ca^{2+} has a greater inhibitory effect on the shear stress-dependent release of NO than does the blockade of the $InsP_3$-mediated mobilization of $[Ca^{2+}]_i$ (Griffith *et al.*, 1986). In contrast, removal of extracellular Ca^{2+} progressively and completely inhibits the release of NO elicited by receptor-dependent agonists, further supporting the notion that the inhibitory effect of thapsigargin is brought about by emptying specific intracellular Ca^{2+} pools (Hecker *et al.*, 1992).

Unlike NO formation, PGI_2 biosynthesis depends critically on the release of Ca^{2+} from intracellular stores (Lückhoff *et al.*, 1988; White & Martin, 1989). Whether this is true for both agonist-stimulated and shear stress-dependent PGI_2 release, however, remains to be established. Our observation that thapsigargin did not affect the shear stress-induced release of PGI_2 argues against the latter hypothesis.

The shear stress-induced release of NO from cultured endothelial cells depends to some extent on the presence of extracellular Ca^{2+} (Hecker *et al.*, 1992). In contrast, it is difficult to evaluate whether the infrequently observed elevation of $[Ca^{2+}]_i$ in response to increased flow (Ando *et al.*, 1988; Geiger *et al.*, 1992; Schwarz *et al.*, 1992; Shen *et al.*, 1992) is simply an epiphenomenon or an integral part of the shear stress-induced signal transduction mechanism. The lack of effect of thapsigargin on the shear stress-dependent release of NO and PGI_2 strongly supports the notion that shear stress does not mobilize Ca^{2+} from intracellular Ca^{2+} stores. This does not exclude the possibility, however, that shear stress activates a set of non-selective Ca^{2+}-permeable cation channels which are different from those required for the refilling of intracellular Ca^{2+} stores (Schwarz *et al.*, 1992). As a consequence, shear stress may elevate endothelial $[Ca^{2+}]_i$ and hence stimulate NO and PGI_2 biosynthesis by a thapsigargin-independent mechanism.

Another explanation for the different sensitivity of agonist- and shear stress-induced NO release is that NO synthase in both human (Hecker *et al.*, unpublished observation) and bovine endothelial cells is membrane-bound (Förstermann *et al.*, 1991). Perturbation of the plasma membrane of endothelial cells exposed to shear stress may, therefore, lead to a $[Ca^{2+}]_i$-independent activation of this enzyme. The agonist-triggered, $InsP_3$-mediated elevation of $[Ca^{2+}]_i$ could then further augment the formation of NO in a Ca^{2+}/calmodulin-dependent manner. Alternatively, endothelial cells may contain both a Ca^{2+}-independent and a Ca^{2+}/calmodulin-dependent constitutive NO synthase (Mülsch *et al.*, 1989), the activity of which could be regulated differently by shear stress and/or receptor-dependent as well as independent agonists.

In conclusion, by emptying and preventing the refilling of intracellular Ca^{2+} stores, thapsigargin selectively inhibits the agonist-stimulated but not the shear stress-induced release of NO and PGI_2 from cultured as well as native endothelial cells. Thus, a principal difference exists in the control by $[Ca^{2+}]_i$ of these two types of stimulation of endothelial auta-

coid release, a finding which may help to unravel the signal transduction mechanism(s) mediating flow-induced endothelium-dependent dilatations *in vivo*.

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