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Modulation of endothelial permeability by 1-O-alkylglycerols

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ABSTRACT

Regulation of endothelial barrier function often occurs through signalling involving phospholipase C activation which produces diacylglycerol (DAG), a lipidic second messenger activator of protein kinase C (PKC). Therefore, modification of lipidic composition of endothelial cell membranes might modify DAG production and, as a result, alter regulation of endothelial permeability. We investigated the *in vitro* effects of natural 1-O-alkylglycerols on porcine aortic endothelial cell permeability to dye-labelled albumin. [^{3}H]-1-O-alkylglycerols (10 μM) were substantially incorporated into phosphatidylcholine (6.6%) and phosphatidylethanolamine (4.4%). Stimulation of endothelial cell monolayer with phorbol-myristate-acetate or with the calcium ionophore A23187 resulted in a raise in permeability to albumin. Pre-treatment with 1-O-alkylglycerols (10 μM, 24 h) had no effect on basal albumin permeability but totally inhibited the effect of phorbol-myristate-acetate, and brought the permeability of A23187-stimulated endothelial cell monolayers below control. After incubation of cells with [^{3}H]-1-O-alkylglycerols (10 μM, 24 h), we detected the production of the analogue of DAG, and PKC inhibitor, [^{3}H]-1-O-alkyl-2-acyl-glycerol, in resting cells. This production was increased by 58% under A23187 stimulation while phorbol-myristate-acetate had no effect. Our data demonstrate that natural 1-O-alkylglycerols modify endothelial permeability, and suggest that this effect could be mediated through alteration of lipidic signalling.

Keywords 1-O-alkylglycerol, endothelium, etherlipids, permeability, protein kinase C.

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Membrane phospholipids have an important role in cell signalling involving phospholipase activation and release of lipidic second messengers. Activation of protein kinase C (PKC) by diacylglycerol (DAG) is a prominent pathway of transduction for many receptors (Nishizuka 1995). Therefore, modification of the composition of cell membrane lipids might modulate cell functions controlled by lipidic signalling. In endothelial cells, PKC activation results in alteration of endothelial barrier function and in increased permeability (Lum & Malik 1994).

1-O-alkylglycerols (alkyl-Gro) are naturally occurring etherlipids with multiple biological activities and therapeutic potential. For instance, alkyl-Gro protect patients from radiotherapy side-effects (Brohult *et al.* 1977) and have anti-cancer properties (Brohult *et al.* 1978, 1986), stimulating immunological responses such as antibody production (Ngwenya & Foster 1990). In a monocyte cell line alkyl-Gro are incorporated into 1-O-alkylglycerophosphocholine, the precursor of platelet-activating factor (PAF), and amplify PAF production (Hichami *et al.* 1997). Alkyl-Gro also increase sperm motility and fertility (Cheminade *et al.* 2002). The molecular basis of these various activities are not fully understood. One of these mechanisms might include incorporation of alkyl-Gro into phospholipids involved in cell signalling as DAG source. This would result in the production of analogues of DAG with an ether bond in position 1 of glycerol. Unlike DAG, these 1-O-alkyl-2-acyl-*sn*–glycerol (alkyl-acyl-Gro) have no or poor effects on PKC (Heymans *et al.* 1987) and inhibit the stimulating effect of DAG on PKC (Daniel *et al.* 1988).

The effect of alkyl-Gro on endothelial cells is unknown, and PKC activation is prominent for modulation of endothelial function. The aim of this

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work was to study the incorporation of alkyl-Gro in membrane phospholipids of cultured porcine aortic endothelial cells (PAEC), and to explore the consequence on cell monolayer permeability to albumin.

MATERIALS AND METHODS

Materials

Foetal bovine serum (FBS), antibiotic–antimycotic solution (10 000 units mL^{-1} penicillin G sodium, 10 000 $\mu g\ mL^{-1}$ streptomycin sulphate, and 25 $\mu g\ mL^{-1}$ amphotericin B as Fungizone®[Bristol-Myers Squibb]), Fungizone® (250 $\mu g\ mL^{-1}$ amphotericin B), trypsin (0.25%), culture medium M-199 with or without phenol red, *N*-2-hydroxyethylpiperazine-*N*-2-ethane-sulphonic acid (25 mM) and Earle's salt medium with glutamin (100 mg mL^{-1}) were purchased from Gibco (Cergy-Pontoise, France). Bovine serum albumin (BSA, V fragment), collagen type I (from rat tail), phorbol-12-myristate-13-acetate (PMA), dimethyl sulphoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and Evan's blue dye were purchased from Sigma (La Verpillière, France). Millicel-CM tissue culture plate inserts (12 mm, 0.4 μm pore size) were purchased from Millipore (St Quentin en Yvelynes, France). The calcium ionophore A23187 was obtained from Calbiochem (Meudon, France). Unlabelled and [^{3}H]-alkyl-Gro were obtained from crude shark liver oil as described previously (Hichami *et al.* 1997) with composition as follows, depending on the batch: 18 : 1(Δ9), 54–65%; 16 : 1(Δ7), 5–15.5%; 16 : 0, 5–10%; 14 : 0, 3%; 18 : 0, 3%; 17 : 1(Δ9), 1.5%.

Cell culture

Primary culture of PAEC were prepared as previously described (Martin-Chouly *et al.* 1999) and cultured in M-199 medium supplemented with 20% FBS, 1.6% antibiotic–antimycotic solution and 0.6% amphotericin B solution. Cell medium was changed after 24 h and then every 48 h until confluency.

Cytotoxicity of alkyl-Gro on porcine aortic endothelial cells

Absence of alkyl-Gro-induced cytotoxicity was assessed by the rate of MTT metabolism into MTT-formazan as described by Mosmann (1983). At subconfluence, cells were trypsinized and seeded at 3×10^3 cells per well, in 96 well plates, with M-199 supplemented with 10% FBS and cultured for 24 h. Endothelial cells were then treated or otherwise with various concentrations of alkyl-Gro (from 2.5 to 50 μM) for 1 day. Then MTT solution was added and cells were further incubated for 4 h at 37 °C. The medium was then replaced by DMSO after centrifugation (5 min, 1000 r.p.m.) and spectrophotometric measurement of MTT-formazan was performed at 540 nm.

Incorporation of [^{3}H]-alkyl-Gro and total lipid extraction

At confluence, cells were trypsinized and seeded at 5×10^5 cells per 60-mm dish. Medium was changed after 24 h and then every 48 h. Subconfluent cells were incubated with medium supplemented with [^{3}H]-alkyl-Gro (10 μM, 5×10^5 dpm) for 6, 12, 24 or 48 h. After the indicated time, cells were washed and harvested for lipid analyses. Total lipids were extracted by the method of Bligh & Dyer (1959). The chloroform fraction was dried under nitrogen and analysed by thin layer chromatography (TLC) on silica gel using chloroform : methanol : acetic acid (35 : 14 : 2.8; v/v) as solvent. Radioactive peaks were then visualized using a radiochromatogram scanner (Bioscan, Washington, DC, USA) and identified by their Rf compared with authentic standards. Silica gel containing each peak was scraped and radioactivity was measured in a scintillation counter (Packard, Les Ulis, France).

Endothelial permeability studies

The permeability of endothelial monolayer was studied at confluence as previously described (Martin-Chouly *et al.* 1999). The PAEC were seeded on collagen-coated filters (5×10^5 cells/filter). At subconfluence, cells were incubated with alkyl-Gro (10 μM) for 24 h. Then the monolayer was rinsed with FBS-free M-199 medium without phenol red, supplemented with 0.5% BSA and incubated at 37 °C during 20 min. Filters were then inserted in a moss ring placed into thermostated beakers (37 °C) containing phosphate buffered saline and 0.5% BSA (abluminal chamber). The experiment began when Evans blue dye-labelled albumin solution [prepared by adding 13.4 $\mu g\ mL^{-1}$ of Evans blue dye to albumin solution (0.5%, w/v)] was added onto the endothelial cell monolayer (luminal chamber). Thirty minutes after the beginning of the experiment, cells were stimulated or otherwise with calcium ionophore A23187 (1 μM) or with PMA (0.2 μM). At regular time, samples were collected from the abluminal chamber. Absorbence was measured at 620 nm with a spectrophotometer (Seconam, Domont, France). Permeability was expressed as clearance rate which was the volume V_l of luminal medium cleared of albumin, calculated using the following formula $V_l = (A_{ab} \times V_{Ab}) \times (A_{Ref})^{-1}$, where V_{Ab} is the abluminal volume, A_{ab} the abluminal sample absorbence and A_{Ref} the absorbence of the luminal medium (with Evans blue) at $t = 0$.

Preparation of 1-O-hexadecyl-2-[^{3}H]-arachidonoyl-glycerol

1-O-hexadecyl-2-lysophosphocholine was acylated with [^{3}H]-arachidonic acid using enzymatic method previously described (Legrand *et al.* 1996). The resulting 1-O-hexadecyl-2-[^{3}H]-arachidonoyl-glycerophosphocholine was then submitted to the action of phospholipase C as described and the product 1-O-hexadecyl-2-[^{3}H]-arachidonoyl-glycerol was isolated by straight phase high performance liquid chromatography using hexane : isopropanol (100 : 1; v/v) as mobile phase.

Production of [^{3}H]-alkyl-acyl-Gro

Porcine aortic endothelial cells were seeded in 60 mm dishes and cultured as described above. At subconfluence, cells were incubated with [^{3}H]-alkyl-Gro (10 μM, 2×10^6 dpm per dish) for 24 h and washed. Cells were then stimulated or otherwise with A23187 (1 μM) or PMA (0.2 μM) during 2 min. The supernatant was withdrawn and reaction was stopped by 2 mL cold methanol. Cells were scraped and neutral lipids were extracted with heptane (seven times, v/v). The heptane fraction was collected and dried under nitrogen. Lipids were separated by TLC on silica gel coated plates using diethyl ether : hexane : acetic acid (35 : 15 : 0.5, v/v) as mobile phase. Radioactive peak migrating with the same Rf than 1-O-hexadecyl-2-[^{3}H]-arachidonoyl-glycerol was visualized with a radiochromatogram scanner (Bioscan), silica of the peak was scraped and radioactivity was counted in a scintillation counter.

Statistical analysis

Data are shown as mean ± SEM of the indicated number of experiments. The results were analysed by *t*-test or by two-way analysis of variance (ANOVA) followed by a Duncan's multiple range test.

RESULTS

Cytotoxicity of alkyl-Gro

Cytotoxicity of alkyl-Gro was evaluated by the decrease of MTT metabolism into MTT-formazan. After 24 h the decrease of MTT metabolism was less than 5%, indicating a minimal cytotoxicity except with the highest dose used (50 μM) for which a 19% decrease in MTT metabolism was observed (Table 1).

[^{3}H]-alkyl-Gro incorporation into endothelial cell phospholipids

Endothelial cells were incubated with [^{3}H]-alkyl-Gro (10 μM) for various periods of time and phospholipids were analysed by TLC. The major radioactive phospholipid classes were identified as phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI). After 24 h, the fractions of [^{3}H]-alkyl-Gro incorporated into PC, PE and PI reached a near-plateau and represented 6.6 ± 0.3, 4.4 ± 0.3 and 0.5 ± 0.1% of the initial radioactivity, respectively (Fig. 1).

Table 1 Cytotoxicity of alkyl-Gro on PAEC. Data represent the per cent of MTT metabolized in MTT-formazan with 100% corresponding to MTT-formazan in control

Alkyl-Gro concentrations (μM)	MTT-formazan (%)
0	100.0 ± 3.3
2.5	108.6 ± 1.7
5	105.3 ± 1.7
10	107.4 ± 2.6
30	95.7 ± 3.3
50	81.0 ± 7.0*

*$P < 0.05$ compared with the control MTT-metabolism. $n = 3$ in seven repetitions.

Effect of alkyl-Gro on permeability of resting and stimulated endothelial cell monolayer

After incubation of endothelial cell monolayers with alkyl-Gro (10 μM, for 24 h), endothelial permeability was evaluated by calculating the clearance rate of dye-labelled albumin.

When endothelial cell monolayers were stimulated with PMA (0.2 μM), we observed an increase of albumin clearance rate. Pre-treatment of monolayers with alkyl-Gro resulted in a complete inhibition of the PMA-induced raise in permeability as no significant difference between control and PMA + alkyl-Gro treated cells appeared (Fig. 2a).

The calcium ionophore A23187 (1 μM), in the same way, induced a raise in albumin permeability which was also prevented by alkyl-Gro pre-treatment, the level of

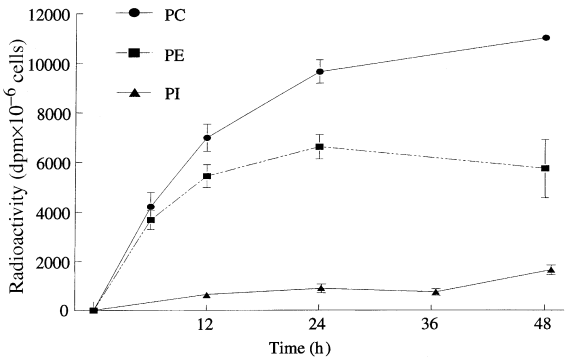

Figure 1 Incorporation of [^{3}H]-alkyl-Gro (10 μM) into phospholipids of endothelial cells ($n = 11$).

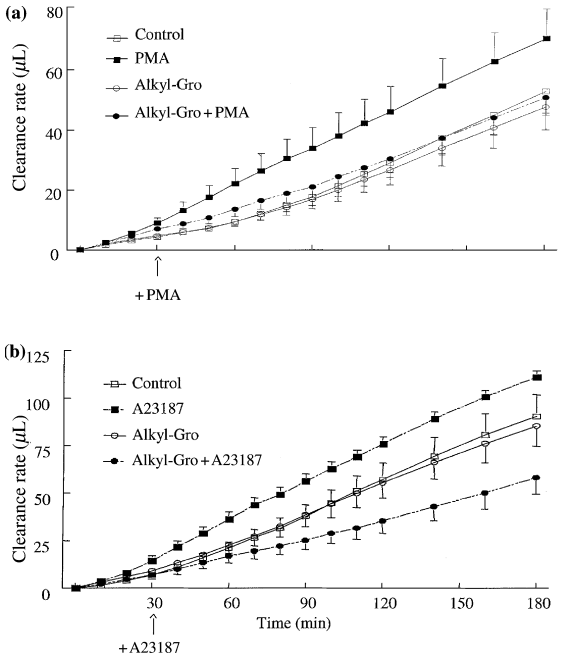

Figure 2 Effect of alkyl-Gro (10 μM, 24 h) on endothelial cell monolayer permeability in resting conditions and during PMA (0.2 μM) or A23187 (1 μM) stimulation. (a) $n = 8$, difference between control and PMA, and between PMA and PMA + alkyl-Gro: $P < 0.05$. (b) $n = 11$, difference between control and A23187: $P < 0.05$, and difference between A23187 and A23187 + alkyl-Gro: $P < 0.01$ (ANOVA at 180 min).

permeability in alkyl-Gro treated cells was even lower than in control experiments (Fig. 2b).

Production of alkyl-acyl-Gro

After incorporation of [^{3}H]-alkyl-Gro (10 μM, 2×10^6 dpm, 24 h), cells were stimulated or otherwise by A23187 (10 μM) or PMA (0.2 μM). After extraction of the neutral lipids, the production of [^{3}H]-alkyl-acyl-Gro was quantified. In resting cells, we detected the production of [^{3}H]-alkyl-acyl-Gro. Furthermore, in A23187-stimulated cells, we observed a very significant increase of this metabolite (+58% over control). By contrast no significant difference was found between unstimulated and PMA-stimulated cells (Fig. 3).

DISCUSSION

Activation of PKC is an important pathway for the modulation of endothelial permeability. In this study, we show that culturing endothelial cells in the presence of alkyl-Gro resulted in inhibition of calcium ionophore- and PMA-induced raise in endothelial permeability. This effect was associated with the production of an ether analogue of DAG described as an inhibitor of DAG-induced PKC activation (Daniel *et al.* 1988).

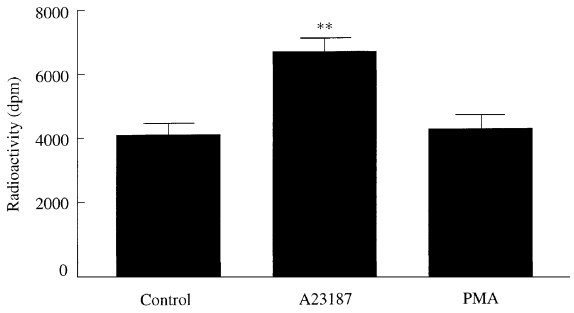

Figure 3 Production of [^{3}H]-alkyl-acyl-Gro by endothelial cells. Cells were incubated with [^{3}H]-alkyl-Gro (10 μM, 24 h) and stimulated with A23187 (1 μM) or PMA (0.2 μM). [^{3}H]-alkyl-acyl-Gro was separated by TLC and quantified. $n = 12$; $^{**}P < 0.01$.

Various mediators, among which pro-inflammatory mediators, increase endothelial permeability. Previous studies have shown that PKC activation was followed by reorganization of actin filaments and by a break in junctional complexes. The protein, PKC-dependent cytoskeletal phosphorylation, would then provoke a change of cell shape and a cellular retraction resulting from phosphorylation of myosin light chains, and increasing endothelial permeability. This endothelial response is associated with cell rounding and intercellular junction widening (Lum & Malik 1994). Calcium mobilization is also involved, triggering the contractile mechanisms that provoke intercellular 'break', and macromolecular leak towards underlying tissues (Siflinger-Birnboim & Malik 1996).

In our experiments, culturing endothelial cells in the presence of 10 μM [^{3}H]-alkyl-Gro had no cytotoxic effects and resulted in the prominent incorporation of radioactive material into PC and PE which may participate in cell signalling as assessed by the production of [^{3}H]-alkyl-acyl-Gro.

Treatment of endothelial cell monolayer with A 23187 or PMA resulted in the raise in albumin permeability. When monolayers were pre-incubated with alkyl-Gro for 24 h, no change of permeability was observed in resting conditions, however, the increase in albumin permeability resulting from PMA-stimulation was totally abolished. Furthermore, when cells were stimulated by the calcium ionophore A23187, endothelial permeability to albumin decreased even to a lower level than in unstimulated cells.

The mechanism of this effect of alkyl-Gro is not totally clear, but these ether lipids may have several sites of actions. By incorporating into membrane phospholipids, they might alter phospholipase-dependent signalling. Our study demonstrated that these compounds were prominently incorporated into 1-O-alkyl-glycerophosphocholine (1-O-alkyl-PC) and to a lesser extent into 1-O-alkyl-glycerophosphoethanolamine (1-O-alkyl-PE). By the action of phospholipase C or D,

PC is a source of DAG (Nishizuka 1995). The stimulation of phospholipase-triggering receptor on 1-O-alkyl-PC has been studied in a murine mast cell line and resulted in the production of 1-O-alkyl-2-acyl-glycerol (Robinson *et al.* 1991). In endothelial cells, after 24-h incubation of cells with [^{3}H]-alkyl-Gro, we observed the production of the DAG analogue [^{3}H]-alkyl-acyl-Gro; furthermore, this metabolite was increased by calcium ionophore stimulation. These data suggest that 1-O-alkyl-PC is a substrate for phopholipases C and/or D in endothelial cells.

Unlike DAG, its ether analogue has no or poor effects on PKC (Heymans *et al.* 1987) and inhibits PKC stimulation by DAG (Daniel *et al.* 1988). Robinson *et al.* (1995) suggested that these compounds may play an inhibitory function on PKC. This inhibitory effect might also occur to prevent PKC stimulation by PMA. This is supported by our data showing the lack of effect of PMA on endothelial permeability and on the ether DAG-analogue production when cells were pre-incubated with alkyl-Gro. In MDCK cells, Robinson & Warne (1991) found that after stimulation with PMA, alkyl-acyl-Gro were not increased, while an important DAG release was observed. These authors explained the low quantity of alkyl-acyl-Gro released by the low level of their precursor in cell membrane. In the same cells, Daniel *et al.* (1986) detected [^{3}H]-alkyl-acyl-Gro after the incubation of the cells by [^{3}H]-hexadecyl-lysoPC and under PMA stimulation. However, considering the very high specific radioactivity of the precursor, a very small mass of alkyl-acyl-Gro produced could explain a lack of inhibition of PKC with these components. In our experiments, the supplementation with alkyl-Gro may raise the level of the precursor 1-O-alkyl-PC and increase the production of the DAG analogue in resting conditions to a level which inhibits PKC. On the other hand, when endothelial cells were stimulated by the calcium ionophore A23187, we observed an increased production of the ether DAG-analogue. This could result from direct activation of phospholipase C and/or D by the raise in cytosolic calcium. We propose the hypothesis that this raise in the DAG analogue and PKC-inhibitor alkyl-acyl-Gro explains that the A23187 decreased endothelial permeability below the basal level in alkyl-Gro-treated cells. Our data show that permeability of cultured endothelial cell monolayer in resting conditions is not at its lowest level. Increased permeability in culture conditions could result from deficit in mediators such as prostacyclin or adrenaline, coupled to adenylate cyclase activation, because cyclic adenosine monophosphate (cAMP) has an inhibitory effect on permeability (Lum & Malik 1994). Culture conditions also suppress flow-induced NO production which may reduce as well endothelial permeability (Arnhold *et al.* 1999). Then the inhibition of PKC by alkyl-acyl-Gro could unmask calcium ionophore inhibitory effect through the stimulation of prostacyclin or NO production by the raise in cytosolic calcium.

Our results suggest that endothelial permeability modulation through PKC regulation may occur in a large range. The diversity of endothelial cells in various vascular beds and in vessels of different diameters raises the question whether aortic endothelium reflects capillary endothelium with respect to permeability modulation. As microvascular permeability is also regulated by PKC (Murray *et al.* 1991, Yuan *et al.* 2000), one would expect that alkyl-Gro exert similar inhibiting activity on capillary endothelium permeability. Our data also confirm that modification of lipidic composition of cells may result in physiological effects through alteration of signals in which lipids play a prominent role.

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