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1-*O*-Alkylglycerols reduce the stimulating effects of bFGF on endothelial cell proliferation *in vitro*

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Abstract

1-*O*-Alkylglycerols (alkyl-Gro) are natural etherlipids with multiple biological activities. We previously demonstrated that alkyl-Gro reduce endothelial permeability. Here we showed that alkyl-Gro reduced the basic Fibroblast Growth Factor (bFGF)-stimulated endothelial cell proliferation in a concentration-dependent manner. The effects of 0.5 and 5 ng/ml bFGF on growth were completely suppressed after 72 h-treatment by 50 μM alkyl-Gro. Since bFGF greatly increased (+56% ± 15) the production of 1-*O*-alkyl-2-acyl-*sn*-glycerophosphate in alkyl-Gro-treated endothelial cells, our data suggest that the observed effects of alkyl-Gro could be mediated through PLD activation. Inhibition of bFGF-stimulated endothelial proliferation could support anti-angiogenic activity of alkyl-Gro.

Keywords: 1-*O*-Alkylglycerols; Endothelial cells; Basic fibroblast growth factor; Etherlipids; Cell proliferation; Alkylglycerols; Endothelium; bFGF; Angiogenesis

1. Introduction

Tumour angiogenesis is a biological process leading to the formation of new blood vessels which is a crucial step for tumour growth and metastasis dissemination [1]. This neovascularisation is controlled by many angiogenic factors such as the basic Fibroblast Growth Factor (bFGF) [2,3]. bFGF is a multifunctional growth factor which stimulates either endothelial proliferation, migration or secretion [4]. The signalling pathways of bFGF receptors involve phospholipases C or D, leading to the production of second messengers such as diacylglycerol (DAG) from phosphatidylinositol or phosphatidic acid from phosphatidylcholine, both known to activate protein kinase C (PKC) [5,6]. Therefore, alteration of signal transduction related to DAG and phosphatidic acid might affect bFGF-induced effects on endothelial cells.

1-*O*-Alkyl-*sn*-glycerols (alkyl-Gro) are etherlipids which are present in hematopoietic organs [7] and particularly abundant in shark liver oils [8]. They have multiple biological activities such as an anti-tumour effect [9], hematopoiesis modulation [10], and

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stimulation of immune responses [11]. Alkyl-Gro are incorporated in cell phospholipids, and may act as precursors of etherlipid analogues of lipid second messengers and mediators. For instance, incorporation of alkyl-Gro in phospholipids of endothelial cells leads to the production of 1-*O*-alkyl-2-acyl-*sn*-glycerol (alkyl-acyl-Gro) [12], while they increase Platelet-Activating Factor (PAF) production in monocyte-like cell line THP-1 [13]. We have previously demonstrated that alkyl-Gro exert an inhibiting activity on endothelial permeability [12], while up-regulation of permeability is involved in the angiogenic processes [14]. This effect could stem from the production of alkyl-acyl-Gro, an analogue of DAG known to inhibit PKC activation [15]. Moreover, we have observed that alkyl-Gro, when administrated orally, reduce an endothelial marker: von Willebrand factor in tumours grafted to mice, suggesting a potential anti-angiogenic effect of these etherlipids [16].

The aim of this study was to determine the effect of alkyl-Gro on bFGF-stimulated growth in cultured calf aortic endothelial cells.

2. Materials and methods

2.1. Reagents

Fetal calf serum (FCS), antibiotics–antimycotics (penicillin G sodium, streptomycin sulfate and amphotericin B), Fungizone® [Bristol-Myers Squibb] (amphotericin B) and culture medium MEM D-Valine were obtained from Gibco (Cergy-Pontoise, France). [methyl-^{3}H]thymidine and [^{3}H]1-*O*-hexadecyl-*sn*-glycerophosphocholine were purchased from Amersham International (Les Ulis, France). Phospholipase D (PLD) from *Arachis hypogaea* and lysoPLD from *Streptococcus aureus* were purchased from ICN (Orsay, France). Bovine bFGF was obtained from R&D System Europe (Oxon, United Kingdom). Arachidonic acid, phosphatidylcholine, phosphatidylinositol, phosphatidylethanolamine, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were procured from Sigma–Aldrich (La Verpillère, France). All solvents, chemical products and Silica gel 60 Å LK6 plates were obtained from Merck (Darmstadt, Germany).

2.2. Alkylglycerols

Alkyl-Gro from *Centrophorus squamosus* liver oil were obtained from the Centre Technique ID-MER (Lorient, France). The alkyl-Gro were prepared and purified from shark liver oil as described previously [13]. Alkyl-Gro species varied according to the alkyl-chain length. The composition was as follows: 14:0 = 0.7%, 16:0 = 9.1%, 16:1n-7 = 12.5%, 18:1n-9 = 68.1%, 18:1n-7 = 4.8% and other minor species (<1%) = 4.8%. Tritiation of alkyl-Gro was performed by ^{3}H-labelling on the *sn*-3 position of glycerol [13].

2.3. Cell culture

Aortas were removed from calves (6 months old). Calf Aortic Thoracic Endothelial Cells (CAEC) were isolated as described previously [17,18]. CAEC were cultured at 37 °C, in a humidified atmosphere of air (95%) and CO_2 (5%) in MEM D-Valine [19], containing antibiotics–antimycotics (160 U/mL penicillin, 160 μg/mL streptomycin, 1.9 μg/mL amphotericin B) and supplemented with 20% FCS.

2.4. Incorporation of [^{3}H]alkyl-Gro

CAEC were sparsely seeded at subconfluence (80%) in 6-well plates (100,000 cells per well) in MEM D-Valine supplemented with 10% FCS. Twenty four hours later, cells were incubated with [^{3}H]alkyl-Gro (3.4 MBq/mmol, 10μM) for 24, 48 and 72 h. At the end of each incubation time, the culture medium was removed and cells were washed with phosphate buffer saline, which contained KCl (2.7 mM), KH_2PO_4 (1.5 mM), NaCl (137 mM) and Na_2HPO_4 (8.1 mM) (pH 7.4). Total lipids were extracted according to the method of Bligh and Dyer [20]. Neutral lipids were separated by thin layer chromatography (TLC) on silica gel plate, first in diethyl ether:acetone 60:20 (v/v) until there was a 10 cm migration and then in hexane:diethyl ether:acetic acid 80:20:1 (v/v). Radioactive material was visualized using a radio chromatogram scanner (Bioscan, Washington, USA). The zones on silica gel corresponding to neutral lipids were scraped off and radioactivity was measured in a liquid scintillation counter (Packard, Rungis, France). The zone corresponding to total phospholipids was scraped off and radioactive lipids were extracted using the Bligh and Dyer method. Phospholipids were separated by TLC with the following solvent system chloroform:methanol:acetic acid 35:14:2.7 (v/v). The radioactive zones corresponding to the different classes of phospholipids identified by authentic standards (phosphatidylcholine, phosphatidylinositol, and phosphatidylethanolamine) were scraped off and radioactivity was determined as described above. Each neutral lipid was identified by the migration of authentic standards, [^{3}H]alkyl-acyl-Gro obtained as described elsewhere [12] and 1-*O*-alkyl-2,3-diacyl-*sn*-glycerol (alkyl-diacyl-Gro) obtained as follow: components of shark liver oil devoid of squalene were separated by TLC using the solvent system hexane: diethyl ether:acetic acid 80:20:1 (v/v). Two major bands were observed corresponding to the triacylglycerol ($R_f = 0.49$) and the alkyl-diacyl-Gro ($R_f = 0.63$). The zone on silica gel corresponding to the alkyl-diacyl-Gro was scrapped off, lipids were extracted [20] and used as an authentic standard.

2.5. Cytotoxicity of alkyl-Gro

Absence of alkyl-Gro-induced cytotoxicity was assessed by the rate of MTT metabolism into MTT–formazan [21]. CAEC were seeded at subconfluence in 96-well plates (1000 cells per well) in MEM D-Valine supplemented with 10% FCS and cultured for 24 h. Cells were treated with various concentrations of alkyl-Gro for 24, 48 and 72 h. For the treatment period, MTT (50 μg per well) was added and cells were further incubated for 4 h at 37 °C. The medium was then replaced by DMSO after centrifugation (1000 rpm for 5 min) and spectrophotometric measurement of MTT–formazan was performed at 540 nm.

2.6. Cell growth study

CAEC were sparsely seeded at subconfluence in 24-wells plates (10,000 cells per well) in MEM D-valine supplemented with 10% FCS. In some experiments, cells were incubated 24 h later with alkyl-Gro for 24, 48 and 72 h. [^{3}H]thymidine (1.6 TBq/mmol, 3.66 kBq/mL) was added into wells 24 h before revelation. In other experiments, cells were incubated with alkyl-Gro, stimulated with bFGF and treated with [^{3}H]thymidine for 72 h. The [^{3}H]thymidine incorporation was stopped by cold methanol. Labelled DNA was precipitated using 10% perchloric acid, dissolved in 0.5 N NaOH and incorporated radioactivity was quantified by liquid scintillation counting.

2.7. Synthesis of [^{3}H]1-O-hexadecyl-2-arachidonoyl-sn-glycerophosphate and [^{3}H]1-O-hexadecyl-sn-glycerophosphate

[^{3}H]1-*O*-hexadecyl-*sn*-glycerophosphocholine (2.3 GBq/mmol, 17 μM) was acylated with arachidonic acid (55 μM) as described previously [22]. [^{3}H]1-*O*-hexadecyl-*sn*-glycerophosphocholine (2.3 GBq/mmol, 7 μM) and the resulting [^{3}H]1-*O*-hexadecyl-2-arachidonoyl-*sn*-glycerophosphocholine was submitted to the action of lysoPLD and PLD respectively, according to Christie's method [23]. The resulting [^{3}H]1-*O*-hexadecyl-*sn*-glycerophosphate and [^{3}H]1-*O*-hexadecyl-2-arachidonoyl-*sn*-glycerophosphate were then extracted and separated by TLC as described above. [^{3}H]1-*O*-Alkyl-*sn*-glycerophosphate (alkyl-lysoPA) and [^{3}H]1-*O*-alkyl-2-acyl-*sn*-glycerophosphate (alkyl-PA), ether analogues of lysophosphatidic acid and phosphatidic acid respectively, were used as standards for TLC identification of [^{3}H]alkyl-Gro metabolites.

2.8. [^{3}H]alkyl-PA, [^{3}H]alkyl-lysoPA and [^{3}H]alkyl-acyl-Gro production

Cells were seeded and treated with [^{3}H]alkyl-Gro for 72 h as described above. CAEC were then washed with phosphate buffer saline and stimulated with bFGF (10 ng/mL) for 10 min in MEM D-valine containing 10% FCS. Lipids were then analysed as described above and identified by their R_f compared to authentic standards, which synthesis is described above or elsewhere [12].

2.9. Statistical analysis

All data are presented as the means ± SEM of the indicated number of separate experiments performed for the indicated number of repetitions. Significance of the differences observed was assessed by the Mann and Whitney non parametric test: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

3. Results

3.1. [^{3}H]alkyl-Gro incorporation into CAEC

The incorporation of [^{3}H]alkyl-Gro into CAEC was determined as a function of time (Fig. 1). [^{3}H]alkyl-Gro were incorporated into both neutral lipids and phospholipids. In neutral lipids, [^{3}H]alkyl-Gro remained essentially unmodified (Fig. 1a). [^{3}H]alkyl-Gro were also incorporated into phospholipids. After 72 h, the major radioactive metabolites of [^{3}H]alkyl-Gro were identified as [^{3}H]1-*O*-alkyl-2-acyl-*sn*-glycerophosphocholine and to a lesser extent as [^{3}H]1-*O*-alkyl-2-acyl-*sn*-glycerophosphoinositol and [^{3}H]1-*O*-alkyl-2-acyl-*sn*-glycerophosphoethanolamine, which represents 24.0 ± 1.4%, 7.7 ± 0.9% and 4.6 ± 0.8% of the incorporated radioactivity after 72 h, respectively (Fig. 1b).

3.2. Alkyl-Gro effect on basal growth of CAEC

Alkyl-Gro exerted an inhibiting effect on the basal growth of CAEC, in a time- and concentration-dependent manner (Fig. 2). Low concentrations of alkyl-Gro (5 μM and 10 μM) had no effect on CAEC growth after 24 and 48 h whereas growth inhibition was observed for longer incubations (72 h) or with higher concentrations of alkyl-Gro. This inhibition reached 32.9 ± 6.4% below control with 50 μM ($p < 0.001$). All concentrations of alkyl-Gro used were devoid of cytotoxicity as measured by MTT test (Fig. 3).

3.3. Alkyl-Gro effect on bFGF-stimulated proliferation of CAEC

bFGF had a concentration-dependent stimulating effect on [^{3}H]thymidine incorporation by CAEC. After 24 h alkyl-Gro curtailed the bFGF-induced stimulation of proliferation of CAEC in a dose-dependent manner (Fig. 4). No significant effect was observed with 10 μM alkyl-Gro for whatever the concentration of bFGF. Fifty micromolar alkyl-Gro evoked the greatest inhibition of CAEC proliferation. This inhibition reached

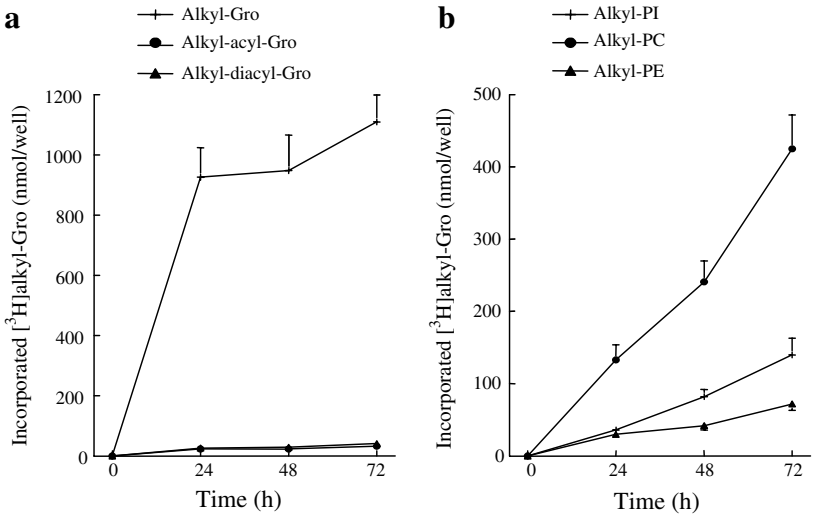

Fig. 1. Incorporation of [^{3}H]alkyl-Gro in (a) neutral lipids and (b) phospholipids of CAEC. CAEC were treated with [^{3}H]alkyl-Gro (10 μM) for indicated times. At each incubation time, total lipids were extracted and separated by TLC as described in Section 2. The radiolabelled zones corresponding to each lipid species were scraped off and radioactivity was determined by liquid scintillation. Means ± SEM ($n = 4$, 3 repetitions). [^{3}H]alkyl-PI: [^{3}H]1-*O*-alkyl-2-acyl-*sn*-glycerophosphoinositol; [^{3}H]alkyl-PC: [^{3}H]1-*O*-alkyl-2-acyl-*sn*-glycerophosphocholine; [^{3}H]alkyl-PE: [^{3}H]1-*O*-alkyl-2-acyl-*sn*-glycerophosphoethanolamine.

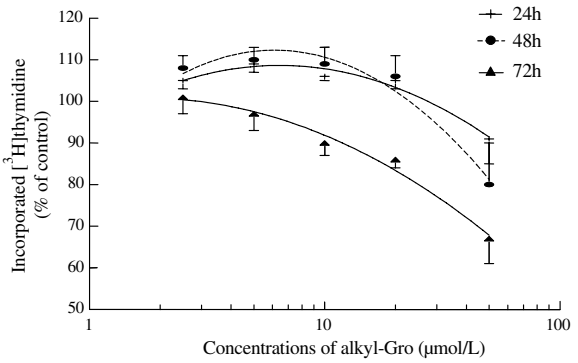

Fig. 2. Effect of alkyl-Gro on basal growth of CAEC. CAEC were incubated with different concentrations of alkyl-Gro for the indicated times. [^{3}H] thymidine was added 24 h before determination of radioactivity by liquid scintillation counting as described in Section 2. Means ± SEM ($n = 5$, 4 repetitions).

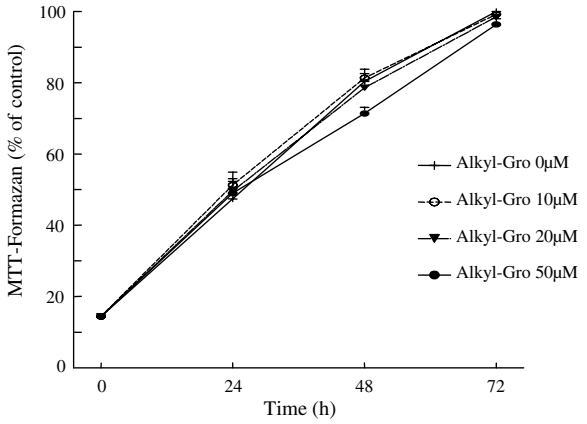

Fig. 3. Cytotoxicity of alkyl-Gro on CAEC. CAEC were treated with different concentrations of alkyl-Gro for 24, 48 and 72 h. For each indicated time, cells were incubated with MTT for 4 h. Formed MTT-formazan was then quantified by spectrophotometry at 540 nm. Means ± SEM ($n = 3$, 7 repetitions).

47.5 ± 10.5% under control when cells were stimulated with 5 ng/mL bFGF and was not overcome by increasing bFGF concentrations.

3.4. Production of [^{3}H]alkyl-Gro metabolites in CAEC

[^{3}H]alkyl-acyl-Gro, [^{3}H]alkyl-PA and [^{3}H]alkyl-lysoPA were produced after [^{3}H]alkyl-Gro incubation and represented 2.0 ± 0.2%, 2.2 ± 0.2% and 2.9 ± 0.2% of the incorporated radioactivity, respectively (Fig. 5). Cell stimulation with 10 ng/mL of bFGF resulted in an increased production of [^{3}H]alkyl-PA (up to 55.9 ± 14.9% over control) whereas no significant effect was observed on the [^{3}H]alkyl-lysoPA and the [^{3}H]alkyl-acyl-Gro levels.

4. Discussion

Tumour angiogenesis is characterised by an increase of endothelial permeability and proliferation, both enhanced by angiogenic factors. We have

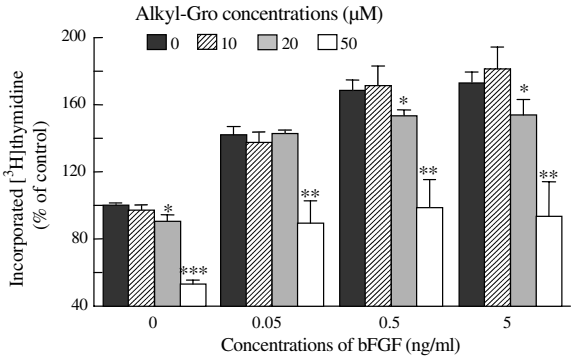

Fig. 4. Effect of alkyl-Gro on bFGF-stimulated proliferation of CAEC. CAEC were simultaneously incubated with different concentrations of alkyl-Gro and stimulated with various concentrations of bFGF for 72 h. CAEC proliferation was measured by the incorporation of [^{3}H]thymidine as described in Section 2. Means ± SEM ($n = 3$, 3 repetitions).

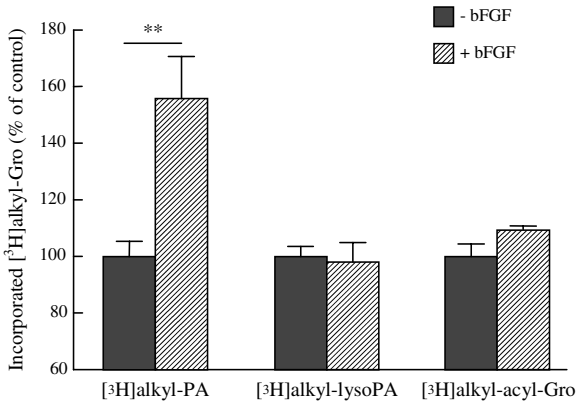

Fig. 5. [^{3}H]Alkyl-PA, [^{3}H]alkyl-lysoPA and [^{3}H]alkyl-acyl-Gro production in CAEC stimulated by bFGF after [^{3}H]alkyl-Gro incorporation. CAEC were incubated with [^{3}H]alkyl-Gro (10 μM) for 72 h. Cells were then stimulated with bFGF (10 ng/mL) for 10 min. Total lipids were then extracted and separated by TLC as described in Section 2. The radiolabelled zones corresponding to each lipid species were scraped off and radioactivity was determined by liquid scintillation. Means ± SEM ($n = 3$, 2 repetitions).

previously demonstrated that alkyl-Gro may inhibit the increases in permeability induced by phorbol myristate acetate and calcium ionophore A23187 [12]. Here we examined the effects of alkyl-Gro on the proliferation of bFGF stimulated endothelial cells.

Alkyl-Gro were metabolised into alkyl-glycerophosphocholine and alkyl-glycerophosphoinositol, two ether analogues of phospholipids potentially involved in cell signalling. We also found that alkyl-Gro were metabolised into several lipids that could influence signal transduction, namely alkyl-PA, alkyl-lysoPA and alkyl-acyl-Gro.

We observed that alkyl-Gro influenced endothelial cell growth, without any cytotoxic effects. Alkyl-Gro decreased the cell proliferation in a concentration- and a time-dependent manner. They also reduced the stimulating effect of bFGF. However, in contrast to calcium ionophore A 23187 [12], bFGF did not induce an increase in alkyl-acyl-Gro, suggesting that activation of phospholipase C in bFGF signalling did not affect the metabolism of alkyl-phospholipids. Furthermore, previous data showed that the mutation of Tyr^{766}, a residue essential for phosphatidylinositol hydrolysis did not affect FGF-receptor mediated mitogenesis [24], indicating an alternative pathway for mitogenesis signalling. By contrast, after incubation of endothelial cells with alkylGro, bFGF greatly increased the production of alkyl-PA confirming an involvement of PLD in bFGF signalling [6]. Recent studies demonstrated that phosphatidic acid may regulate tyrosine phosphorylation [25]. A phosphatidic acid-activated protein kinase was also described [26] and phosphatidic acid activates cell proliferation [27,28]. Alkyl-PA properties are poorly documented but its production is correlated with biological effects [29]. The structural particularity of alkyl-PA could confer potential and singular activities to this molecule, which could be distinct to those of phosphatidic acid. This hypothesis needs further studies. Since neither alkyl-acyl-Gro nor alkyl-lysoPA were increased with bFGF, this could indicate that phospholipase A2 and phosphatidic acid phosphatase had no effect on alkyl-PA. This could be an other explanation for the inhibiting effect of alkyl-Gro, by interfering with the production of potent bioactive lipids involved in proliferation. One of the critical pathways controlling tumour growth and metastasis is related to the regulation of the phospholipid homeostasis. PLD has emerged as a good candidate implied in tumour cell proliferation and survival [30]. Elevated PLD activity has been reported in several cancer tissues and contributes to cell motility and invasiveness. Altering the PLD-dependent formation of nascent second messenger such as phosphatidic acid could thus represent an anti-tumour target. In conclusion, our data suggest that alkyl-Gro could modify endothelial cell response by modulating PLD signalling. This would be a new pathway for alkyl-Gro-mediated responses in endothelial cells, with new therapeutic perspectives.

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