British Journal of Pharmacology (1996) 119, 185-186

SPECIAL REPORT

The identification of DL-*threo* dihydrosphingosine and sphingosine as novel inhibitors of extracellular signal-regulated kinase signalling in airway smooth muscle

Deborah Tolan, Ann-Marie Conway, Leslie Steele*, Susan Pyne & [1]Nigel J. Pyne

Department of Physiology and Pharmacology, University of Strathclyde, Glasgow, G1 1XW and *Knoll Pharmaceuticals, 1 Thane Road West, Nottingham, NG2 3AA

We present entirely novel evidence that DL-*threo* dihydrosphingosine and sphingosine are inhibitors of the extracellular signal-regulated kinase (ERK) signalling cassette in mammalian cells. We show that DL-*threo* dihydrosphingosine is effective against both growth factor- and G-protein-dependent activation of ERK. We conclude that DL-*threo* dihydrosphingosine may represent an important pharmacological cell-permeable agent that may be usefully employed to block smooth muscle cell proliferation.

Keywords: Extracellular signal regulated kinase; DL-*threo* dihydrosphingosine; phosphatase; DNA synthesis; platelet-derived growth factor; bradykinin

Introduction A number of 'so-called' mitogen-activated kinase (MAPK) cascades function to regulate a variety of mammalian cell responses, such as proliferation, differentiation and cell survival. For instance, activation of extracellular signal-regulated kinase-1 (ERK-1 and ERK-2) is critical for the initiation of immediate early gene expression and cell cycle progression leading to mitogenesis (see Marshall, 1995 for review). An additional signalling pathway involving $p70^{S6K}$ results in the phosphorylation of S6 ribosomal protein via a PI-3 kinase-dependent pathway (Downward, 1994). Therefore, $p70^{S6K}$ functions in concert with ERK-regulated transcription factor activation to provoke cell progression through G1. Finally, JNK is activated by a distinct kinase cascade (MEK kinase, SEK-1/MEK-4) that results in N-terminal directed phosphorylation of c-Jun (Yan *et al.*, 1994) and growth arrest in response to cellular stress.

Growth factors and cytokines can also induce spingomyelin hydrolysis leading to the sequential formation of intracellular ceramide, sphingosine and sphingosine phosphate (Olivera & Spiegel, 1993). In this context, we have previously shown that PDGF stimulates transient sphingosine phosphate formation in airway smooth muscle cells (Pyne *et al.*, 1996). Spingosine is an effective activator of the JNK cascade in ASM cells, whereas spingosine phosphate induces ERK activation (Pyne *et al.*, 1996; Pyne & Pyne, 1996). We have therefore, assessed whether a sphingosine and its saturated equivalent, DL-*threo* dihydrosphingosine, have any inhibitory effect upon ERK signalling in these cells.

Methods *Cell culture and DNA synthesis* The preparation of the primary cultures of guinea-pig airway smooth muscle cells was achieved as described previously (Pyne & Pyne, 1993). Their identity was confirmed to be smooth muscle by the presence of α-actin using a smooth muscle-specific mouse anti α-actin monoclonal antibody (Pyne & Pyne, 1993). DL-*threo* dihydrosphingosine and sphingosine were assessed for their effect on PDGF-stimulated [^{3}H]-thymidine incorporation into DNA. This was performed according to Pyne *et al.* (1996).

Detection of ERK Cells were placed in Dulbecco's modified Eagles medium containing 1% (v/v) foetal calf serum and 1% (v/v) donor horse serum for 24 h. After agonist treatment, the medium was removed and boiling sample buffer (mM: Tris/HCl 125, pH 6.7, $Na_4P_2O_7$ 0.5, EDTA 1.25, DTT 25, glycerol 5% (v/v), SDS 0.5% (w/v), bromophenol blue 0.05% (w/v)) was added and the cell lysates harvested. Samples were boiled for 5 min and then subjected to SDS-PAGE and transferred to nitrocellulose sheets. These were subsequently probed with anti-ERK-2 antibody according to Pyne & Pyne (1996). Detection of immunoreactivity was achieved by incubating nitrocellulose sheets with reporter horseradish peroxidase-linked anti-mouse antibody. Immunoreactive bands were visualised with the ECL detection kit.

ERK activity assays in isolated cell homogenates Homogenate ERK activity was assessed by use of the radioactive Biotrak (Amersham) peptide activity assay. This employs an EGF receptor peptide substrate (containing one phosphorylation site) that is specifically phosphorylated by ERK. Homogenates were isolated according to the Biotrak protocol. Assays were performed using 0.5 μCi [γ-^{32}P]-ATP (final concentration 0.2 mM).

Materials All biochemicals, including collagenase, elastase and soya bean trypsin inhibitor were from Sigma Chemical Co. (U.K.). [γ-^{32}P]ATP (3000 Ci mmol^{-1}) and the ERK Biotrak assay kit were from Amersham International (U.K.). Cell culture supplies were from Gibco (U.K.). DL-*threo* dihydrosphingosine, sphingosine and the specific anti-ERK-2 antibody were from Affiniti (U.K.). Male Dunkin-Hartly guinea-pigs (200-400g) were used for isolation of tracheal smooth muscle.

Results Growth factors and G-protein-linked receptor agonists regulate ERK by a mechanism that involves its Thr/Tyr phosphorylation and activation by 'up-stream' dual-specific kinase(s). This results in its slower mobility on SDS-PAGE. Using shift assays, we show in Figure 1 that a maximal concentration of PDGF elicited the rapid activation of ERK-2. This was confirmed by use of an ERK specific peptide substrate assay (Biotrak). PDGF and bradykinin induced substantial stimulation of ERK activity (Table 1).

DL-*threo* dihydrosphingosine (125 μM) was ineffective at eliciting the activation of ERK (Table 1). However, the prior treatment of cells with DL-*threo* dihydrosphingosine elicited inhibition of bradykinin- and PDGF-stimulated ERK activity (Table 1). This was confirmed by shift blot analysis, where DL-*threo* dihydrosphingosine induced the appearance of non-activated ERK-2 in lysates isolated from PDGF-treated cells

[1] Author for correspondence.

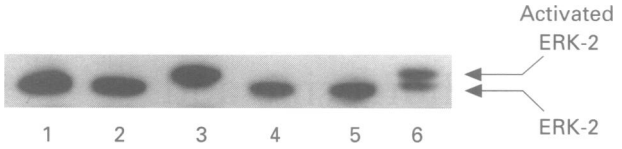

Figure 1 The effect of DL-*threo* dihydrosphingosine on the PDGF-induced activation of ERK-2 in airway smooth muscle cells. Shift blot analysis demonstrating the inhibition of PDGF-induced activation of ERK-2 by DL-*threo* dihydrosphingosine. Lane 1, unstimulated cells; lane 2, PDGF (10 ng ml^{-1}, 7.5 min) plus DL-*threo* dihydrosphingosine (125 μM); lane 3 PDGF (10 ng ml^{-1}, 7.5 min); lane 4, unstimulated cells; lane 5, PDGF (1 ng ml^{-1}, 7.5 min plus DL-*threo* dihydrosphingosine (125 μM); lane 6, PDGF (1 ng ml^{-1}, 7.5 min). In all cases cells were pretreated with DL-*threo* dihydrosphingosine for 10 min before agonist challenge for the indicated time. This is a representative result of an experiment performed on three separate cell preparations. ERK-2 and activated ERK-2 are denoted by arrows.

(Figure 1). At concentrations of either 1 or 10 ng ml^{-1} PDGF, DL-*threo* dihydrosphingosine (125 μM) induced the complete inhibition of PDGF-stimulated ERK activity (Figure 1, Table 1). A sub-maximal concentration of sphingosine (62.5 μM) also induced inhibition of PDGF-stimulated ERK activity (Table 1). Both sphingosine and DL-*threo* dihydrosphingosine (62.5 μM) inhibited PDGF-stimulated DNA synthesis, reducing it to below basal levels ([^{3}H]-thymidine incorporation expressed as means $\pm$ s.d.; untreated cells, 1915 $\pm$ 143 d.p.m.; PDGF (10 ng ml^{-1})-treated, 43603 $\pm$ 3715 d.p.m.; DL-*threo* dihydrosphingosine-treated plus PDGF, 342 $\pm$ 40 d.p.m.; sphingosine-treated plus PDGF, 674 $\pm$ 109 d.p.m., $n = 3$ separate experiments).

Table 1 The concentration-dependent effect of DL-*threo* dihydrosphingosine on PDGF-stimulated ERK activity

Treatments	*ΔERK activity (d.p.m.)*
PDGF	12431 $\pm$ 979
Dihydro SPH (125 μM)	-209 ± 48
PDGF plus dihydro SPH	
125 μM	163 $\pm$ 10
62.5 μM	4010 $\pm$ 353
12.5 μM	11054 $\pm$ 1058
BK	3893 $\pm$ 187
BK plus dihydro SPH (125 μM)	438 $\pm$ 137
PGDF plus SPH (62.5 μM)	6329 $\pm$ 1597

ERK assays were performed with a specific peptide assay kit provided by Amersham (U.K.). Cell extracts were normalised for protein (10 μg per assay) and activity measured for 15 min at 30°C. Cells were stimulated with either DL-*threo* dihydrosphingosine (dihydro SPH) or sphingosine (SPH) for 10 min before challenge with a maximal concentration of either PDGF (10 ng ml^{-1}) or bradykinin (BK, 1 μM) for t = 7.5 min. Results are expressed as the change in ERK activity (d.p.m.) above basal levels. This is a representative result of one performed on three separate cell preparations.

Discussion We present evidence that DL-*threo* dihydrosphingosine negatively regulates growth factor- and G-protein-linked receptor-stimulated ERK signalling in mammalian cells. We also show that this can be mimicked by sphingosine. DL-*threo* dihydrosphingosine can induce the complete inhibition of PDGF-stimulated ERK activity in ASM cells.

It is well established that sphingosine can inhibit PKC (Hannun & Bell, 1989), an enzyme that catalyses phosphorylation of c-Raf. Preliminary evidence indicates that approx. 50% of the PDGF-stimulated ERK activity is PKC-dependent, indicating that DL-*threo* dihydrosphingosine must act at additional site(s). Other possible candidates as sites for interaction are: (1) the Shc family members which are tyrosyl phosphorylated in response to both G-protein linked receptor agonists and growth factors and which may function to regulate different ERK modules, or (2) ERK-specific phosphatase (i.e. MKP1 or a related phosphatase), an enzyme family that dephosphorylates ERK, irrespective of its route of activation.

We conclude that DL-*threo* dihydrosphingosine and sphingosine may be usefully employed to block smooth muscle cell proliferation.

This study was supported by the Wellcome Trust and an MRC collaborative award. S.P. is a Wellcome Trust Senior Biomedical Research Fellow.

References

DOWNWARD, J. (1994). Regulating S6 kinase. *Nature*, **371**, 378–379.

HANNUN, Y.A. & BELL, R.M. (1989). Functions of sphingolipids and sphingolipid breakdown products in cellular regulation. *Science*, **243**, 500–506.

MARSHALL, C.J. (1995). Specificity of receptor tyrosine kinase signalling: transient versus sustained extracellular signal-regulated kinase activation. *Cell*, **80**, 179–185.

OLIVERA, A. & SPIEGEL, S. (1993). Sphingosine 1-phosphate as a second messenger in cell proliferation induced by PDGF and FCS mitogens. *Nature*, **365**, 557–560.

PYNE, S., CHAPMAN, J., STEELE, L. & PYNE, N.J. (1996). Sphingomyelin-derived lipids differentially regulate ERK-2 and JNK signal cascades in airway smooth muscle. *Eur. J. Biochem.*, **237**, 819–826.

PYNE, S. & PYNE, N.J. (1993). Bradykinin stimulates phospholipase D in primary cultures of guinea-pig tracheal smooth muscle. *Biochem. Pharmacol.*, **45**, 593–603.

PYNE, S. & PYNE, N.J (1996). The differential regulation of cyclic AMP by sphingomyelin-derived lipids and the modulation of sphingolipid-stimulated extracellular signal regulated kinase-2 in airway smooth muscle. *Biochem. J.*, **315**, 917–923.

YAN, M., DAI, T., DEAK, J.C., KYRIAKAS, J.M., ZON, L.I., WOODGETT, J.R. & TEMPLETON, D.J. (1994). Activation of stress-activated protein kinase by MEKK1 phosphorylation of its activator SEK1. *Nature*, **372**, 798–800.

(Received April 11, 1996
Accepted June 20, 1996)