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The involvement of p38 mitogen-activated protein kinase in the α-melanocyte stimulating hormone (α-MSH)-induced melanogenic and anti-proliferative effects in B16 murine melanoma cells

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Abstract Activation of p38 or p44/42 mitogen-activated protein (MAP) kinases has been shown to trigger differentiation in a number of cell types. The present study has investigated the roles of these kinases in the α-melanocyte stimulating hormone (α-MSH)-induced melanogenic and proliferative responses in B16 melanoma cells. Treatment of cells with α-MSH led to the time-dependent phosphorylation of both p38 and p44/42 MAP kinases. However, only inhibition of p38 MAP kinase activity with SB 203580 blocked both the α-MSH-induced melanogenic and anti-proliferative effects. It therefore appears that activation of the p38 pathway can promote melanogenesis and inhibit growth of B16 melanoma cells.

Key words: B16 melanoma; p38 mitogen-activated protein kinase; α-Melanocyte stimulating hormone; Differentiation; Melanocyte

1. Introduction

α-Melanocyte stimulating hormone (α-MSH) is a tridecapeptide which is derived from corticotrophin, a cleavage product of the larger precursor proopiomelanocortin [1]. α-MSH exerts its effects through interaction with a family of five heptahelical, G protein-coupled receptors (MC_1–MC_5) which have a wide range of effects in the skin, as well as the central nervous and immune systems [1–3]. Melanocortin receptors have been identified on the surface of both melanocytes and melanoma cells, as detected by ligand-conjugated beads and fluorescent markers [4,5]. The finding that melanoma cells are able to secrete autocrine α-MSH [6], coupled with the fact that patients with malignant melanoma have elevated plasma α-MSH levels [2], suggests that this hormone may be involved in the pathophysiology of melanoma. A number of agents, including cholera toxin, forskolin, α-MSH, and ultraviolet (UV) light, have been reported to trigger melanoma/melanocyte differentiation [7–11], a process which involves both dendrite outgrowth and the activation of melanin production (melanogenesis) [8–10,12].

A growing number of G protein-coupled receptors have been shown to exert their effects via activation of the mitogen-activated protein (MAP) kinases [13]. The p44/42 MAP kinases are activated almost universally in response to growth factors and can either phosphorylate cytoplasmic targets or translocate to the nucleus, where they activate transcription factors such as Elk-1 and SAP-2 [14]. Other members of the MAP kinase family, such as p38 or the stress-activated protein kinases, are stimulated by inflammatory cytokines and environmental stresses leading to apoptosis or differentiation [15–17]. At present, little is known about the ability of melanocortin receptors to activate either p38 or p44/42 MAP kinases. The aim of the present study was to examine the role of these MAP kinases in the growth and differentiation of B16 murine melanoma cells, which are known to endogenously express MC_1 receptors [18].

2. Materials and methods

2.1. p38 and p44/42 MAP kinase stimulation studies

Unless otherwise stated, all reagents were from Sigma (Dorset, UK). B16 murine melanoma cells (obtained from Drs Ulrike Sahm and Colin Pouton, School of Pharmacy and Pharmacology, University of Bath) were seeded at a density of 100 000 cells per well into six-well plates (Falcon) and incubated overnight in RPMI 1640 media (Autogen BioClear, Wiltshire, UK) supplemented with 10% foetal calf serum, penicillin (5000 IU), streptomycin (50 μg ml^{-1}) and L-glutamine (2 mM). In time-course studies, α-MSH (Calbiochem, UK) (10 nM) or forskolin (10 μM) was added to B16 melanoma cells for increasing periods of time (4–120 min). Experiments were terminated by aspiration of the media and the addition of ice-cold phosphate-buffered saline. In some studies, cells were treated with inhibitors (all Calbiochem, UK) of either p38 MAP kinase (SB 203580, 10 μM), MEK1 (PD 98059, 10 μM), protein kinase C (PKC) (Ro 31-8220, 2 μM), or phosphoinositide (PI) 3-kinase (LY 294002, 10 μM), for 30 min, prior to addition of either α-MSH (10 nM), or phorbol-12-myristate-13-acetate (TPA, 500 nM) for 10 min.

2.2. Western analysis

Cells were solubilised in 200 μl SDS–PAGE sample buffer (4% sodium dodecyl sulphate (SDS), 60 mM Tris pH 6.8, 5% glycerol, 0.01% bromophenol blue and 50 mM mercaptoethanol), diluted with 400 μl distilled water and heated to 95°C for 5 min. Whole cell protein extracts were separated on 10% SDS-polyacrylamide gels. Proteins were electrophoretically transferred onto PVDF membranes (Millipore) overnight, washed in Tris-buffered saline (TBS; 100 mM Tris–HCl, pH 7.5 and 150 mM NaCl), before being blocked in TBS containing 0.1% Tween-20 and 5% milk (TBST-milk). Antibody incubations were for 1 h at 21°C in TBST-milk with either anti-phospho specific p38 or anti-phospho specific p44/p42 MAP kinase antibodies as the primaries (both 1:1000 dilutions, from New England Biolabs), followed by an anti-rabbit horseradish peroxidase-conjugated secondary antibody (New England Biolabs). Immunocomplexes were visualised using the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, UK) and detected on photographic film (Kodak, UK). In order to confirm equal protein loading, blots were stripped by treatment for 1 h in stripping buffer (50°C, 0.1 M mercaptoethanol, 62.5 mM Tris–HCl). Blots were then washed three times in TBST and blocked in TBST-milk followed by incubation

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with an anti-α-tubulin antibody (Sigma, UK). Blots were then visualised by ECL, as described previously. In some studies, equal protein loading was confirmed by staining blots with Coomassie blue solution (0.1% Coomassie brilliant blue, 45% methanol, 10% acetic acid), followed by destaining (45% methanol, 10% acetic acid).

2.3. Melanogenesis studies

Extracellular melanin release was measured as previously described [19]. Briefly, B16 cells were seeded at a density of 5×10^4 cells per ml into six-well plates and incubated overnight, before the administration of increasing concentrations of α-MSH (0.1 nM–1 μM). Plates were then incubated for 72 h, after which 100 μl of the media was taken and read spectrophotometrically at 404 nm using a plate reader. Cells were then scraped from the plates and re-suspended in cell culture media containing trypan blue (final concentration 0.1% w/v) and counted using a haemocytometer. Mean increases in extracellular melanin production per cell were expressed as a percentage of basal melanin production in control cells. In some studies, cells were treated with inhibitors of PKC (Ro 31-8220 at 2 μM), PI 3-kinase (LY 294002 at 10 μM), MEK1 (PD 98059, at 10 μM) or p38 (SB 203580, at 10 μM) for 30 min prior to the addition of α-MSH (10 nM). Control experiments were also performed where cells were incubated with kinase inhibitors alone (as above).

2.4. Growth inhibition studies

Cells were seeded at a density of 5×10^4 cells per ml into six-well plates and incubated overnight before being treated with either inhibitors of PKC (Ro 31-8220 at 2 μM), PI 3-kinase (LY 294002, at 10 μM), MEK1 (PD 98059, at 10 μM) or p38 MAP kinase (SB 203580, at 10 μM) for 30 min prior to the addition of α-MSH (10 nM). Control wells were either treated with α-MSH only, or received no drug treatment. In other studies, individual wells were incubated with increasing concentrations of α-MSH (0.1 nM–1 μM) in the absence or presence of SB 203580 (10 μM). In all experiments, plates were left to incubate for 72 h and cells were removed by scraping. Cells were centrifuged and re-suspended in cell culture media containing trypan blue (final concentration 0.1% w/v) and were counted using a haemocytometer.

2.5. Statistical analysis

Unless otherwise stated all results are taken from three experiments performed in duplicate ± S.E.M. All pEC_{50} values were determined from individual experiments by non-linear regression, using a four-parameter logistic equation (GraphPad Prism). Results were analysed using Student's *t*-test and noted to be significantly different where $P<0.05$.

3. Results

Treatment of B16 melanoma cells with α-MSH (10 nM) induced a time-dependent stimulation of p38 MAP kinase (Fig. 1A). Increases in p38 MAP kinase activity were slow in onset with increases in activity not being detected until 60 min after stimulation. Equal loading of protein samples was confirmed by stripping of the original blots and detection with an anti-α-tubulin antibody (Fig. 1B). Exposure of cells to increasing concentrations of α-MSH (0.1 nM–1 μM for 60 min) led to a concentration-dependent increase in p38 MAP kinase phosphorylation (Fig. 1C). Pre-treatment of cells with the MEK1 inhibitor (PD 98059, 10 μM) or PI 3-kinase inhibitor (LY 294002, 10 μM) were found to potentiate the α-MSH-induced phosphorylation of p38 MAP kinase (Fig. 1D). In contrast, pre-treatment with the PKC inhibitor (Ro 31-8220, 2 μM) did not significantly alter the ability of α-MSH to activate p38 MAP kinase. In other studies, treatment of cells with the adenylyl cyclase activator forskolin (10 μM) was shown to activate p38 MAP kinase (Fig. 1E), with increases in phosphorylation only being detected after 120 min of drug treatment (Fig. 1E). In contrast to p38 MAP kinase, α-MSH was shown to rapidly activate p44/42 MAP kinase (4 min) in a concentration-dependent manner for sustained periods of time (>120 min) (data not shown). Treatment of B16 cells with the protein kinase C activator, TPA, 500 nM, was also shown to activate p44/42 MAP kinase (data not shown). In this instance, pre-treatment cells with PD-98059 and Ro

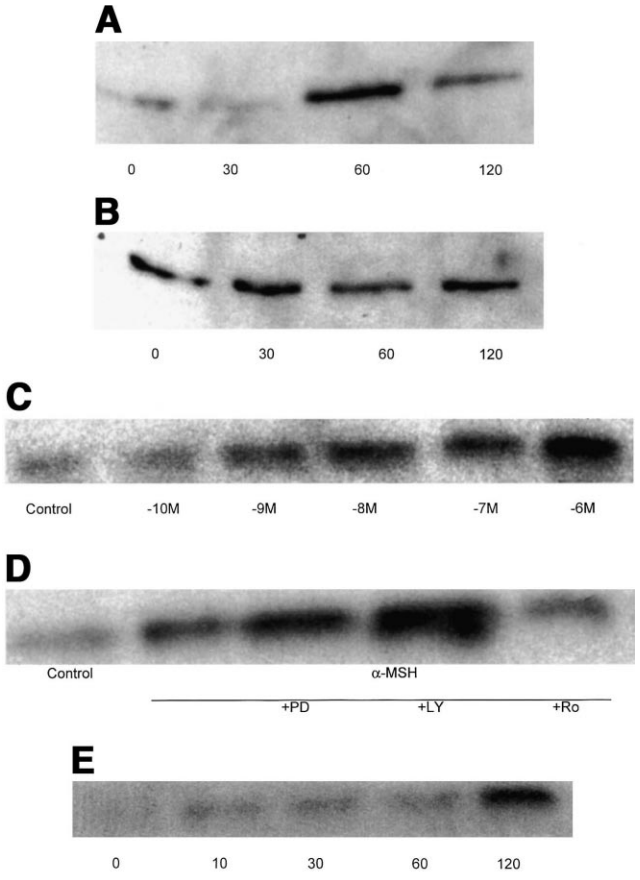

Fig. 1. A: Time-course (in min) of the α-MSH-stimulated phosphorylation of p38 MAP kinase in B16 cells. Cells were incubated in the presence of α-MSH for the times shown in min and then analysed using Western blotting using an antibody specific for the dually phosphorylated forms of p38 MAP kinase (Thr^{180} and Tyr^{182}). The blot is representative of three separate experiments, and represents a single immunoblot. B: Re-probe of the blot from A, demonstrating equal protein loading. The blot from A was stripped and probed with an antibody specific for α-tubulin. C: α-MSH-stimulated phosphorylation of p38 MAP kinase in B16 murine melanoma cells. Cells were incubated in the presence of increasing concentrations of α-MSH (60 min) and analysed using Western blotting using an antibody specific for the dually phosphorylated forms of p38 MAP kinase (Thr^{180} and Tyr^{182}). The blot is representative of three separate experiments, and represents a single immunoblot. D: Effect of various kinase inhibitors on α-MSH-induced phosphorylation of p38 MAP kinase in B16 cells. Cells were incubated in the presence of α-MSH or absence (labelled control) for 60 min either with or without pre-incubation with PD 98059 (PD, 10 μM), LY 294002 (LY, 10 μM) and Ro 31-8220 (Ro, 2 μM) (all 30 min) then analysed using Western blotting using an antibody specific for the dually phosphorylated forms of p38 MAP kinase (Thr^{180} and Tyr^{182}). The blot is representative of three separate experiments and represents a single immunoblot. E: Time-course (in min) of the forskolin-stimulated phosphorylation of p38 MAP kinase in B16 cells. Cells were incubated in the presence of forskolin (10 μM) for the times shown in min and then analysed using Western blotting using an antibody specific for the dually phosphorylated forms of p38 MAP kinase (Thr^{180} and Tyr^{182}). The blot is representative of three separate experiments and represents a single immunoblot.

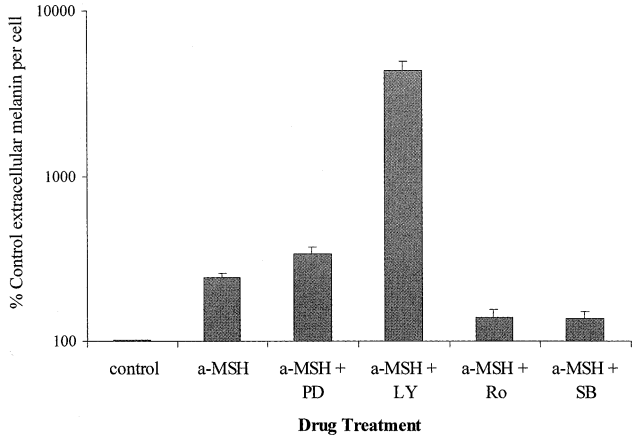

Fig. 2. Percentage increase in extracellular melanin production in response to α-MSH (10 nM) in B16 melanoma cells, in the absence and presence of PD-98059 (PD, 10 μM), LY 294002 (LY, 10 μM) Ro 31-8220 (Ro, 2 μM) or SB 203580 (SB, 10 μM). Increases in absorbance were measured spectrophotometrically (at 404 nm) and expressed as a percentage of basal melanin release per cell. Data show the mean ± S.E.M. of three experiments performed in duplicate.

31-8220, but not LY 294002, were shown to block the TPA-induced p44/42 MAP kinase activation.

Studies were undertaken to determine the involvement of p38 and p44/42 MAP kinases in the α-MSH-mediated stimulation of melanogenesis. Addition of increasing concentrations of α-MSH increased the amount of melanin detected in the extracellular media, as measured by spectrophotometry (Fig. 2). In the absence of α-MSH, absorbance levels were 0.25 ± 0.02 AU (wavelength: 404 nm), which increased to 0.54 ± 0.03 AU after a 72 h treatment with α-MSH (10 nM). After correction for cell number, α-MSH (10 nM) induced significant ($P < 0.05$) increases in extracellular melanin equivalent to 243 ± 17% of basal melanin production. Pre-treatment of cells with either the protein kinase C inhibitor, Ro 31-8220, or the p38 MAP kinase inhibitor, SB 203580, led to significant ($P < 0.05$) reductions in α-MSH-induced extracellular melanin accumulation to 140 ± 14% and 137 ± 13% of basal melanin release, respectively (Fig. 2). This was in contrast to pre-treatment of the cells with either PD 98059 or

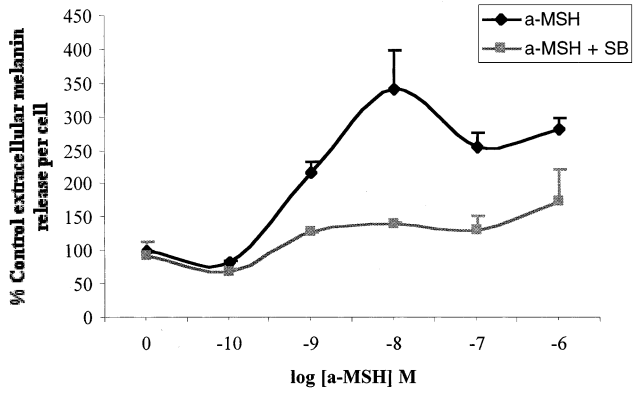

Fig. 3. Increases in extracellular melanin accumulation in response to increasing concentrations of α-MSH, in the absence (♦) and presence (■) of SB 203580 (10 μM). Increases in absorbance were measured spectrophotometrically (at 404 nm) and expressed as a percentage of basal melanin release per cell. Data show the mean ± S.E.M. of three experiments performed in duplicate.

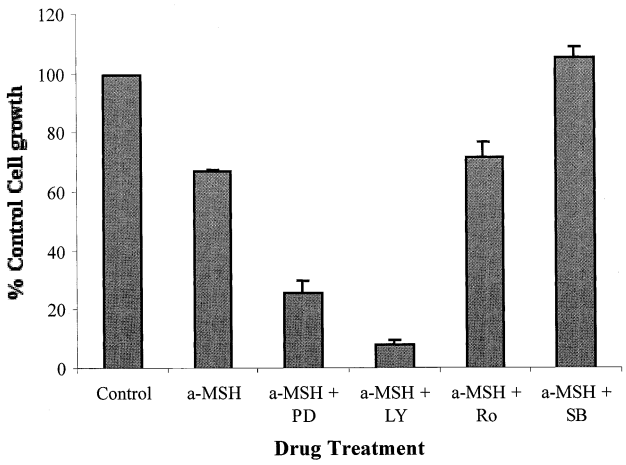

Fig. 4. The effects of α-MSH and various kinase inhibitors upon B16 melanoma cell growth. Data show cell growth after 72 h in untreated cells (Control), cells treated with α-MSH (10 nM) and cells in the continuous presence of either PD 98059 (PD, 10 μM), LY 294002 (LY, 10 μM), Ro-31 8220 (Ro, 2 μM) or SB 203580 (SB, 10 μM) followed by α-MSH treatment (10 nM). Cell numbers were expressed as a percentage of control cell growth. Data show mean ± S.E.M. of three experiments performed in duplicate.

LY-294002 which significantly ($P < 0.05$) increased levels of α-MSH-induced melanin production to 338 ± 36% and 4411 ± 558% of basal extracellular melanin levels per cell, respectively. In control studies it was found that administration of PD 98059 or LY 294002 alone significantly ($P < 0.05$) increased extracellular melanin production per cell to 418 ± 69% and 1288 ± 140% of basal values, respectively. However, incubation of cells with either Ro 31-8220 or SB 203580 alone did not significantly alter levels of extracellular melanin.

In order to investigate the role of p38 MAP kinase in the α-MSH-induced melanogenic response, concentration–effect curves to α-MSH were generated in the absence and presence of SB 203580 (10 μM). In the absence of SB 203580, α-MSH was highly potent at inducing melanin production (pEC_{50} of 9.20 ± 0.05) (Fig. 3), whereas in the continuous presence of SB 203580 the α-MSH-induced increases in extracellular melanin were significantly inhibited ($P < 0.05$).

The effects of α-MSH upon cell growth were examined by directly counting viable cells using a trypan blue exclusion protocol. In the absence of drug, incubation of cells for 72 h gave mean cell counts of $2.02 \pm 0.09 \times 10^6$ cells/well. Treatment of cells with α-MSH (10 nM) led to a significant ($P < 0.05$) reduction in cell numbers of 33.0 ± 1.0% relative to control values (Fig. 4). Pre-treatment of cells with PD 98059 (10 μM) or LY 294002 (10 μM), had inhibitory effects on cell growth that were more marked than α-MSH alone; reducing cell numbers by 74.3 ± 4.5% and 91.8 ± 1.4% of control values, respectively (Fig. 4). Other studies showed that administration of PD 98059 or LY-294002 alone led to a significant ($P < 0.05$) reduction in cell growth to 53.2 ± 2.3% and 90.2 ± 1.6% of control values, respectively. In contrast, treatment of the cells with the PKC inhibitor, Ro 31-8220, had no significant effect upon on the inhibition of growth mediated by α-MSH, but did induce a slight proliferative effect when applied alone equivalent to 127.3 ± 7.8% of basal growth. Pre-treatment of the cells with the p38 MAP kinase inhibitor, SB 203580 (10 μM), blocked the anti-proliferative effect seen in response to increasing concentrations of α-MSH.

Incubation of B16 cells in the continuous presence of increasing concentrations of α-MSH led to concentration-dependent inhibition of cell growth (Fig. 5). Maximal inhibition reduced cell numbers to 47.9 ± 4.1% of control cell growth (pIC_{50} of 8.22 ± 0.08) (Fig. 5). However in the continuous presence of SB 203580 (10 μM) the α-MSH-induced inhibition of B16 cell growth was abolished. It was further noted that incubation of cells with SB 203580 alone had no significant effects upon growth.

4. Discussion

Activation of the p38 and p44/42 MAP kinases has been shown to trigger cell differentiation [16,17,20]. Recent work has demonstrated that both forskolin and α-MSH activate p44/42 MAP kinase in B16 melanoma cells [21,22]. However, little is known about the ability of these agents to stimulate p38 MAP kinase. The aim of the present study was to investigate whether α-MSH activated p38 MAP kinase and whether the evoked activity of p38 and p44/42 MAP kinases were involved in B16 melanoma-induced melanogenesis or proliferation.

Administration of α-MSH induced a time-dependent increase in the phosphorylation of p38 MAP kinase, which was much slower in onset than that of p44/42 MAP kinase. Treatment of B16 cells with the adenylyl cyclase activator, forskolin, also induced a time-dependent increase in p38 MAP kinase activation, with a similar kinetic profile as that of α-MSH. Previous studies have demonstrated that forskolin can induce melanin production in B16 melanoma cells independently of G-protein activation [12]. This may suggest that the α-MSH-induced stimulation of p38 kinase is downstream of adenylyl cyclase activation. Pre-treatment of cells with inhibitors of MEK1 and PI 3-kinase were found to potentiate the α-MSH-induced stimulation of p38 MAP kinase, suggesting that there may be some feedback between PI 3-kinase/p44/42 MAP kinase inhibition and p38 MAP kinase activation.

A primary aim of this study was to determine the role of p38 and p44/42 MAP kinases in B16 cell differentiation. Upon differentiation, melanoma cells undergo morphological changes, and induce melanin production [8–10,12]. This study has utilised the accumulation of extracellular melanin as a marker of B16 differentiation. In common with previous work [7,19,28], it was demonstrated that administration of α-MSH induced significant levels of extracellular melanin in the media of B16 cells. Pre-treatment with the p38 inhibitor, SB 203580, abolished the α-MSH-induced increases in extracellular melanin accumulation, implicating the involvement of p38 in the α-MSH-induced melanogenic response. Melanogenesis is a multistage process involving melanin synthesis, melanosome transport to dendrite tips and melanosome release. As this study has looked at extracellular melanin accumulation only we have been unable to determine the stage(s) of melanogenesis which involve p38 MAP kinase activation. Pre-treatment of cells with inhibitors of either PI 3-kinase (LY 294002) or MEK1 (PD 98059) were found to potentiate the α-MSH-induced increases in extracellular melanin. Moreover, when cells were treated with either PD 98059 or LY 294002 alone, significant melanogenesis was induced, possibly indicating that these pathways were constitutively active and negatively regulating melanogenesis. These findings agree with previous studies that have also shown that inhibition of either the PI 3-kinase or p44/42 MAP kinase pathways induced melanin production in B16 melanoma cells [12,21].

The observed increases in α-MSH-stimulated extracellular melanin accumulation were found to be significantly inhibited following pre-treatment with the PKC inhibitor, Ro 31-8220. Differentiation of B16 melanoma cells, as measured by the α-MSH-induced melanogenic response has been shown previously to be PKC-dependent, in that both PKC downregulation via treatment with phorbol esters [23] and PKC inhibitors such as calphostin C, were found to block α-MSH-induced melanogenesis [24]. The concentration of Ro 31-8220 (2 μM) used in these studies were demonstrated to partially inhibit TPA-induced p44/42 MAP kinase phosphorylation. Concentrations of Ro 31-8220 in excess of 2 μM were not used as they were found to affect the ability of B16 cells to adhere to tissue culture plates (unpublished observations).

Treatment of B16 melanoma cells for 72 h with α-MSH led to a concentration-dependent inhibition of cell growth. Time-course studies revealed that cell growth was not significantly inhibited until after 48 h of α-MSH treatment (unpublished observations), suggesting that α-MSH treatment was not inducing apoptosis in this cell line. The actions of α-MSH upon melanoma growth are subject to controversy, with both proliferative [25,26] and anti-proliferative effects [27,28] being reported. It has been suggested that the anti-proliferative effects of α-MSH are a result of melanogenesis being induced, and the subsequent accumulation of toxic melanin by-products [25]. There is, however, evidence that other factors may be involved; in amelanotic Bomirski hamster melanoma cells, α-MSH inhibits cell growth independently of melanogenesis [26]. Furthermore, studies using B16 melanoma cells, have shown that the naturally occurring α-MSH antagonist, agouti protein, is able to antagonise melanogenesis as well as inhibiting cell growth in a similar manner as α-MSH [29]. Pre-treatment of B16 cells with SB 203580 in this study, abolished the anti-proliferative effect of α-MSH, and suggests for the first time that p38 MAP kinase may be involved in mediating this effect. Further evidence for the involvement of p38 MAP kinase in these processes comes from studies demonstrating that agents which induce B16 cell differentiation, such as for-

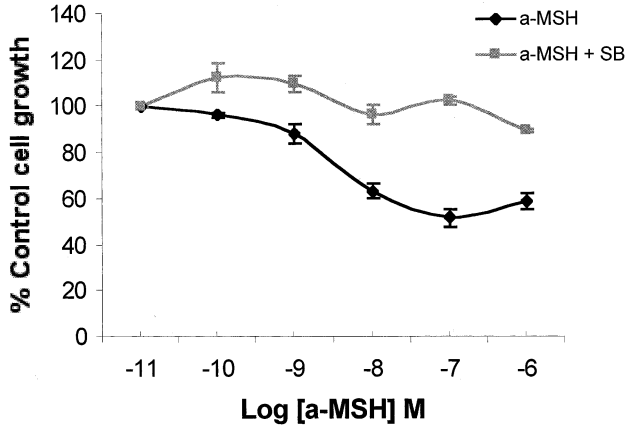

Fig. 5. Inhibition of B16 melanoma growth in response to increasing concentrations of α-MSH. Cells were incubated with increasing concentrations of α-MSH in the absence (♦) and presence (■) of SB 203580 (10 μM), for 72 h. Cell numbers were expressed as a percentage of control cell growth. Data shows mean ± S.E.M. of three experiments performed in duplicate.

skolin, LY 294002 and PD 98059 also activate p38 MAP kinase in these cells. Although p38 MAP kinase seems to be involved in both the anti-proliferative and melanogenic effects of α-MSH, a differential requirement seems to be needed for PKC. Treatment of cells with the PKC inhibitor, Ro 31-8220, was found to have no significant effect upon the inhibition of cell proliferation by α-MSH. This would suggest that a build-up of melanin or toxic products by these cells does not contribute significantly to an anti-proliferative function. However, studies on basal melanocyte growth have shown that PKC down-regulation (by phorbol ester treatment) is proliferative [11,30,31].

Treatment of cells with either PD 98059 or LY 294002 potentiated the α-MSH-mediated anti-proliferative effect. Indeed, when either agent was added alone there were marked inhibitory effects on cell growth; suggesting that these pathways may be constitutively active in melanoma cells.

Melanocyte differentiation and proliferation are believed to be opposing cellular phenomena. In melanocytes, differentiation induced by UV light is accompanied by G_1 cell-cycle arrest, via the expression of cyclin-dependent kinase inhibitor $p21^{Waf-1/SD-1/Cip-1}$ [9]. An intriguing finding of the present study is that the anti-proliferative activity of α-MSH correlated with p38 activation and not with that of p44/42 MAP kinases. Previous studies on 3T3-L1 [32] and ATDC5 cells [17] in which both p44/42 and p38 MAP kinase were simultaneously active showed that inhibition of p38 alone blocked differentiation. Moreover, in common with the present study, administration of PD 98059 alone was sufficient to induce differentiation in ATDC5 cells [17].

In summary, we have demonstrated for the first time that α-MSH activates p38 MAP kinase in B16 murine melanoma cells. Furthermore, activation of p38 MAP kinase by α-MSH may be important for both its anti-proliferative effects and the production of melanin.

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