α–Melanocyte-Stimulating Hormone Modulates Nitric Oxide Production in Melanocytes[1]

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We have previously observed that melanocytes produce nitric oxide in response to ultraviolet radiation and lipopolysaccharide and in this study have examined how these responses are affected by α–melanocyte-stimulating hormone. Nitric oxide production by cultured cells was measured electrochemically in real time using an ISO-nitric oxide sensor probe. B16 mouse melanoma cells released nitric oxide in response to lipopolysaccharide and the effects were enhanced in cells that had been grown in the presence of 10^{-11}–10^{-9} M α–melanocyte-stimulating hormone prior to stimulation. At concentrations in excess of 10^{-9} M α–melanocyte-stimulating hormone decreased nitric oxide production. Preincubation with lipopolysaccharide, a well-known inducer of inducible nitric oxide synthase, also increased nitric oxide production but this response was reduced by α–melanocyte-stimulating hormone. α–Melanocyte-stimulating hormone also increased the levels of nitric oxide produced in response to ultraviolet radiation (20–100 mJ per cm²) in B16 cells. The same effect was seen in human melanocytes and as this was inhibited by aminoguanidine would appear to involve an induction of inducible nitric oxide synthase. Reverse transcription–polymerase chain reaction showed that melanocytic cells express inducible nitric oxide synthase mRNA. Western blotting analysis and immunocytochemistry confirmed the presence of inducible nitric oxide synthase protein in B16 cells and FM55 human melanoma cells and that the levels were increased in response to α–melanocyte-stimulating hormone. α–Melanocyte-stimulating hormone, however, decreased inducible nitric oxide synthase protein expression, which occurred in response to lipopolysaccharide. These results suggest that α–melanocyte-stimulating hormone regulates nitric oxide production in melanocytic cells by modulating the induction of inducible nitric oxide synthase. Additional experiments showed that nitric oxide increased melanin production by B16 cells and human melanocytes. This is in keeping with a melanogenic role for nitric oxide but whether its production by melanocytes in response to α–melanocyte-stimulating hormone is associated with such a role or whether it has some other significance relating to melanocyte differentiation or in mediating immunomodulatory actions of α–melanocyte-stimulating hormone remains to be seen. *Key words: melanogenesis/nitric oxide synthase/ultraviolet radiation. J Invest Dermatol 114:520–526, 2000*

α–Melanocyte-stimulating hormone (α-MSH) is a tridecapeptide that is derived, together with numerous other peptides from the 31–36 kDa protein proopiomelanocortin (POMC) (Eipper and Mains, 1980). The main site of production of POMC is the

pituitary, although it is now recognized that α-MSH and other POMC derivatives are produced at other sites including the skin (Thody *et al*, 1983). In the skin α-MSH is found in keratinocytes (Slominski *et al*, 1993; Schauer *et al*, 1994; Chakraborty *et al*, 1995; Liu *et al*, 1995), although there are greater concentrations of the peptide in melanocytes (Lunec *et al*, 1990; Wakamatsu *et al*, 1997). The molar concentrations of α-MSH in human skin are several magnitudes higher than those in the circulation and this is consistent with the view that in human skin the peptide acts locally through paracrine and/or autocrine mechanisms.

α-MSH has numerous effects in the skin. For instance, it has been shown to stimulate sebaceous glands (for review see Thody and Shuster, 1989). It also interacts with different cytokines to have immunomodulatory and anti-inflammatory actions (Catania and Lipton, 1993; Bhardwaj and Luger, 1994). The peptide is, however, best known for its actions on melanocytes where it regulates melanogenesis through its activation of tyrosinase, the rate-limiting enzyme in the melanin pathway (Hunt *et al*, 1994; Abdel-Malek *et al*, 1995) and more specifically stimulates a

preferential increase in the synthesis of eumelanin (Burchill *et al*, 1986, 1993; Hunt *et al*, 1995). The MSH receptor that mediates these effects has been cloned and shown to be a member of a subfamily of the G protein-coupled receptors (Chhajlani and Wikberg, 1992; Mountjoy *et al*, 1992). Several subtypes have been characterized, and one, namely melanocortin-1 (MC-1) receptor, is functional in human melanocytes (Donatien *et al*, 1992; De Luca *et al*, 1993). Activation of this receptor also has other effects and in human melanocytes for instance, has been shown to increase dendricity and attachment to extracellular matrix proteins (Hunt *et al*, 1993, 1994).

We have recently observed that α-MSH protects melanocytes from oxidative stress and while we have proposed that this could involve an activation of tyrosinase (Valverde *et al*, 1996a, b), the precise mechanisms are still unclear. Compared with other cells in the skin, e.g., keratinocytes and fibroblasts, melanocytes are especially vulnerable to oxidative stress (Valverde *et al*, 1996c). The reasons for this are not known, but it could be related to their ability to generate nitric oxide (NO). This reactive molecule has many physiologic functions (for reviews see Ånggård, 1994; Bruch-Gerharz *et al*, 1998), but also has cell-damaging effects through its ability to react with the superoxide anion to produce the highly cytotoxic hydroxyl radical. In preliminary experiments we have observed that melanocytes and melanoma cells produce NO in response to ultraviolet radiation (UVR) and lipopolysaccharide (LPS). In this study we have extended these studies and now report that the production of NO by melanocytic cells is modulated by α-MSH.

MATERIALS AND METHODS

Cell culture Human epidermal melanocytes and keratinocytes were isolated from samples of skin obtained from children and young adults undergoing surgery and cultured in supplemented MCDB 153 medium (Sigma, Poole, U.K.) as previously described (Hunt and Thody, 1994). Human fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Paisley, U.K.) containing 10% fetal bovine serum (ICN Flow, Basingstoke, U.K.) and penicillin (50 IU per ml)/streptomycin (50 μg per ml) (ICN Flow). B16F1 mouse melanoma cells were maintained in DMEM (Gibco BRL) containing 10% fetal bovine serum (ICN Flow) and Penicillin (50 IU per ml)/streptomycin (50 μg per ml) (ICN Flow) in a humidified 5% CO_2 atmosphere as described previously (Thody *et al*, 1988). FM55 human melanoma cells were maintained in the same way except RPMI 1640 medium (Gibco BRL) was used in place of DMEM.

Experimental procedures Cells were seeded on to 24-well plates (Costar, High Wycombe, U.K.) at a density of 2×10^4 per well and incubated in the presence or absence of α-MSH (Bachem, Essex, U.K.) at concentrations ranging from 10^{-11} to 10^{-7} M for varying times up to 72 h. Controlled cultures were incubated in the absence of α-MSH. The cells were then stimulated with either UVR or LPS (60 ng per ml) to release NO, which was measured as described below. Prior to irradiation the culture medium was replaced by phosphate-buffered saline. The source of UVR was a Helarium 40 W lamp (Wolff B1.01) which emits predominantly in the UVB range. A preliminary experiment was carried out in order to determine the concentration of LPS required to stimulate NO release. The inducible nitric oxide synthase (iNOS) inhibitor aminoguanidine hydrochloride (Sigma) was used at a concentration of 25 mM; this was added at the same time as α-MSH.

The effect of NO on melanogenesis was examined in B16 melanoma cells. In these experiments the cells were seeded in six-well plates at a density of 2×10^5 per well and cultured in the presence of varying concentrations of the NO donor sodium nitroprusside (SNP) for 3 d and then harvested for the measurement of melanin content.

Measurement of NO NO production was measured in real-time using a commercially available ISO-NO sensor probe. The principle of the NO electrode (ISO-NO meter, World Precision Instruments Ltd, Sarasota, FL) is based on oxidation of NO to NO^+ at a platinum anode covered by a NO-selective membrane. The electrode was calibrated in a stirred solution of a 0.1 M KI, acidified with 0.1 M H_2SO_4 (Tsukahara *et al*, 1993; Brodenick and Taha, 1995). NO was generated in solution by adding a known volume of 1 M KNO_2. A peak current response of 350 pA at the NO electrode corresponded to approximately 75 nM NO. Measurements

were started immediately after beginning the UVR or after the addition of LPS and continued for up to 30 min.

Immunocytochemical staining of NO Cells were seeded on to a Lab-Tek chamber slide in DMEM, allowed to attach overnight at 37°C, then incubated for 6 h in the presence or absence of α-MSH (10^{-9} M) or LPS (60 ng per ml). The cells were washed three times in phosphate-buffered saline before fixing in 1% glutaraldehyde for 30 min and immunostained using polyclonal antibodies to iNOS (Affiniti Laboratories, Mamhead, U.K.) and a standard avidin–biotin horseradish peroxidase technique.

Western blot analysis Cell lysates were prepared by solubilizing cells in lysis buffer (2% sodium dodecyl sulfate, 10% glycerol, 100 mM dithiothreitol, 0.063 M Tris–HCl, 2 mM sodium pyrophosphate, 5 mM ethylenediamine tetraacetic acid). Protein concentrations were determined with an assay kit, based on the Lowry assay (BioRad, Hemel Hempstead, U.K.). Aliquots of the lysates containing 80 μg protein were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis at 100 mV for 2 h, and transferred to a nitrocellulose membrane (Amersham, Little Chalfont, U.K.). After transfer the gel was stained with Coomassie blue to verify even loading and transfer. The membranes were blocked in 0.05% Tween 20–Tris-buffered saline (150 mM NaCl, 20 mM Tris base, pH 7.4) containing 3% bovine serum albumin. iNOS was detected using a monoclonal mouse antibody (Affiniti Laboratories) diluted at 1:1000 in 0.05% Tween 20–Tris-buffered saline containing 1% bovine serum albumin and a horseradish peroxidase-tagged sheep antimouse IgG second antibody (Amersham) followed by autoradiography (Hyperfilm ECL, Amersham).

Reverse transcriptase–polymerase chain reaction (RT–PCR) Total RNA was extracted using Tri Reagent according to the supplier's instructions. For RT–PCR assays 1–5 μg total RNA was reverse transcribed using oligo dT as primers and Super Script II reverse transcriptase (Gibco BRL). The products were amplified using gene specific primers: (i) iNOS sense (5′ atgtgaggatcaaaaactggg 3′) and iNOS anti-sense (5′ cctggccagatgttcatctat 3′); (ii) brain nitric oxide synthase (bNOS) sense (5′ ggaatccaggtggacagaga 3′) and bNOS anti-sense (5′ gcatgatggagcccatgag 3′). For both iNOS and bNOS samples contained 10 pmol of each primer, 200 μM dNTP, 2 mM $MgCl_2$, 1 × PCR buffer, 1 U *Taq* Polymerase and 4 μl cDNA to a final volume of 50 μl. Samples were heated for 5 min at 94°C and then amplified for 40 cycles at 94°C, 60°C for 30 min each. After a final extension at 75°C for 30 min the PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide and photographed under UV.

Measurement of melanin Cells (5×10^5) were solubilized in 100 μl 1 M NaOH and diluted in 400 μl distilled water. Absorbency was compared with a standard curve of synthetic melanin (Sigma) prepared in a final NaOH concentration of 0.2 M.

RESULTS

α-MSH modulates NO production in LPS stimulated melanoma cells The addition of LPS to B16 cells produced immediate concentration-related increases in the release of NO. On the basis of these findings a concentration of 60 ng per ml was used in subsequent experiments in order to stimulate NO release. Pre-incubation with α-MSH for 3, 6, 24, or 72 h enhanced the levels of NO released by B16 cells in response to LPS and at all times in a concentration-related manner (**Fig 1**). The peak effect was seen at 24 h in response to 10^{-9} M α-MSH. The same concentration of α-MSH also produced the greatest response at most of the other times studied. With increasing concentrations of α-MSH the levels of NO decreased.

Similar increases in NO production were observed in FM55 human melanoma cells that had been grown in the presence of 10^{-10} M and 10^{-9} M α-MSH for 6 or 24 h (**Table I**). Higher concentrations of α-MSH were not examined in the human cell line.

α-MSH and LPS are mutually antagonistic in their effects on NO production in melanoma cells The above experiments demonstrate that α-MSH modulates the production of NO in melanocytic cells. There are reports that in other cells, e.g., macrophages α-MSH acts to inhibit LPS-induced NO production
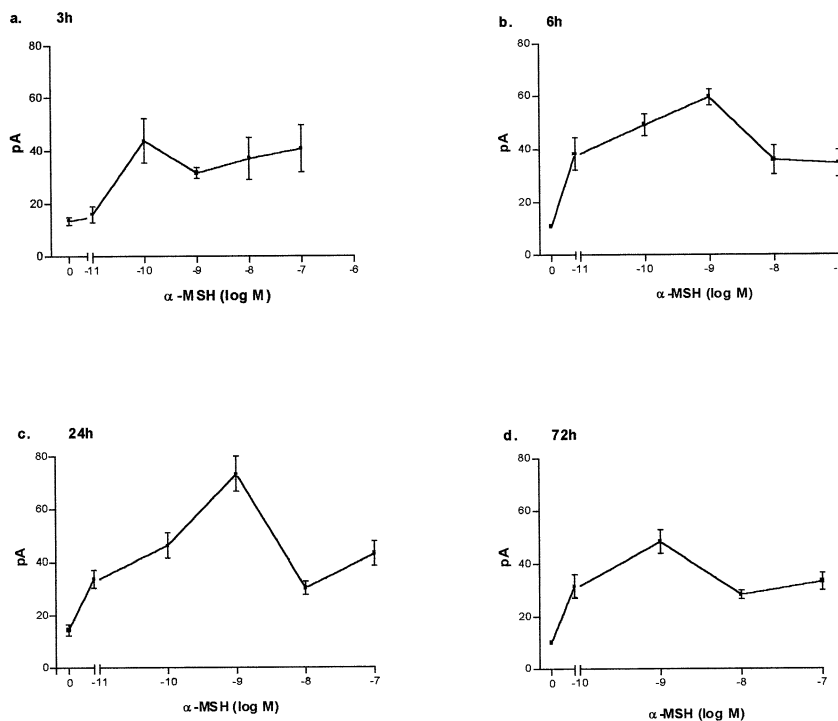
Figure 1. Effect of α–MSH on NO production in LPS-stimulated B16 cells. Current (pA) is proportional to NO produced. α–MSH was added to the cells at (*a*) 3 h, (*b*) 6 h, (*c*) 24 h, and (*d*) 72 h prior to stimulation with LPS. The results are expressed as mean ± SEM of at least three determinations. At all times the peak current was significantly greater than the control p < 0.01.

Table I. Effect of α–MSH, alone and in the presence of LPS, on the production of NO in B16 mouse and FM55 human melanoma cells[a]

	α–MSH		
	0	10^{-10} M	10^{-9} M
B16			
6 hours			
control	10.8 ± 0.4	49.1 ± 3.6*	59.5 ± 2.8*
LPS 60 ng per ml	28.0 ± 6.0*	49.6 ± 4.2	67.5 ± 8.7
24 hours			
control	15.0 ± 2.2	46.3 ± 4.5*	73.3 ± 6.2*
LPS 60 ng per ml	30.0 ± 2.5*	35.0 ± 2.3	47.0 ± 4.2**
FM55			
6 hours			
control	23.3 ± 6.6	55.0 ± 5.0*	65.0 ± 15.0*
LPS 60 ng per ml	90.0 ± 8.3*	40.0 ± 10.0*	35.0 ± 5.0*
24 hours			
control	26.6 ± 3.3	43.3 ± 8.8*	75.0 ± 5.0
LPS 60 ng per ml	95.0 ± 11.9*	40.0 ± 10.0	20.0 ± 10.0

[a]NO was measured as described in the *Materials and Methods*. The results are shown as peak current and are the mean of at least three determinations ± SEM. *p < 0.01 *vs* control cells; **p < 0.01 *vs* cells stimulated with α-MSH.
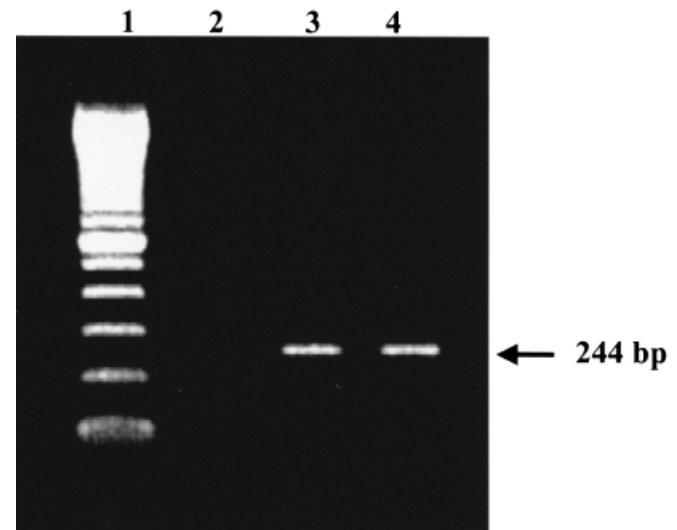

Figure 2. Expression of iNOS mRNA in unstimulated melanoma cells. Molecular weight marker (*lane 1*), negative control (*lane 2*), FM55 human melanoma cells (*lane 3*), B16 mouse melanoma cells (*lane 4*).

Expression of iNOS Using RT–PCR, iNOS mRNA was detected in B16 and FM55 cells (**Fig 2**). In preliminary experiments iNOS was also detected in human melanocytes.

Protein lysates from B16 and FM55 cells were found to contain iNOS protein and the levels were higher in cells that had been exposed to α–MSH for 6 or 24 h. Cells that had been exposed to LPS for the same periods of time showed similar enhanced expression of iNOS. Cells that had been exposed to LPS and α–MSH together, however, had similar or lower levels of iNOS compared with those exposed to LPS alone. This was particularly obvious in FM55 cells (**Fig 3**).

Immunostaining confirmed the presence of iNOS in B16 melanoma cells. The staining which was cytoplasmic and generally perinuclear was seen in control cells and was increased in response to α–MSH or LPS. The intensity of staining varied from cell to cell

(Star *et al*, 1995). In order to see whether α–MSH acts in the same way in cells of melanocyte lineage experiments were carried out using B16 and FM55 cells. As before cells grown in the presence of 10^{-10} M and 10^{-9} M α–MSH showed increased production of NO. Preincubation with LPS (60 ng per ml) for 6 h and 24 h also increased the levels of NO but in the presence of α–MSH this effect was reduced (**Table I**). In the FM55 cells the levels of NO produced in response to α–MSH and LPS together were actually lower than those from cells grown in the presence of α–MSH or LPS alone (**Table I**). This suggests that α–MSH and LPS are mutually antagonistic in their effects on NO production in B16 and FM55 cells.
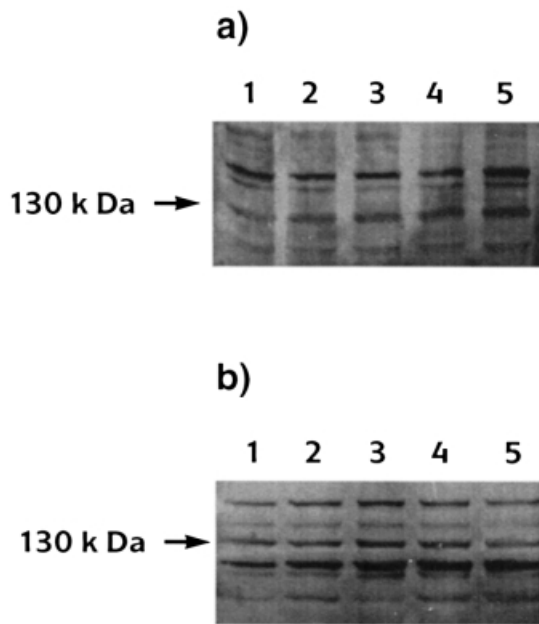
a)

b)

Figure 3. Expression of iNOS protein in stimulated and unstimulated B16 and FM55 melanoma cells. (*a*) B16 melanoma cells; (*b*) FM55 melanoma cells. Cells were incubated with α-MSH 10^{-9} M for 6 h (*lane 2*), 24 h (*lane 3*), with LPS 60 ng per ml for 6 h (*lane 4*), with α-MSH 10^{-9} M and LPS 60 ng per ml together for 6 h (*lane 5*). Control cells are shown in *lane 1*.

with some cells showing a particularly strong reaction, especially in response to α-MSH (**Fig 4**).

RT–PCR also revealed the presence of bNOS mRNA in melanoma cells and human melanocytes but no protein could be detected by western analysis (results not shown).

NO increases melanin production   NO has been shown to increase melanin production (Roméro-Graillet *et al*, 1997). In order to examine this B16 cells and human melanocytes were grown in the presence of SNP, a NO donor, for 3 d. SNP produced concentration–related increases in the melanin content of the B16 cells and human melanocytes (**Table II**). Higher levels of melanin were found in human melanocytes and as in the B16 cells these were increased in response to SNP (**Table II**). In the presence of SNP the numbers of B16 and human melanocytes were lower than those of controls (**Table II**), but greater than the numbers initially seeded, which would suggest a cytostatic effect of NO. At concentrations in excess of 150 μM SNP was cytotoxic to B16 cells and human melanocytes.

α-MSH increases NO production in UV-irradiated cells   As NO may serve as a mediator of melanogenesis it is important to establish whether UVR, which is an important stimulator of melanogenesis, is capable of increasing its production by melanocytes and whether this is affected by α-MSH.

As shown in **Fig 5**, UVR, over the dose range of 20–100 mJ per cm^2, increased the levels of NO released from B16 cells and the responses were enhanced in cells that had been grown in the presence of α-MSH for 24 h. Similar results were obtained with human melanocytes (**Fig 6**). For comparison similar experiments were carried out using human keratinocytes and fibroblasts. UVR produced small in the levels of NO release from those cells but in both cases the responses were unaffected by α-MSH (only the results for keratinocytes are shown; see **Fig 6**).

α-MSH had no effect on NO release when added immediately prior to UVR and under these conditions failed to potentiate UVR–induced NO production, suggesting that its effect involved

an induction, rather than a stimulation of NOS. Further experiments using aminoguanidine hydrochloride, a specific inhibitor of iNOS, confirmed that the increase of NO production by α-MSH in human melanocytes was mediated via iNOS (**Fig 7**). Thus as before α-MSH significantly increased NO production when added 24 h prior to UV stimulation; however, in the presence of aminoguanidine hydrochloride this effect was blocked (**Fig 7**).

DISCUSSION

NO is produced from L-arginine through the action of the enzyme NOS. Several isoforms of the enzyme exist and the most important are the constitutive forms (cNOS) which are present in endothelial cells and neurons and iNOS, which is found in a variety of cells, including macrophages and neutrophils. The latter is not normally expressed, but is induced by inflammatory cytokines and bacterial LPS. There is evidence of cNOS and iNOS in the skin with the former being present in keratinocytes (Baudouin and Tachon, 1996) and the latter in Langerhans cells (Marletta, 1994; Nathan and Xie, 1994; Qureshi *et al*, 1996).

In this study we have demonstrated that melanocytes are capable of producing NO and this implies that these cells express NOS. This effect was seen in response to UVR and this confirms our earlier findings.[3,4] The increase in NO in response to UVR was immediate and this suggests an activation of constitutively expressed NOS. In this study we detected bNOS mRNA in melanocytic cells but found no evidence of this constitutive form of NOS at the protein level.

The iNOS was, however, detected in these cells at both the RNA and protein levels. Moreover, expression was seen in unstimulated cells and this would suggest that contrary to the situation in most cell types, iNOS may be constitutively expressed in melanocytic cells. It should be mentioned, however, that whereas the immunoreactive iNOS in FM55 cells had a molecular size of 130 kDa which is comparable with that found in other cells, our results would suggest that a slightly smaller form of iNOS exists in B16 cells.

In this study we confirmed that α-MSH modulates the production of NO by melanocytic cells and our results would suggest that the actions of α-MSH are mediated by iNOS. This was supported by our findings that the effect of α-MSH was blocked by aminoguanidine, a weak, but selective inhibitor of iNOS. The increase in NO production in response to α-MSH was maximal at 24 h and this is consistent with an induction process.

Further confirmation that α-MSH was acting by modulating the induction of iNOS came from the immunocytochemical experiments and western blot analyses.

These results further demonstrate that α-MSH has two effects on NO production and these appear to be concentration dependent. Thus at concentrations up to 10^{-9} M α-MSH produced dose related increases in NO production but at higher concentrations of the peptide the production of NO decreased. Why α-MSH has these two opposite effects is not clear but this is reminiscent of many α-MSH actions, e.g., melanogenesis (Hunt *et al*, 1994) and is consistent with the view that α-MSH functions as a modulatory peptide.

It has been reported that α-MSH is able to affect the induction of iNOS in other cells, e.g., macrophages (Star *et al*, 1995). Star *et al* observed that α-MSH inhibited the induction of iNOS which occurred with IFN-γ or LPS. To see whether α-MSH could have a similar inhibitory action in pigment cells we examined its effects in the presence of LPS. Our results show that under these conditions α-MSH has an inhibitory effect and blocks the stimulatory action

[3]Valverde P, Manning P, Graham A., McNeil CJ, Thody AJ: Melanocytes produce superoxide anion and nitric oxide in response to low doses of ultraviolet radiation. *J Invest Dermatol* 107:509, 1996 (abstr.)

[4]Graham A, Manning P, Atif U, McNeil CJ, Thody AJ: α-MSH induces nitric oxide production in melanocytes. *Pigment Cell Res* 10:327, 1997 (abstr.)
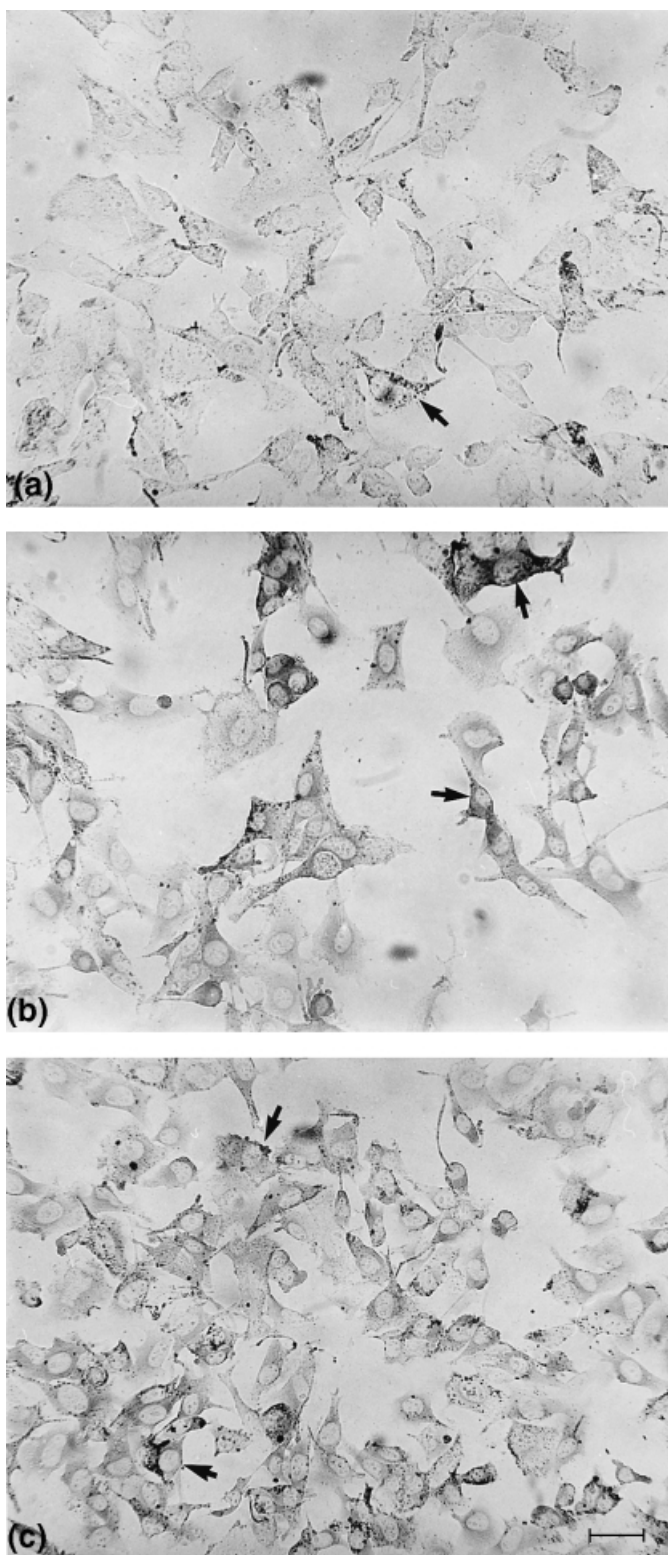
Figure 4. Immunocytochemical staining of iNOS in B16 cells. B16 cells were stained with iNOS, after incubation for 6 h with α-MSH 10^{-9} M (*b*) and LPS 60 ng per ml (*c*). Control cells are shown in (*a*). *Arrows* indicate strongly positive cells. *Scale bar:* 10 μm

Table II. Effect of NO on melanin synthesis in B16 melanoma cells and human melanocytes

SNP (μM)	Number of viable cells	Melanin content (μg 10^6 cells^{-1})
B16		
0	4.5 ± 0.33 ($\times 10^6$)	2.81 ± 0.28
100	1.52 ± 0.11 ($\times 10^6$)	5.49 ± 0.26
125	0.68 ± 0.05 ($\times 10^6$)	8.89 ± 0.98
150	0.25 ± 0.06 ($\times 10^6$)	13.87
Human melanocytes		
0	14.36 ± 1.39 ($\times 10^4$)	54.8 ± 3.8
100	10.10 ± 0.98 ($\times 10^4$)	85.0 ± 6.1
125	4.16 ± 0.98 ($\times 10^4$)	193.8

^{a}Melanin content was assayed as described in *Materials and Methods*. Cell numbers were counted using the Trypan blue exclusion assay. Results are expressed as mean ± SEM for three experiments, except for the melanin contents in response to 150 μM SNP in B16 cells and 125 μM in human melanocytes, where the results are the mean of two experiments. *p < 0.05, **p < 0.01 *versus* melanin content in the absence of SNP.
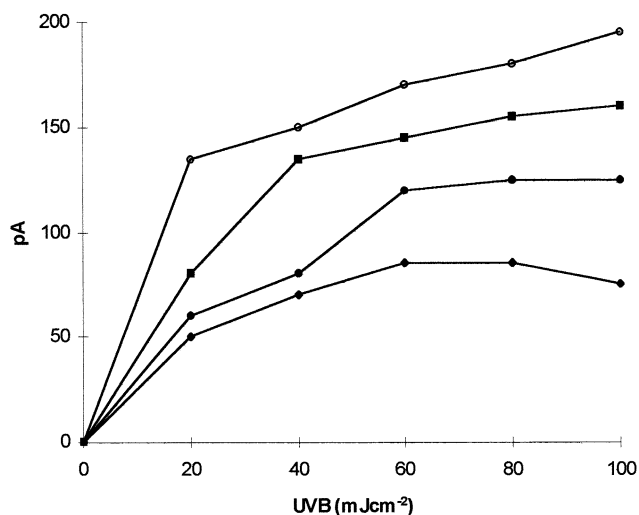

Figure 5. Effect of α-MSH on NO production in UV-stimulated B16 cells. ◇; control; ●, 10^{-9} M α-MSH; ■, 10^{-8} M α-MSH; ○, 10^{-7} M α-MSH. Current (pA) is proportional to NO produced. α-MSH was added to the cells 24 h prior to UV irradiation. This experiment was carried out three times with similar results. A representative experiment is shown.
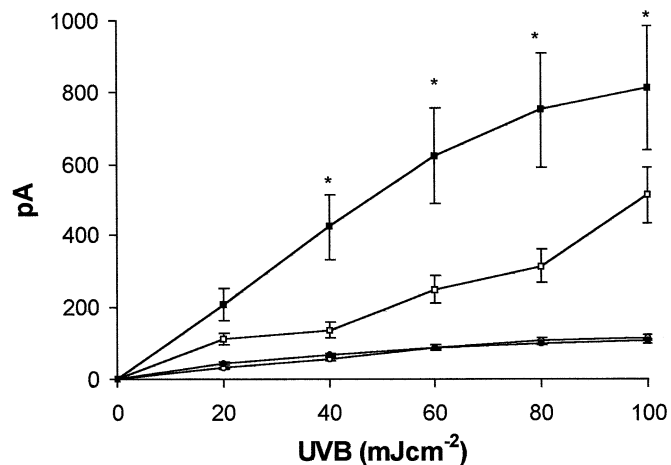

Figure 6. Effect of α-MSH on NO production in UV-stimulated human melanocytes. □, melanocytes; ■, melanocytes + α-MSH. Current is proportional to the NO produced. α-MSH 10^{-8} M was added to the cells 24 h prior to UV irradiation. The results are expressed as the mean ± SEM of three determinations. *p < 0.01 *versus* NO production by melanocytes alone. The effects in keratinocytes are shown for comparison ○, keratinocytes; ●, keratinocytes + α-MSH.

of LPS on NO production. Our results, however, indicate that in B16 cells LPS also inhibits the action of α-MSH. It would therefore appear that α-MSH and LPS are mutually antagonistic in their effects on NO production in B16 cells. How this occurs is not yet clear but it would appear that α-MSH has different effects on NO
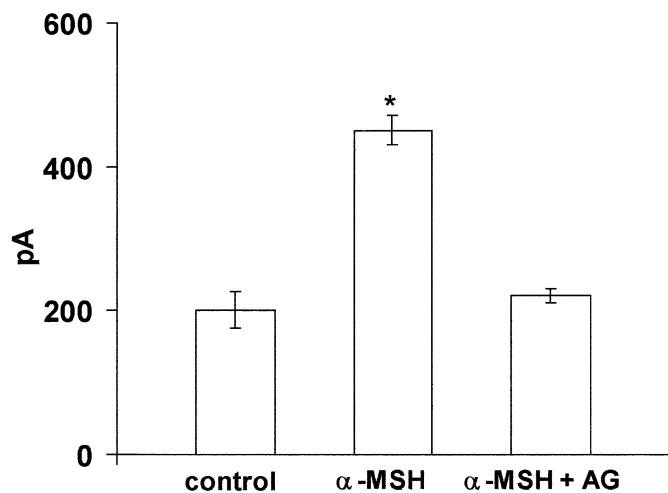
Figure 7. Effect of iNOS inhibitor on NO production by human melanocytes. The iNOS inhibitor aminoguanidine hydrochloride (AG) 25 mM, was added to the cells along with α–MSH 10^{-8} M 24 h prior to UV irradiation. Current (pA) is proportional to NO produced. The results are shown as the peak current and are the mean ± SEM of three determinations. *$p < 0.005$ *versus* control.

production depending upon whether it is present on its own or whether other inducers of iNOS are also present.

How α–MSH acts to affect the induction of iNOS in melanocytes is not clear. It seems reasonable to suppose that the action is mediated by the melanocortin-1 (MC-1) receptor, which is expressed by melanocytes and other cell types, including macrophages (Luger *et al*, 1997). This receptor is coupled to the cyclic adenosine monophosphate signaling pathway and there is evidence that activation of this pathway increases NO production in macrophages and endothelial cells (Bulut *et al*, 1993). There is evidence that the MC-1 receptor couples to other signaling pathways and these could also be involved in mediating the actions of α–MSH on iNOS. An example could be the NFκB pathway as there is recent evidence that this pathway is affected by α–MSH (Haycock *et al*, 1999). We are, however, unable to rule out the possibility that α–MSH affects iNOS through a mechanism independent of the MC-1 receptor. There is, for instance, evidence that α–MSH can act intracellularly to regulate melanogenesis through interactions with tetrahydrobiopterin (Schallreuter *et al*, 1997). Tetrahydrobiopterin is a cofactor for iNOS and, if as suggested, α–MSH complexes with this pterin then this could have the effect of reducing iNOS activity and hence NO production.

It is still not clear why melanocytes produce NO. Roméro-Graillet *et al* (1997) have shown that NO stimulates melanin production and as it is released from keratinocytes in response to UVR suggested that it could serve as a paracrine factor in UVR-induced melanogenesis. Our results confirm that keratinocytes produce NO in response to UV and that NO is capable of stimulating melanin production. As melanocytes are capable of producing NO, and apparently in greater amounts than keratinocytes, however, it seems reasonable to suppose that the molecule acts as an autocrine factor to regulate melanogenesis and, furthermore, may mediate the melanogenic actions of α–MSH.

An alternative possibility is that the NO produced by melanocytes serves as a second messenger in regulating their differentiation analogous to the situation in neuroneal cells where the induction of NOS by nerve growth factor has been shown to cause growth arrest and differentiation (Peunova and Enikolopov, 1995). α–MSH has similar effects in melanocytes (Eberle, 1988) and it is possible that these effects are mediated by NO.

It is generally considered that whereas NO has a role in cell signaling, the larger amounts that are generated following the induction of iNOS are involved in host defense (for reviews see

Änggård, 1994 and Bruch-Gerharz *et al*, 1998). Such a role is evident in macrophages where the released NO contributes to the phagocytic process through its antimicrobial and cytotoxic actions. The NO produced by melanocytes could have a similar role as melanocytes are capable of phagocytosis (Le Poole *et al*, 1993). There is evidence that NO has other actions in the immune system (see Bruch-Gerharz *et al*, 1998) and its production by melanoma cells has been associated with a suppression of tumorigenesis and metastasis (Xie *et al*, 1995). Whether the NO which is produced by normal melanocytes acts to regulate immune responses is not known. In this respect, however, it is worth recalling that α–MSH has many immunomodulatory actions (Catania and Lipton, 1993; Bhardwaj and Luger, 1994) and it is tempting to suggest that at least some of these are mediated by NO released from melanocytes.

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