α-Melanocyte-stimulating Hormone Reduces Impact of Proinflammatory Cytokine and Peroxide-generated Oxidative Stress on Keratinocyte and Melanoma Cell Lines*

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We have previously shown that α-melanocyte-stimulating hormone (α-MSH) can oppose tumor necrosis factor α activation of NF-κB (1–2 h) and intercellular adhesion molecule 1 up-regulation (mRNA by 3 h and protein by 24 h) in melanocytes and melanoma cells. The present study reports on the ability of four MSH peptides to control intracellular peroxide levels and glutathione peroxidase (GPx) activity in pigmented and nonpigmentary cells. In human HBL melanoma and HaCaT keratinocytes tumor necrosis factor α and H2O2 both activated GPx in a time- and concentration-dependent manner (by 30–45 min). α-MSH peptides were found to inhibit the stimulated GPx activity and had biphasic dose-response curves. MSH 1–13 and MSH [Nle3,α-Phe7] achieved maximum inhibition at 10–10 and 10 M respectively. Higher concentrations (10–100 fold) of MSH 4–10 and MSH 11–13 were required to produce equivalent levels of inhibition. α-MSH was also capable of reducing peroxide accumulation within 15 min, and again this inhibition was biphasic. The data support a role of α-MSH in acute protection of cells to oxidative/cytokine action that precedes NF-κB and GPx activation. The rapidity and potency of the response to α-MSH in pigmented and nonpigmentary cells suggest this to be a central role of this peptide in cutaneous cells.

α-Melanocyte-stimulating hormone (α-MSH)1 is a 13-amino acid peptide that arises by proteolytic processing of the proopiomelanocortin precursor molecule and is produced in several vertebrate tissues, including the pituitary, gut, and skin (1, 2). It is best known for its role in the control of melanogenesis in vertebrate tissues, including the pituitary, gut, and skin (1, 2). However, more recent work has demonstrated potent and broad acting roles as an antipyretic, anti-inflammatory, and immunomodulatory peptide (2–7). The action of α-MSH is transmitted via a family of specific MC G-protein receptors. Five different MC receptors (MC-1R to MC-5R) have been cloned and are located on pigmentary cells (e.g. melanocytes and melanoma cells) but also on several dissimilar nonpigmentary cell types (e.g. monocytes, keratinocytes, fibroblasts, endothelial cells, neural cells, and adipocytes) (8).

The mechanism by which α-MSH acts as an anti-inflammatory peptide is not completely understood, but a number of studies suggest inhibition of proinflammatory cytokine production/action (e.g. TNF-α and IL-1 (5)) or an increase in the synthesis of anti-inflammatory cytokines (e.g. IL-10 (9)). Consistent with these reports, our group has previously suggested α-MSH to have a role in cutaneous immunomodulation. Thus α-MSH was demonstrated to inhibit the ability of the proinflammatory cytokine TNF-α to up-regulate ICAM-1 expression on melanocytes and melanoma cells (10, 11). Surface ICAM-1 expression is a necessary requirement for T-lymphocyte binding to target cells. It is known that melanoma cells are able to produce α-MSH/ACTH, and therefore it is possible that α-MSH may prevent immune system recognition of melanoma cells (10, 11). α-MSH autocrine control may similarly extend to regulating inflammation in skin (e.g. following UV exposure) and may include autocrine/paracrine regulation on other cutaneous cells, such as keratinocytes and Langerhans cells in addition to melanocytes.

In investigating the mechanism of action of α-MSH inhibition of TNF-α-stimulated up-regulation of ICAM-1 (which was evident after a 24-h exposure to TNF-α and α-MSH), we have recently reported that α-MSH acts as a potent inhibitor of TNF-α-stimulated NF-κB transcription factor in melanoma cells and melanocytes (12). NF-κB is responsible for expression of several inflammatory and immune system genes, and hence inhibition of its activity by α-MSH is likely to be one important signaling pathway by which α-MSH exhibits anti-inflammatory behavior. Maximum inhibition of NF-κB activity by α-MSH occurred after 2 h. The rapid activation of NF-κB is triggered by several stimuli, in particular, cytokines, lipopolysaccharides, and UV light, but also by ROS or ‘oxidative stress’ (13–15). Previous studies have suggested that the transient intracellular generation of ROS, especially as peroxide, has the potential to act as second signaling messengers although the precise identity of a particular ROS species remains to be identified (15). TNF-α signaling is known to generate intracellular peroxide species because of “leakage” of electrons via complexes I and III of the mitochondrial electron respiratory chain. Release of superoxide anions and rapid dismutation...
to peroxide by manganese-superoxide dismutase is thought to occur, and a number of studies suggest that this oxidative generation is an important step in the TNF-α postreceptor signaling pathway (16–18). Levels of intracellular peroxide are controlled by the GPx family of antioxidant enzymes (19). Cellular, extracellular and a phospholipid hydroperoxide GPx isoforms exist, and each contains a seleno-cysteine catalytic center. Together, these have the ability to metabolize H$_2$O$_2$ and organic and lipid peroxide species (catalase is also able to scavenge hydrogen peroxide but is present at lower levels than GPx in most cell types). Accordingly, GPx is thought to be of primary importance in ROS metabolism in most tissues and has therefore been suggested to have a role in controlling transcription factor activity and gene expression by regulating the availability of those peroxide derivatives reported to have second messenger potential (15).

The aim of the present study was to investigate the mechanism of how α-MSH assists cells to resist pro-inflammatory cytokines and oxidative stress. We examined TNF-α and hydrogen peroxide activation of GPx and generation of intracellular peroxide (as GPx substrate) in a cutaneous melanoma cell line and a keratinocyte cell line, investigating to what extent α-MSH is able to oppose these early events in the response of cells to proinflammatory and oxidative species.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**HBL is a human cutaneous melanoma cell line established in one of our laboratories (20). Cells were cultured in Ham P-10 (Life Technologies, Inc.) supplemented with 5% fetal calf serum, 5% new born calf serum (Advanced Protein Products, West Midlands, UK), 2 mM t-glutamine (Life Technologies, Inc.), 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate. HaCaT is a human keratinocyte cell line obtained as a generous gift from Professor N. E. Fusieng (Institute of Biochemistry, German Cancer Research Center, Heidelberg, Germany). Cells were cultured in Dulbecco’s modified Eagle medium (Sigma, Poole, UK), supplemented with 5% fetal calf serum and 2 mM l-glutamine. Cells were incubated in a humidified atmosphere of 5% CO$_2$ and 95% air at 37 °C.

**Measurement of Glutathione Peroxidase—**Melanoma cells and keratinocytes were seeded into T75 flasks at 2 × 10$^6$ cells/flask and grown to ∼80% confluence in 6-well plates (50 μM K$_2$HPO$_4$, KH$_2$PO$_4$, pH 7.0, and placed on ice. Cell lysis was obtained by repeated needle aspiration, and the membrane fraction was then removed by centrifugation at 11,000 × g for 5 min. 20 μl of supernatant was used for each assay. Test mixtures for the determination of GPx contained 1 mM GSH, 0.15 mM β-NADPH, 0.24 units/ml glutathione reductase in 0.1 M K$_2$HPO$_4$, KH$_2$PO$_4$, pH 7.0, 1.0 mM EDTA. Reactions were started by the addition of t-butyl hydroperoxide (0.12 mM final concentration), and oxidation of β-NADPH was followed for 5 min at 340 nm using a Labsystems iEMS 96-well plate reader spectrophotometer at 25 °C. Enzyme activities were calculated from the linear part of the kinetic curve. One unit was defined as the amount of enzyme oxidizing 0.5 μM of β-NADPH (corresponding to 1 μM of GSH)/min.

**Measurement of Intracellular Peroxide Accumulation—**To assess levels of intracellular peroxide formation, flow cytometric analysis was carried out using an oxygen-sensitive fluorescent probe DCFH-DA (λ$_{ex}$ = 488, λ$_{em}$ = 510) (22). Briefly, cells (HBL or HaCaT) were cultured to ∼60% confluence in 6-well plates (∼5 × 10$^6$ cells). Cell medium was removed, and cells were washed with PBS (without Ca$^{2+}$ or Mg$^{2+}$ ions) two times, and then PBS (without Ca$^{2+}$ or Mg$^{2+}$ ions) containing 5 μM DCFH-DA was added to the cells for 10 min. DCFH-DA was removed, and cells were washed thoroughly with PBS (without Ca$^{2+}$ or Mg$^{2+}$ ions) five times. Medium was added back to the cells for experimenta-

**RESULTS**

**TNF-α and Hydrogen Peroxide Activation of Total GPx—**In both HBL cutaneous melanoma and HaCaT keratinocyte cell lines the addition of the proinflammatory cytokine TNF-α caused a transient increase in GPx enzyme activity in comparison with untreated cells. A very similar increase in GPx activity was observed when cells were stimulated exogenously with hydrogen peroxide, and increases in activity were found to be time- and concentration-dependent. A maximum increase in enzyme activity was identified after a 30-min incubation period for HBL cutaneous melanoma cells, when stimulated with either TNF-α (Fig. 1a) or hydrogen peroxide (Fig. 1b). GPx activity increased 2.2-fold over control levels after 30 min of stimulation with 400 units/ml TNF-α (Fig. 1a, p = 0.001, n = 3), in comparison with increases of 1.9-fold after a 15-min incubation (p = 0.008, n = 3) and 1.6-fold after a 45-min incubation (p = 0.04, n = 3). When HBL cells were stimulated with 600 μmol/liter hydrogen peroxide, a maximum GPx increase of 2-fold over control levels was observed (Fig. 1b, p = 0.019, n = 3), in comparison with 1.1-fold after 15 min (p = 0.68, n = 3) and 1.6-fold after 45 min (p = 0.05, n = 3). However, GPx activity was observed to be maximum (3.3-fold over control levels) in the HaCaT keratinocyte cell line after a 45-min incubation with TNF-α (400 units/ml) (Fig. 1a, p = 0.001, n = 3). In this cell line, activity was less after a 30-min (1.7-fold, p = 0.100, n = 3) and 60-min incubation (0.81-fold, p = 0.46, n = 3). Similarly, when HaCaT cells were stimulated with hydrogen peroxide (300 μmol/liter) a maximum increase in GPx activity of 3-fold was measured after a 45-min incubation (Fig. 1b, p = 0.010, n = 3). Activity was also found to be lower after 30-min (1.7-fold, p = 0.100, n = 3) and 60-min incubation periods (0.81-fold, p = 0.456, n = 3), respectively.

Using the incubation times where maximum GPx activity was achieved, a concentration-dependent increase in activity was observed when cells were stimulated with TNF-α or hydrogen peroxide. This was maximum for HBL cells when stimulated with 900 μM hydrogen peroxide (Fig. 1c, 2.8-fold, p = 0.01, n = 3) or 400 units/ml TNF-α (Fig. 1d, 2.3-fold, p = 0.0009, n = 3). Maximum GPx activity was measured when HaCaT cells were stimulated with 1200 μM hydrogen peroxide (Fig. 1c, 6.25-fold, p = 0.001, n = 3) or 800 units/ml TNF-α (Fig. 1d, 2.3-fold, p = 0.0008, n = 3). A significant increase in GPx activity of 2-fold was observed for HBL cells when using 400 units/ml TNF-α or 600 μM hydrogen peroxide and 400 units/ml TNF-α or 300 μM hydrogen peroxide for HaCaT cells. These respective concentrations of TNF-α and hydrogen peroxide were used for all subsequent investigations.

**Effect of α-MSH and Related Peptides on TNF-α and Hydrogen Peroxide Stimulation of Total GPx—**We investigated the ability of four α-MSH peptides to inhibit TNF-α or hydrogen peroxide.
peroxide-stimulated increases in GPx activity, using the respective incubation times determined for each cell type. The four MSH peptides investigated were: (i) α-MSH 1–13, (ii) α-MSH 4–10, (iii) α-MSH [Nle4-D-Phe7], and (iv) α-MSH 11–13. Cells were preincubated for 15 min with the MSH peptides prior to the addition of either TNF-α or hydrogen peroxide. MSH peptides alone did not affect unstimulated cell GPx activity (data not shown), but they did inhibit GPx enzyme activity stimulated by TNF-α or hydrogen peroxide. In general terms, the ability of the MSH peptides to inhibit enzyme activity was similar irrespective of whether the cells were stimulated with TNF-α or hydrogen peroxide. However, the two cell types differed slightly in their response to the peptides α-MSH 4–10 and α-MSH 11–13. The inhibitory data for all MSH peptides are shown in Fig. 2.

GPx enzyme activity stimulated by TNF-α in the human HBL melanoma cell line was inhibited by α-MSH 1–13 in a slightly biphasic manner (Fig. 2a). The most effective inhibitory concentration was 10⁻¹⁰ M (84% inhibition, p < 0.0001, n = 3), and it was less effective at 10⁻¹¹ M (51% inhibition, p = 0.0001, n = 3), 10⁻⁹ M (79% inhibition, p < 0.0001, n = 3), and 10⁻⁸ M (79% inhibition, p = 0.0001, n = 3). A similar biphasic inhibitory profile for this peptide was also observed when cells were stimulated with hydrogen peroxide (Fig. 2b), with maximum inhibition observed at 10⁻¹² M (83%, p = 0.0003, n = 3). The α-MSH 4–10 peptide also inhibited GPx activity in a biphasic manner when HBL cells were stimulated with TNF-α (Fig. 2a), with maximum inhibition at 10⁻¹⁰ M (90%, p < 0.0001, n = 3). Biphasic inhibition was also noted when the cells were stimulated with hydrogen peroxide (Fig. 2b; with maximum inhibition at 10⁻¹⁰ M, 81% inhibition, p = 0.0003, n = 3). In contrast, the “superpotent” peptide α-MSH [Nle₄-D-Phe₇] was most effective at inhibiting TNF-α-induced GPx activity at 10⁻¹² M (Fig. 2a, 87%, p < 0.0001, n = 3). This peptide was also found to inhibit GPx activity in a biphasic manner, as concentrations of α-MSH [Nle₄-D-Phe₇] higher than 10⁻¹² M were progressively less effective in inhibiting GPx activity (although all concentrations investigated achieved significant inhibition). When HBL cells were stimulated with hydrogen peroxide, the inhibitory profile was broadly similar (Fig. 2b), with 10⁻¹² M as the most potent concentration. Higher or lower concentrations were less effective. The α-MSH 11–13 tripeptide also had a biphasic action in inhibiting GPx activity. However, the most effective concentration at inhibiting GPx activity was 10⁻⁹ M when cells were stimulated with either TNF-α (Fig. 2a, 78%, p = 0.0002, n = 3) or hydrogen peroxide (Fig. 2b, 95%, p =
When HaCaT human keratinocytes cells were stimulated with TNF-α or hydrogen peroxide, a similar GPx inhibition was observed with α-MSH 1–13 and α-MSH [Nle 4-D-Phe7]. The most effective inhibitory concentration for α-MSH 1–13 was $10^{-10}$ M when cells were stimulated with either TNF-α (Fig. 2c, 87% mean inhibition, $p < 0.0001, n = 3$) or hydrogen peroxide (Fig. 2d, 133% mean inhibition, $p < 0.0001, n = 3$; this was the only instance when an MSH peptide inhibited unstimulated GPx activity). As noted for the HBL melanoma cell line, inhibition was biphasic in terms of effective concentration, with $10^{-11}$, $10^{-10}$, and $10^{-9}$ M being less effective than $10^{-8}$ M, irrespective of the stimulus (TNF-α or hydrogen peroxide). The α-MSH [Nle4-D-Phe7] peptide was most effective at $10^{-12}$ M, achieving 100% GPx inhibition ($p < 0.0001, n = 3$) when stimulated with TNF-α (Fig. 2c) and 74% inhibition ($p < 0.0001, n = 3$) when stimulated with hydrogen peroxide (Fig. 2d). In contrast to the biphasic response to α-MSH 4–10 and 11–13 in the HBL melanoma cell line, α-MSH 4–10 achieved 62% inhibition ($p < 0.0001, n = 3$) (Fig. 2c) in inhibiting TNF-α-induced GPx activity at $10^{-8}$ M. The same concentration was also most effective for inhibiting hydrogen peroxide-induced GPx activity (Fig. 2d, 71% inhibition, $p = 0.01, n = 3$). A summary of the most effective inhibitory concentrations of each MSH peptide on total GPx activity arising in HBL and HaCaT cells when stimulated with TNF-α or hydrogen peroxide is shown in Table I.

**Effect of Forskolin and IBMX on Total GPx Activity—**As α-MSH is known to operate through the G-protein activation of cyclic AMP, we substituted forskolin (which directly activates adenylate cyclase independent of the presence of the receptor) or IBMX (an inhibitor of the adenylate cyclase-generating phosphodiesterase, which degrades cyclic AMP) for α-MSH. Forskolin at $10^{-4}$ M and IBMX at $10^{-3}$ M both achieved inhibition of 60–80% against TNF-α- and peroxide-stimulated GPx activity in both cells (Table II).

**Effect of α-MSH, IBMX, and Forskolin on Peroxide Accumulation—**HBL and HaCaT cells loaded intracellularly with DCFH-DA were compared with unloaded cells by fluorescence-activated cell sorting analysis. Typical results are shown in Fig. 3 for HaCaT keratinocytes, where an increase in basal fluorescence was observed (very similar results were obtained for HBL melanoma cells (not shown)). Cells were then incubated exogenously with hydrogen peroxide for 15 min. Fluorescence-activated cell sorting analysis demonstrated reproducible in-
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することがintrinsic fluorescence after exposure, interpreted as an increase in the level of peroxide species (potentially as lipid peroxide, organic peroxide, or hydrogen peroxide species) within the cell (Fig. 3a) (we were unable to detect any significant effect of TNF-α on peroxide accumulation in either cell line). When cells were preincubated with α-MSH for 5 min, followed by a 15-min incubation period with hydrogen peroxide, intracellular fluorescence compared with hydrogen peroxide alone stimulation was found to decrease (Fig. 3b). Fig. 4 shows a comparison of the sensitivity of HaCaT and HBL cells to α-MSH. For HaCaT cells a decrease of ~50% in fluorescence was observed using concentrations ranging from 10⁻¹¹ to 10⁻⁸ M α-MSH (Fig. 4a); concentrations greater than 10⁻⁷ M were less effective. In HBL cells the α-MSH inhibition of peroxide accumulation was concentration-dependent in that increasing concentrations of α-MSH increased the inhibition to a maximum of 50% achieved at 10⁻⁷ M (Fig. 4b). Again, higher concentrations of α-MSH were less effective. When forskolin or IBMX were substituted for α-MSH, both were effective in inhibiting the peroxide-initiated increase in intracellular fluorescence in a manner similar to that observed for α-MSH (Fig. 3c). As with the inhibition of stimulated GPx activity, the concentrations of forskolin and IBMX required for effective inhibition were 10⁻⁶ and 10⁻⁵ M, respectively (Table III), for both HBL and HaCaT cell lines. Forskolin at 10⁻⁴ M achieved greater than 60% inhibition of the response to hydrogen peroxide.

**DISCUSSION**

Our objective in this study was to learn more about acute signaling events that α-MSH initiates enabling it to oppose the actions of proinflammatory cytokines. α-MSH is produced in the pituitary gland, brain, and various extraneural tissues including immunocompetent cells (Langerhan’s and murine TH1 dendritic epidermal cells), melanocytes, keratinocytes, and melanoma cells (23, 26, 27, 28). Generation arises via proteolysis of proopiomelanocortin, which is also a precursor for β-endorphin, β-lipotrophin, and ACTH (24, 25). MSH signaling is transmitted via the MC receptor family. Of the five different MC receptors (MC-1R to MC-5R) MC-1R has high affinity for α-MSH and is expressed on surface melanocytes, melanoma cells, monocytes, keratinocytes, fibroblasts, and endothelial cells (8, 29–31). This receptor activates adenyl cyclase and elevates cAMP. MC-2R is found in adrenal cortical cells, and MC-3R is found in placenta, gut, and brain (30, 32, 33). MC-4R is found in brain and gut, and MC-5R is found in thymus, spleen, bone marrow, skeletal muscle, adipocytes, adrenal glands, and brain (34, 35).

The different tissues in which α-MSH is released and where melanocortin receptors have been expressed have led a number of investigators to suggest alternative extrapigmentary roles for MSH in controlling host reactions such as the control of fever, inflammation, and secretion of C reactive protein (2). In support of this, *in vivo* studies demonstrate α-MSH to have potent anti-inflammatory and antipyretic activities (3, 4, 6, 36–39) which are suggested to act by two major routes: (i) in the brain via neural melanocortin receptors, which generate efferent signaling events that act at distant tissue sites by a glucocorticoid-dependent mechanism but by inhibiting production/action of proinflammatory cytokines (e.g. TNF-α (11), IL-1β (5), and IL-6 (4)) and by stimulating production of anti-inflammatory cytokines (e.g. IL-10 (9)).

**Comparison of the potency of α-MSH and related peptides in inhibiting TNF-α and hydrogen peroxide-stimulated GPx activity**

The table shows the concentration of peptide that produced maximum inhibition of TNF-α and hydrogen peroxide-stimulated GPx activity in HBL melanoma cells and HaCaT keratinocyte cells. Figures in parentheses show the mean percentage inhibition ± S.E. achieved based on three separate experiments. HaCaT keratinocytes were exposed to 300 μM hydrogen peroxide or 400 units/ml TNF-α, and HBL melanoma cells were exposed to 600 μM hydrogen peroxide or 400 units/ml TNF-α. ***, p < 0.001.

### Table I

| Peptide          | TNF-α    | H₂O₂     |
|------------------|----------|----------|
| α-MSH 1–13       | HaCaT    | 10⁻¹⁰ (87 ± 17%)*** | 10⁻¹⁰ (133 ± 14%)*** |
|                  | HBL      | 10⁻¹⁰ (84 ± 3%)***  | 10⁻¹⁰ (83 ± 10%)***  |
| α-MSH 4–10       | HaCaT    | 10⁻⁹ (62 ± 9%)***   | 10⁻⁸ (71 ± 9%)***    |
|                  | HBL      | 10⁻¹⁰ (90 ± 5%)***   | 10⁻¹⁰ (81 ± 10%)***   |
| α-MSH 11–13      | HaCaT    | 10⁻⁸ (89 ± 10%)***   | 10⁻⁸ (79 ± 8%)***    |
|                  | HBL      | 10⁻⁹ (78 ± 10%)***   | 10⁻⁹ (95 ± 2%)***    |
| α-MSH [Nle₂-D-Phe₃] | HaCaT | 10⁻¹² (100 ± 7%)*** | 10⁻¹² (74 ± 3%)*** |
|                  | HBL      | 10⁻¹² (87 ± 5%)***   | 10⁻¹² (92 ± 6%)***   |

### Table II

**Inhibition of glutathione peroxidase by forskolin and IBMX**

The influence of forskolin and IBMX on TNF-α- or hydrogen peroxide-stimulated GPx activity in HaCaT keratinocytes and HBL melanoma cells. Cells were preincubated with forskolin or IBMX for 15 min prior to stimulation with either TNF-α or hydrogen peroxide (concentrations as stated) for 45 min (HaCaT) or 30 min (HBL). Values shown are percentage inhibition of maximum stimulated GPx activity (TNF-α or H₂O₂ alone); mean ± S.E. *, p < 0.05; **, p < 0.01.

| Cell treatment | HaCaT keratinocytes | HBL melanoma cells |
|----------------|---------------------|--------------------|
|                | TNF-α               | H₂O₂               | TNF-α               | H₂O₂               |
|                | 400 units/ml        | 300 μM             | 400 units/ml        | 600 μM             |
| IBMX (10⁻⁴ M)  | 0.67 ± 0.3          | 3.3 ± 1.9          | 3.5 ± 2.0           | 36.7 ± 6.5*        |
| IBMX (10⁻³ M)  | 66.8 ± 3.6*         | 55.4 ± 3.5**       | 18.1 ± 2.3*         | 54.2 ± 5.9*        |
| Forskolin (10⁻⁵ M) | 20.0 ± 3.5      | 46.9 ± 3.2**       | 17.8 ± 3.2          | 34.2 ± 2.9*        |
| Forskolin (10⁻⁴ M) | 84.4 ± 5.8**      | 54.4 ± 4.1**       | 62.0 ± 6.8**        | 56.1 ± 6.5*        |
α-stimulated up-regulation of ICAM-1 in melanocytes (10) and melanoma cells (11). Expression is thought to be related to the metastatic potential of cutaneous melanoma cells, where metastatic cells express more ICAM-1 than primary melanoma (44). However, ICAM-1 expression is also relevant for lymphocytic and macrophage interactions at primary tumor sites. Thus, the α-MSH attenuation of TNF-α may also extend to melanoma progression, especially as melanoma cells produce α-MSH, raising the possibility of autocrine/paracrine control of melanoma cell interaction with immune cells.

As ICAM-1 expression is central to many aspects of inflammation, we next investigated the ability of α-MSH to inhibit the NF-κB transcription factor, which regulates expression of ICAM-1 and several other inflammatory mediators (45, 46). α-MSH significantly reduced the activation of NF-κB by TNF-α, and p50-p65 was identified as the complex predominantly inhibited, implying functional inhibition of transcription (12). Concentrations of around $10^{-9}$ M were most effective with higher and lower concentrations proving less effective. This biphasic concentration dependence has previously been noted by ourselves (10, 11) and by others (2) with respect to anti-inflammatory responses.
Activation of NF-κB requires phosphorylation, ubiquitination, and proteosomal degradation of the inhibitor κB subunit (47). Inhibitor κB release allows the p50-p65 complex to migrate to the nucleus and induce transcription. Several studies indicate that activation of NF-κB can be controlled by cellular redox status (13, 18, 48, 49). Evidence is based on observations that most agents activating NF-κB trigger the formation of ROS, or are oxidants themselves (e.g. superoxide, lipoxygenase products, or H₂O₂). For instance, TNF-α is known to increase the generation and leakage of superoxide species between complexes I and III of the mitochondria (17, 18). Superoxide is then rapidly dismutized to H₂O₂ by resident manganese-superoxide dismutases (50). Rotenone (a mitochondrial complex I inhibitor) can decrease TNF-α-stimulated NF-κB activity, whereas antimycin A (a complex III inhibitor) can amplify NF-κB activity (18). Activation may also arise directly by organic hydroperoxides or be induced by the addition of H₂O₂ (used as a nonphysiological stimulus). NF-κB activation is also inhibited by a number of antioxidants, either as exogenous agents or by transfection with overexpressing antioxidant enzyme vectors (15). The GPx family may therefore have a role in the control of transcription factor/NF-κB activity as H₂O₂ and organic/lipid hydroperoxides reduce intracellular glutathione levels (19), and many common antioxidants (e.g. N-acetyl cysteine and α-lipoic acid) elevate these levels (51, 52). A wide range of substrates for GPx exist including H₂O₂, hydroperoxides, products of multiple lipoxygenases, and 15-lipoxygenase products, and many of these have NF-κB-activating potential (19).

In this study we focused specifically on the events relevant to the immunomodulatory activities of MSH pre-NF-κB activation, as we have previously shown α-MSH to be capable of attenuating the response to TNF-α on NF-κB by 1–2 h (12). Thus, we looked specifically at total GPx and the generation of intracellular peroxide as factors arising before and strongly implicated in NF-κB activation. We found that TNF-α- and H₂O₂-stimulated total GPx activity in both the HBL and HaCaT cell line and that α-MSH and related peptides inhibited this activation for both cell types.

We investigated full-length and MSH peptide derivatives to learn more about the nature of the peptide receptor interaction. Full-length α-MSH has previously been reported to have pigmentary (1) and anti-inflammatory actions (2), whereas α-MSH [Nle₄-d-Phe₇] is similar in this respect but differs from α-MSH in that a higher receptor binding affinity and cAMP response (up to 24-fold) is observed. α-MSH 4–10 contains the essential peptide core sequence necessary for cAMP activation in melanoma cells and is the minimum sequence required for pigmentary action. However, the anti-inflammatory properties of α-MSH 4–10 have not been fully investigated. Results obtained for α-MSH 11–13 contribute to a somewhat confused understanding on how this peptide works. Present data show that this tripeptide responds in a similar manner to the other three MSH peptides in terms of inhibiting GPx activity, albeit at higher concentrations. Studies have previously reported α-MSH 11–13 to be ineffective in the lizard skin assay and adenylyl cyclase assay in Cloudman S91 mouse melanoma cells (53). Also, melphalan-conjugated α-MSH 11–13 is reported not to bind to the MSH receptor in HBL cells, although data here are for a conjugated peptide (54). However, other studies report on MSH 11–13 being effective at opposing inflammation in vivo (36), albeit at higher concentrations than α-MSH. The latter is consistent with results from the current study. Indeed, very low concentrations of MSH 11–13 (10⁻¹⁵ m) have been reported to stimulate IL-10 production in human peripheral blood mononuclear cells, although less effectively than α-MSH (9).

The predominant features of MSH inhibition were: (i) it was effective at nanomolar concentrations, (ii) the extent of the inhibition was partial rather than complete (although inhibition in the order of 70–80% was often seen), and (iii) the response to α-MSH and related peptides was often biphasic (with higher concentrations proving less effective than lower concentrations) in inhibiting the stimulated GPx activity. The HBL cutaneous melanoma cells (with pigmentary potential) and the keratinocyte HaCaT cell line (which lack pigmentation ability) showed very similar responses to the full-length peptide with 10⁻¹⁰ m proving the most effective concentration for inhibition of GPx activity stimulated by either TNF-α or H₂O₂. Cells were extremely sensitive to α-MSH [Nle₄-d-Phe₇] with concentrations of 10⁻¹² m achieving greater than 70% inhibition. In the HBL cell line α-MSH 4–10 was equipotent with α-MSH 1–13, and α-MSH 11–13 was only 10-fold less potent than α-MSH 1–13. In contrast, the HaCaT cell line required 100 times more of α-MSH 4–10 or α-MSH 11–13 to achieve equivalent inhibition to that seen with the full-length peptide. The observed differences may be attributable to the number of MC-1Rs expressed/cell (which ~2000–3000 for HBL cells (20)). There are, as yet, no reports of MC-1R number for HaCaT cells to the best of our knowledge.

Inhibition of total GPx was also achieved using receptor-independent methodologies for elevating cAMP, namely forskolin or IBMX, strongly suggesting the role of cAMP in the inhibitory actions of α-MSH. We suggest that the reduced GPx response to TNF-α or H₂O₂ when MSH was present is explained by MSH acting upstream of GPx on substrate availability, rather than direct enzyme inhibition as the MSH peptides alone had no effect on basal GPx activity. This finding was substantiated by the reduction in DCFH intracellular fluorescence when MSH was present in H₂O₂-stimulated cells. cAMP elevation also reduced peroxide detection. In this system the

**Table III**

**Inhibition of intracellular peroxide/oxidative stress by forskolin and IBMX**

The influence of forskolin and IBMX on intracellular peroxide/oxidative stress generation in HaCaT keratinocytes and HBL melanoma cells, as determined by DCFH fluorescence and flow cytometric analysis. Cells were preincubated with forskolin or IBMX for 5 min prior to stimulation with exogenous hydrogen peroxide for 15 min. HaCaT keratinocytes were exposed to 300 μM hydrogen peroxide, and HBL melanoma cells were exposed to 600 μM hydrogen peroxide. Values in parentheses show percentage inhibition for respective median and geo-mean values. Results shown are typical of three repeat analyses.

| Cell treatment | Median | Geo mean | Median | Geo mean |
|----------------|--------|----------|--------|----------|
| HaCaT keratinocytes | | | | |
| Control | 2.67 (100) | 3.54 (100) | 3.01 (100) | 3.42 (100) |
| H₂O₂ | 8.13 (0) | 10.81 (0) | 10.54 (0) | 13.26 (0) |
| H₂O₂ + IBMX (10⁻⁴ m) | 8.35 (24) | 8.86 (27) | 7.96 (45) | 9.26 (41) |
| H₂O₂ + IBMX (10⁻³ m) | 6.44 (31) | 8.21 (36) | 7.39 (42) | 9.68 (36) |
| H₂O₂ + forskolin (10⁻⁴ m) | 6.26 (34) | 8.13 (37) | 6.91 (48) | 9.14 (42) |
| H₂O₂ + forskolin (10⁻³ m) | 4.37 (69) | 5.87 (68) | 5.66 (65) | 7.32 (61) |
| HBL melanoma cells | | | | |
| Control | | | | |
| H₂O₂ | | | | |
| H₂O₂ + IBMX (10⁻⁴ m) | | | | |
| H₂O₂ + IBMX (10⁻³ m) | | | | |
| H₂O₂ + forskolin (10⁻⁴ m) | | | | |
| H₂O₂ + forskolin (10⁻³ m) | | | | |
peptides. We conclude that α-MSH and related peptides are extremely potent in opposing the oxidative stress.

In summary, the present investigation clearly shows that MSH and related peptides are extremely potent in opposing the generation of peroxide and inhibiting GPX activation. The rapidity of action of the peptides and their potency in pigmented and nonpigmented cells suggest that these events are central to MSH biology rather than peripheral to the activity of these peptides. We conclude that α-MSH is likely to have a major role in assisting keratinocyte and melanocytic cells to cope with oxidative stress.

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