Prostaglandin production by melanocytic cells and the effect of α-melanocyte stimulating hormone

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Received 12 May 2004; revised 18 June 2004; accepted 21 June 2004

Available online 2 July 2004

Edited by Veli-Pekka Lehto

Abstract Prostaglandins are potent mediators of the inflammatory response and are also involved in cancer development. In this study, we show that human melanocytes and FM55 melanoma cells express cyclooxygenase-1 and -2 (COX-1 and -2) and thus have the capability to produce prostaglandins. The FM55 cells produced predominantly PGE_2 and $PGF_{2\alpha}$, whereas the HaCaT keratinocyte cell line produced mainly PGE_2. The anti-inflammatory peptide, α-melanocyte stimulating hormone (α-MSH), reduced prostaglandin production in FM55 and HaCaT cells and reversed the effect of the pro-inflammatory cytokine TNF-α in the former. These results indicate that melanocytes produce prostaglandins and that α-MSH, by inhibiting this response, may play an important role in regulating inflammatory responses in the skin.

Keywords: Prostaglandin; Melanocytic cell; Cyclooxygenase; α-Melanocyte stimulating hormone; FM55; HaCaT

1. Introduction

Prostaglandins are lipid-derived mediators that have a wide spectrum of biological activities that is determined by their tissue of origin [1,2]. In the skin they mediate inflammatory reactions, although the sequence of events involved in these responses is poorly understood. The cellular origin of these mediators in the skin is also not clear, although it is generally suspected that a major source is the keratinocyte. The possibility should be considered that melanocytes are a source of prostaglandins. These cells, which are best known for their pigment production, are capable of secreting inflammatory mediators such as nitric oxide (NO) and numerous cytokines [3]. Recently, human malignant melanoma cells were shown to over-express cyclooxygenase-2 (COX-2) [4–6], the inducible isoform of COX which is the rate-limiting enzyme in the production of prostanoids. Increased expression of COX-2 has been linked to the development and progression of malignant epithelial cancers [7], raising the possibility that prostaglandins produced by melanocytic cells have a functional role in skin carcinogenesis.

Melanocyte function is regulated by α-melanocyte stimulating hormone (α-MSH). It is also recognized that α-MSH and related proopiomelanocortin-derived peptides are produced in the skin where they act as autocrine and paracrine regulators of skin pigmentary and immune systems [8]. α-MSH stimulates melanin synthesis in addition to other actions in melanocytic cells [3]. Thus, in human melanocytes it inhibits UV-induced apoptosis [9]. In addition, it has been shown to protect against proinflammatory cytokine attack and reduce invasion in melanoma cells [10]. α-MSH also modulates NO production by melanocytes and melanoma cells by regulating the expression of iNOS [11]. Assuming that melanocytes produce prostaglandins, it is then possible that this process will be regulated by α-MSH. Such an action would be in keeping with its role as an anti-inflammatory, anti-pyretic and immunoregulatory peptide [12]. Interestingly, these immunoregulatory actions of α-MSH are mediated by the same melanocortin receptor subtype, namely the MC1R, that is expressed on melanocytes.

The purpose of the present study was to examine the production of prostaglandins by melanocytes and to see whether this function is affected by α-MSH.

2. Materials and methods

2.1. Materials

Phosphate-buffered saline, dimethylsulfoxide, calcium ionophore A23187, dithiothreitol, phospholipid standards and anhydrous sodium sulfate were obtained from Sigma, Poole, UK. Primary prostaglandin HPLC mixture and PGB_2 were from Cayman Chemicals, Ann Arbor, USA. ^{14}C-Arachidonic acid was purchased from Amersham Biosciences, Chalfont St Giles, UK. HPLC grade methanol, glacial acetic acid, acetonitrile, ethyl acetate, and absolute ethanol were purchased from VWR, Lutterworth, UK. Detergent-compatible protein assay kit was from Bio-Rad, Hemel Hampstead, UK.

2.2. Cell culture

Human epidermal melanocytes and keratinocytes were isolated from skin samples from adults undergoing plastic surgery at the Bradford Royal Infirmary (according to the protocol approved by the local ethics committee). The cells were cultured in supplemented MCDB 153

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Abbreviations: COX, cyclooxygenase; α-MSH, α-melanocyte stimulating hormone

0014-5793/$22.00 © 2004 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2004.06.041

medium as previously described [13]. Human melanoma cell line FM55 established from metastatic melanoma nodules was a gift from Dr. A.F. Kirkin (Danish Cancer Society, Copenhagen, Denmark). FM55 human melanoma cells were maintained in RPMI-1640 medium (Gibco-BRL, UK) and HaCaT cells in DMEM (Gibco-BRL) containing 10% foetal bovine serum (ICN Flow, Basingstoke, UK) and penicillin (50 IU/ml)/streptomycin (50 μg/ml) (Gibco-BRL). All cultures were kept in a humidified atmosphere at 37 °C, 5% CO_2.

2.3. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from approximately 5×10^5 cells using Tri-reagent according to the manufacturer's instructions (Invitrogen, Paisley, UK). About 1–5 μg of 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) COX-1 sense 5′-tgcccagctcctggcccgccgct-3′ and COX-1 antisense 5′-gtgcatcaacacaggcgcctctt-3′; (ii) COX-2 sense 5′-ttcaaatgagattgtggaaaaattgct-3′ and COX-2 antisense 5′-agatcatctctgcctgagtatctt-3′. The primer sets yield PCR products of 303 and 304 bp for COX-1 and COX-2, respectively. For both COX-1 and COX-2 gene amplification, samples contained 10 pmol of each primer, 200 μM dNTP, 2 mM $MgCl_2$, 1× PCR buffer, 1U *Taq* polymerase and 1 μl cDNA to a final volume of 50 μl. Reaction mixtures were incubated in a thermal cycler (PCR Sprint temperature cycling system, Hybaid, UK) for 38 cycles (10 cycles of denaturation for 30 s at 94 °C, annealing for 45 s at 65 °C and extension for 1 min at 72 °C and 28 cycles of denaturation for 30 s at 94 °C, annealing for 45 s at 58 °C and extension for 1 min at 72 °C). Negative control experiments were performed using the reaction mixture and primers without cDNA. Finally, the PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide and photographed under UV.

2.4. Resting production of ^{14}C-prostaglandins

Cells were cultured to approximately 80% confluence. They were then transferred to fresh serum free medium and quiesed for 3 h. At the end of this period, the medium was changed to serum free medium containing ^{14}C-arachidonic acid (^{14}C-AA) (1 μCi) and the cells were incubated for 3 h. The medium containing ^{14}C-prostaglandins produced during the ^{14}C-AA incubation was removed and stored at −20 °C awaiting analysis.

2.5. Stimulated production of ^{14}C-eicosanoids

Cells were cultured to approximately 80% confluence and treated with ^{14}C-AA (1 μCi) as previously described. The medium was then removed and the cells washed twice with serum free medium to ensure complete removal of excess ^{14}C-AA. Serum free medium (5 ml) containing calcium chloride (2 mM) and calcium ionophore A23187 (3 M) was then added to stimulate prostaglandin production and the cells were incubated for a further 30 min. The medium, containing ^{14}C-prostaglandins, was removed and stored at −20 °C awaiting analysis.

2.6. Inhibition of ^{14}C-prostaglandin production by indomethacin

Production of ^{14}C-prostaglandins was inhibited by indomethacin (3 mM) [14]. Inhibition of basal ^{14}C-eicosanoids was achieved by adding the indomethacin solution and ^{14}C-AA to the cells simultaneously. Inhibition of stimulated ^{14}C-prostaglandins was achieved by adding the indomethacin solution to the labelled cells 10 min prior to adding calcium ionophore. In both cases, cells and media were treated in the same way as already described for basal and stimulated production of ^{14}C-prostaglandins.

2.7. Effect of TNF-α and α-MSH on ^{14}C-prostaglandin production

Cells were labelled with ^{14}C-AA (1 μCi) for 3 h as previously described. TNF-α (500 IU) and/or α-MSH (10^{-8} M) were then added and the cells were incubated for a further 24 h. The ^{14}C-prostaglandin containing medium was collected and stored at −20 °C awaiting analysis.

2.8. Extraction of ^{14}C-prostaglandins

Internal standard (PGB_2 500 ng) was added to each sample of medium containing ^{14}C-prostaglandins [14]. The resulting solution was acidified with 1 M citric acid to pH 3.0 and the ^{14}C-prostaglandins were extracted by adding 2 vol of ethyl acetate. The ethyl acetate extract was dried with anhydrous Na_2SO_4, the organic solvent evaporated under nitrogen and stored at −20 °C awaiting analysis. Appropriate control experiments using commercially available prostaglandin standards were performed to ensure the reliability of the extraction procedure.

2.9. HPLC analysis of ^{14}C-prostaglandins

^{14}C-prostaglandins were separated by HPLC performed on a C18 Spherisorb ODS2 column (4.5 × 250 mm, 5 mm, Waters) with a C18 ODS2 (5 μm) guard-column (Alltech). The mobile phase consisted of acetonitrile:water:glacial acetic acid (45:55:0.02 v/v). The ^{14}C-prostaglandin extracts were reconstituted in mobile phase. The flow rate was 0.5 ml/min. Fractions were collected at 30s intervals and ^{14}C-metabolites were detected by liquid scintillation counting. ^{14}C-prostaglandins were identified by co-elution with unlabelled standards as detected at 192 nm (Waters 486 UV detector). The internal standard (PGB_2) was detected at 280 nm [14].

2.10. Measurement of cell viability

At the end of all experiments, cells were trypsinized and counted using a heamocytometer. Cell viability was assessed by the trypan blue exclusion assay and was found to be greater than 95% for all experiments.

2.11. Protein assay

Cell pellets from all experiments were dissolved in 1% SDS, boiled for 10 min and protein measurements were carried out using the DC protein assay kit (Bio-Rad, UK) according to the manufacturer's instructions.

2.12. Calculations and statistical analysis

All data are expressed as mean values ± S.D. of at least three independent determinations unless otherwise stated. Statistical analyses were performed using Student's independent samples two-tailed *t*-test. All tests were performed with the SPSS 11.0 Software.

3. Results

3.1. Melanocytes express COX-1 and COX-2

Using RT-PCR, the presence of mRNA for both COX-1 and COX-2 was detected in the primary cultures of human melanocytes and keratinocytes, as well as in human FM55 melanoma and HaCaT keratinocyte cell lines. The results are shown in Fig. 1. Keratinocytes and HaCaT cells are known to express COX-2 [15,16] and were included in the present study for comparison purposes.

3.2. Production and profile of prostaglandins

Because of the limited availability of clinical material for primary cultures, detailed studies on the profile of prostaglandins were carried out in FM55 melanoma and HaCaT cells.

The profile of prostaglandins produced by FM55 melanoma cells and HaCaT keratinocytes was investigated using ^{14}C-AA-labelled cells. Under resting conditions, ^{14}C-AA was converted into three ^{14}C-labelled prostaglandins, which were identified as $PGF_{2\alpha}$, PGE_2 and PGD_2. This production was seen both in FM55 melanoma and HaCaT keratinocytes (Fig. 2). In the FM55 melanoma approximately similar amounts of $PGF_{2\alpha}$, PGE_2 and PGD_2 were produced (Fig. 2A), whereas in the HaCaT cells PGE_2 was found to be the most abundant product (Fig. 2B). The level of incorporation of ^{14}C-AA tended to be slightly higher in the HaCaT cells although there was some variation between experiments (data not shown).

Stimulation of ^{14}C-prostaglandin with calcium ionophore A23187 was examined in FM55 and HaCaT cells. Calcium ionophore stimulates phospholipase A_2 activity with concomitant release of arachidonic acid from the cells leading to acute stimulation of eicosanoid production. This treatment

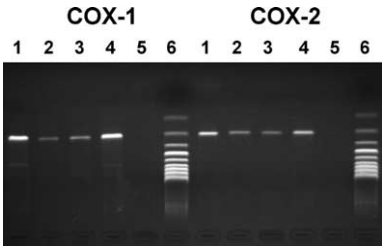

Fig. 1. Expression of COX-1 and COX-2 mRNA in melanocytic cells and keratinocytes. Lane 1: Human melanocytes; lane 2: FM55 melanoma cells; lane 3: human keratinocytes; lane 4: HaCaT keratinocytes; lane 5: negative control; lane 6: molecular weight markers.

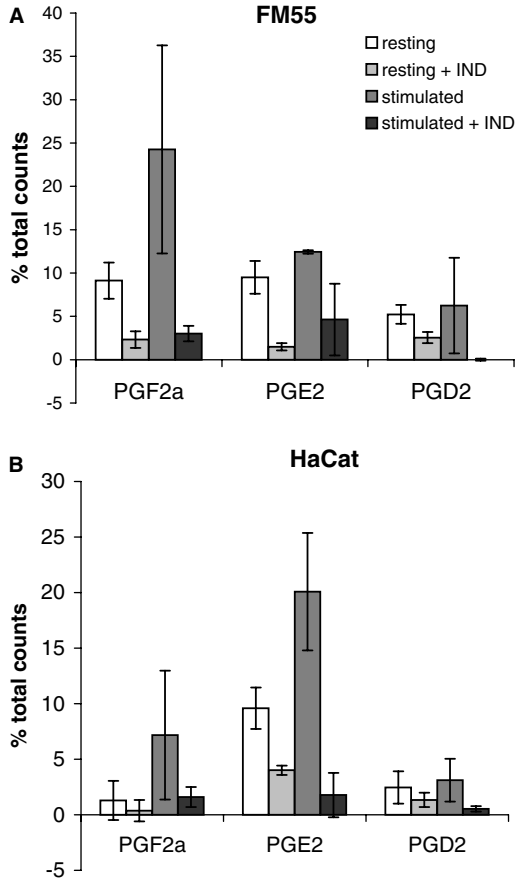

Fig. 2. ^{14}C-Prostaglandins produced by FM55 melanoma (A) and HaCaT keratinocytes (B) under resting and stimulated conditions. Indomethacin (3 mM) inhibited resting (resting + IND) and stimulated (stimulated + IND) ^{14}C-prostaglandin production. The results are expressed as means ± S.D. of at least three independent determinations.

resulted in increased ^{14}C-prostaglandin production as well as changes in their profile (Fig. 2A and B). In the FM55 cells the production of $PGF_{2\alpha}$ was more than doubled, with smaller increases in PGE_2 and PGD_2 (Fig. 2A). In the HaCaT cells, there was a doubling in the production of PGE_2 and $PGF_{2\alpha}$ (Fig. 2B). Furthermore, incubation with indomethacin (COX-1 and COX-2 inhibitor) inhibited resting and stimulated ^{14}C-prostaglandin production in both FM55 and HaCaT cells, thus confirming that the observed ^{14}C-metabolites were COX products (Fig. 2).

3.3. Effect of α-MSH on prostaglandin production

It has been reported that α-MSH can oppose the action of the pro-inflammatory cytokine TNF-α in melanocytes and melanoma cells [17]. Therefore, TNF-α was used as stimuli for prostaglandin production with a view to explore whether inhibition of prostaglandins contributes to the anti-inflammatory action of α-MSH. α-MSH inhibited ^{14}C-$PGF_{2\alpha}$ production in FM55 melanoma cells and reversed the TNF-α-induced PGE_2 production in these cells by approximately 80% (Fig. 3). It also reduced ^{14}C-$PGF_{2\alpha}$ production in HaCaT cells (Fig. 4) but in contrast to its effects in the FM55 cells failed to alter prostaglandin production in the presence of TNF-α. TNF-α alone had no effect on the production of prostaglandins in the HaCaT cells (Fig. 4).

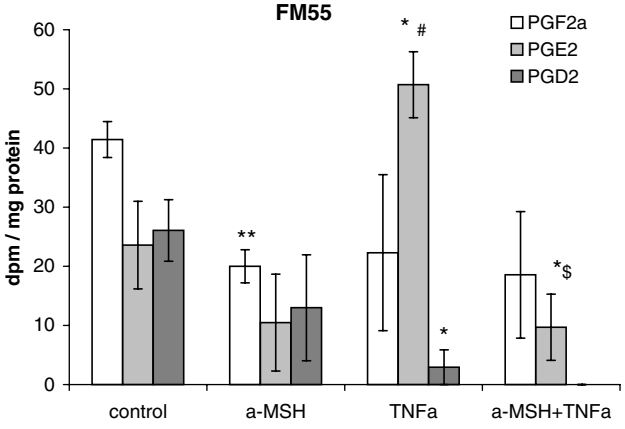

Fig. 3. The effect of α-MSH alone and in the presence of TNF-α on the production of ^{14}C-prostaglandins by FM55 melanocyte cells. The results are expressed as means ± S.D. of at least three independent determinations. $^{*}P < 0.05$ and $^{**}P < 0.01$ comparing all treatments to control; $^{\#}P < 0.005$ comparing TNF-α treatment to α-MSH treatment; $^{\$}P < 0.05$ comparing α-MSH + TNF-α treatment to TNF-α treatment.

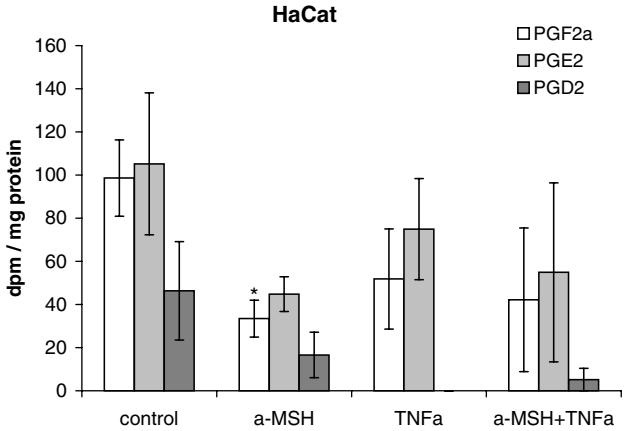

Fig. 4. The effect of α-MSH alone and in the presence of TNF-α on the production of ^{14}C-prostaglandins by HaCaT keratinocyte cells. The results are expressed as means ± S.D. of at least three independent determinations. $^{*}P < 0.05$ comparing all treatments to control.

4. Discussion

It is considered that a major source of prostaglandins in the skin is the keratinocyte. Our findings show that melanocytes express COX-1 and -2, suggesting that these cells are a potential source of prostaglandins. One of the recognized functions of the melanocyte is the production of melanin which is transferred into keratinocytes where it protects against UV induced damage [3]. Prostaglandins may be involved in regulating this function, since they have been shown to stimulate melanogenesis in iridial melanocytes [18]. In addition, it has been suggested that PGE_2 has a role in post-inflammatory pigmentation [19]. Thus, prostaglandins may act as local regulators of skin pigmentation. They could affect melanocyte function in other ways. For instance, since COX-2 is expressed in melanoma cells and is implicated in tumour development [4–6] prostaglandins could affect the progression of melanocytes to malignant phenotypes. Whatever the significance of prostaglandins for melanocyte function, their production by melanocytes could contribute to inflammatory responses in the skin.

Our findings show that FM55 melanocytes express both isoforms of COX (COX-1 and -2), and produce an array of prostaglandins under basal and stimulated conditions. When these cells were stimulated with calcium ionophore, the production of $PGF_{2\alpha}$ was increased with no change in the amounts of PGE_2 and PGD_2. This differed from the situation in HaCaT cells where PGE_2 was the main metabolite. Furthermore, whereas the proinflammatory cytokine TNF-α increased PGE_2 production in the FM55 cells, it was found to have no effect on the prostaglandin production by HaCaT keratinocytes. This lack of effect in HaCaT cells was surprising in view of previous reports that TNF-α will stimulate COX-2 expression and prostaglandin production in transformed human keratinocytes [20], and induce NFκB activation in HaCaT keratinocytes [21]. Nevertheless, our findings indicate that the profile of prostaglandin production in melanocytic cells may differ from that in keratinocytes and especially in response to inflammatory stimuli. This could have important implications in inflammatory situations in the skin and warrants further investigation.

The observed inhibition of prostaglandin production in FM55 melanoma and HaCaT keratinocytes by α-MSH supports the view that this peptide has anti-inflammatory properties. Its ability to reverse the action of TNF-α in FM55 cells is consistent with previous reports that this peptide is able to antagonize the actions of pro-inflammatory cytokines [22,23]. Through its ability to stimulate melanogenesis in melanocytes, α-MSH has an important role as a mediator in UV induced skin pigmentation [3]. This induced tanning can be preceded by erythema and, since PGE_2 is involved [24], α-MSH might be expected to reduce this response. These actions of α-MSH may explain the inverse relationship between tanning ability and UV induced erythema seen in some individuals. It may also explain why individuals with loss of function mutations at the MC1R show an increased risk of erythema following UV. If prostaglandins are involved in the more long term effects of UV, such as photoaging and carcinogenesis [24], then α-MSH could act to suppress these effects. In the long term, UV irradiation can lead to the development of skin cancer [25] and if, as suspected, changes in prostaglandin production are involved (see Introduction) then α-MSH could be protective. Since COX-2 is implicated in tumour development, including malignant melanoma [4–7], then further studies to explore the effect of α-MSH on the expression of this COX would be worthwhile.

Acknowledgements: This work was supported by Stiefel Laboratories (UK) Ltd. and The Wellcome Trust.

References

[1] Nicolaou, A. (2004) in: Nicolaou, A., Kokotos, G., (Eds.), Bioactive Lipids, Oily Press. (in press).
[2] Smith, K.J. and Skelton, H. (2002) J. Cutan. Med. Surg. 6, 241–256.
[3] Tsatmali, M., Ancans, J. and Thody, A.J. (2002) J. Histochem. Cytochem. 50, 125–133.
[4] Goulet, A.C. et al. (2003) Cancer Biol. Ther. 2, 713–718.
[5] Denkert, C., Kobel, M., Berger, S., Siegert, A., Leclere, A., Trefzer, U. and Hauptmann, S. (2001) Cancer Res. 61, 303–308.
[6] Higashi, Y., Kanekura, T. and Kanzaki, T. (2000) Int. J. Cancer 86, 667–671.
[7] Bishop-Bailey, D., Calatayud, S., Warner, T.D., Hla, T. and Mitchell, J.A. (2002) J. Environ. Pathol. Toxicol. Oncol. 21, 93–101.
[8] Slominski, A., Wortsman, J., Luger, T., Paus, R. and Solomon, S. (2000) Physiol. Rev. 80, 979–1020.
[9] Kadekaro, A.L., Kanto, H., Kavanagh, R. and Abdel-Malek, Z.A. (2003) Ann. NY Acad. Sci. 994, 359–365.
[10] Eves, P. et al. (2003) Br. J. Cancer 89, 2004–2015.
[11] Tsatmali, M., Graham, A., Szatkowski, D., Ancans, J., Manning, P., McNeil, C.J., Graham, A.M. and Thody, A.J. (2000) J. Invest. Dermatol. 114, 520–526.
[12] Luger, T.A., Scholzen, T.E., Brzoska, T. and Bohm, M. (2003) Ann. NY Acad. Sci. 994, 133–140.
[13] Hunt, G. and Thody, A.J. (1994) Cell and Tissue Culture: Laboratory Procedures. Wiley, Chistester. Chapter 11.
[14] Urquhart, P., Parkin, S.M., Rogers, J.S., Bosley, J.A. and Nicolaou, A. (2002) Biochim. Biophys. Acta 28, 2–3.
[15] Buckman, S.Y., Gresham, A., Hale, P., Hruza, G., Anast, J., Masferrer, J. and Pentland, A.P. (1998) Carcinogenesis 19, 723–729.
[16] Bachelor, M.A., Silvers, A.L. and Bowden, G.T. (2002) Oncogene 21, 7092–7099.
[17] Hedley, S.J., Gawkrodger, D.J., Weetman, A.P., Morandini, R., Boeynaems, J.M., Ghanem, G. and Neil, S.M. (1998) Br. J. Dermatol. 138, 536–543.
[18] Stjernschantz, J.W., Albert, D.M., Hu, D.N., Drago, F. and Wistrand, P.J. (2002) Surv. Ophthalmol. 47, S162–S175.
[19] Tomita, Y., Maeda, K. and Tagami, H. (1992) Pigm. Cell. Res. 5, 357–361.
[20] Grewe, M., Trefzer, U., Ballhorn, A., Gyufko, K., Henninger, H. and Krutmann, J. (1993) J. Invest. Dermatol. 101, 528–531.
[21] Shiratori, K., Ohgami, K., Ilieva, I.B., Koyama, Y., Yoshida, K. and Ohno, S. (2004) Invest. Ophthalmol. Vis. Sci. 45, 159–164.
[22] Haycock, J.W., Rowe, S.J., Cartledge, S., Wyatt, A., Ghanem, G., Morandini, R., Rennie, I.G. and MacNeil, S. (2000) J. Biol. Chem. 275, 15629–15636.
[23] Moustafa, M., Szabo, M., Ghanem, G.E., Morandini, R., Kemp, E.H., MacNeil, S. and Haycock, J.W. (2002) J. Invest. Dermatol. 119, 1244–1253.
[24] Rhodes, L.E., Belgi, G., Parslew, R., McLoughlin, L., Clough, G.F. and Friedmann, P.S. (2001) J. Invest. Dermatol. 117, 880–885.
[25] Matsumura, Y. and Ananthaswamy, H.N. (2002) Expert Rev. Mol. Med. 2, 1–22.