Arenarol isolated from a marine sponge abrogates endothelin-1-stimulated melanogenesis by interrupting MEK phosphorylation in normal human melanocytes

Bong-Keun Choi · Byung-Yoon Cha ·
Takeshi Fujiwara · Akihiko Kanamoto ·
Je-Tae Woo · Makoto Ojika · Genji Imokawa

Received: 23 December 2012 / Accepted: 7 March 2013 / Published online: 1 May 2013
© Springer Science+Business Media Dordrecht 2013

Abstract Using B16 melanoma cells for screening, we found that a marine sponge extract has a potent anti-pigmenting effect and identified arenarol as its major active compound. In normal human melanocytes (NHMs), arenarol significantly abrogated the endothelin 1 (EDN1) stimulated expression of tyrosinase, tyrosinase-related protein 1 and dopachrome tautomerase at the transcriptional, translational and enzymatic activity (only for tyrosinase) levels. That effect was accompanied by the attenuation of the increased expression level of microphthalmia-associated transcription factor (MITF) protein at the transcriptional and translational levels. Analysis of EDN1 signaling demonstrated that arenarol signifi-

cantly suppressed the EDN1-induced phosphorylation of MEK, ERK, MITF and CREB but not of Raf-1s. In contrast, the forskolin-induced phosphorylation of CREB was not down-regulated by arenarol. As for the mode of action of the suppressed phosphorylation of MEK, Raf-1 activity was not directly inhibited by arenarol in vitro and pretreatment with the protein phosphatase inhibitor okadaic acid did not affect the down-regulated phosphorylation of MEK that was induced by arenarol in NHMs. The sum of these findings suggests that arenarol abrogates the EDN1-stimulated expression of melanocyte-specific proteins by interrupting MEK phosphorylation in an as yet unknown Raf-1 inactivation mechanism.

Keywords Endothelin 1 · Intracellular signaling pathways · Normal human melanocytes · Tyrosinase · Arenarol · Marine sponge

Genji Imokawa and Makoto Ojika as corresponding authors contributed equally to this work.

B.-K. Choi · M. Ojika (✉)
Graduate School of Bioagricultural Sciences, Nagoya
University, Nagoya, Japan
e-mail: ojika@agr.nagoya-u.ac.jp

B.-Y. Cha · J.-T. Woo · G. Imokawa (✉)
Research Institute for Biological Functions, Chubu
University, Kasugai, Japan
e-mail: imokawag@dream.ocn.ne.jp

T. Fujiwara · A. Kanamoto
OP Bio Factory Co. Ltd., Okinawa, Japan

Abbreviations

NHMs	Normal human melanocytes
MITF	Microphthalmia-associated transcription factor
TYR	Tyrosinase
TYRP-1	Tyrosinase-related protein-1
DCT	Dopachrome tautomerase
EDN1	Endothelin 1
SCF	Stem cell factor
PKC	Protein kinase C
CREB	Cyclic AMP responsive element binding protein

Introduction

Hyperpigmented spots are the most serious problem in Asian skin. Abundant studies have elucidated the biological mechanisms involved in epidermal hyperpigmentation in which epidermal keratinocytes are stimulated by several stimuli, such as UV radiation or the primary inflammatory cytokines interleukin (IL)-1 or tumor necrosis factor (TNF) α , to secrete melanogenic cytokines such as endothelin 1 (EDN1) (Imokawa et al. 1992, 1995) and α -melanocyte stimulating hormone (MSH) (Abdel-Malek et al. 1995; Suzuki et al. 1996) or to produce and express stem cell factor (SCF) (Hachiya et al. 2001, 2004). Those factors trigger neighboring melanocytes to accentuate their melanogenesis through the activation of relevant intracellular signaling cascades (Yada et al. 1991; Imokawa et al. 2000). In melanocytes activated by several melanogenic cytokines, tyrosinase (TYR) expression is markedly up-regulated at the transcriptional and translational levels, which facilitates melanization in unique organelles termed melanosomes. Fully melanized melanosomes are transferred from the dendritic tips of melanocytes to surrounding keratinocytes during the phagocitizing process via protein activated receptor (PAR)-2 (Paine et al. 2001). Among these melanogenic cytokines or chemokines, EDN1 is the most potent agonist for stimulating melanocytes to accelerate melanin synthesis. EDN1 plays an essential role in UVB-melanosis (Imokawa et al. 1992, 1995; Hachiya et al. 2001, 2004) as well as in epidermal hyperpigmentary disorders such as seen in solar lentigos (Kadono et al. 2001; Hattori et al. 2004). Based on these epidermal pigmentation mechanisms, several anti-melanogenic agents have been developed and are mechanistically associated with their biological abilities to inhibit TYR activity (Chung et al. 2012), to accelerate the proteolytic degradation of TYR through the ubiquitination/proteasome system (Ando et al. 1999), and/or to interrupt the transfer of melanin granules from melanocytes to keratinocytes via the inactivation of PAR-2 (Paine et al. 2001). These approaches utilizing the interruption of constitutional melanogenic mechanisms toward the development of appropriate therapeutic and anti-melanogenic agents may lead to a serious challenge as follows; even in normal skin color (non-hyperpigmented) conditions, TYR also plays a key role in the

synthesis of melanin in melanosomes to a genetically regulated extent and the fully melanized melanosomes are transferred to keratinocytes, which maintains the normal level of skin color. Thus, owing to the difficulty of confining the topical application to the hyperpigmented skin area, potent anti-melanogenic agents capable of attenuating the natural non-stimulated pigmentation process have the risk of leading to hypopigmentation. In this respect, the general notion that hyperpigmentation should be attenuated by anti-melanogenic agents to a level of pigmentation not less than the normal skin color would be ideal. Since intracellular signaling pathways within melanocytes do not function substantially in normal skin color conditions and are activated only by environmental stimuli such as UV radiation to cause hyperpigmentation, specifically down-regulating the over-activation of melanogenesis to the constitutive level would be an appropriate challenge for developing new potent anti-melanogenic agents.

We have already established a screening system utilizing the recovery process of melanization of glucosamine (GlcN)-induced amelanotic B-16 mouse melanoma cells (Imokawa and Mishima 1982, 1984) by which anti-melanogenic substances can be easily screened by evaluating color of cell pellet (Komiyama et al. 1993). In this study, we found that a marine sponge extract has a potent anti-melanogenic effect and we identified arenarol as its major active compound. Arenarol isolated from an Okinawa marine sponge has been reported to be cytotoxic against P388 lymphocytic leukemia cells (Schmiz et al. 1984) and it serves as a potent inhibitor of several medically relevant protein kinases (Yoo et al. 2003). However, there has been no previous study elucidating its effect on melanogenesis in melanocytes or melanoma cells. Thus, we characterized the anti-melanogenic effect of arenarol in normal human melanocytes (NHMs) by focusing on its ability to interrupt the EDN1-triggered intracellular signaling linkage, as described later in details in “Discussion” section, which is a major intracellular signaling cascade involved in epidermal hyperpigmentary disorders (Imokawa et al. 1992, 1995; Hachiya et al. 2004; Kadono et al. 2001). Here we show that arenarol serves as a potent anti-melanogenic agent that interrupts the EDN1 triggered intracellular signaling linkage at a signaling site between Raf-1 and MEK in NHMs.

Materials and methods

Materials

NHMs, medium 254 and human melanocyte growth supplements (HMGS) were obtained from Kurabo Co. (Osaka, Japan). EDN1 was purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies to phosphorylated or non-phosphorylated Raf-1s, phosphorylated MEK, phosphorylated ERK, phosphorylated CREB and β -actin were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies to human TYR, TYRP1 and DCT were purchased from Santa Cruz (Santa Cruz, CA, USA).

Isolation of arenarol

For the screening, we used the extract library consisting of approximately 700 extracts (350 EtOAc-soluble and 350 water-soluble ones) obtained from 350 Okinawan organisms. As one of the active candidates, the Okinawan marine sponge *Dysidea arenaria* was extracted with MeOH. The extract from the sponge was partitioned between EtOAc and water. The organic fraction (838 mg) was chromatographed on silica gel Flash column (Hi-Flash column, M-size, silica gel-40 μ m, 14 g, Yamazen Science, Osaka, Japan), with a linear gradient from 20 to 100 % EtOAc (20 min) in hexane to give five fractions. The fraction 2 (609 mg) that eluted with 34–40 % EtOAc in hexane was separated on ODS (20 g, Cosmosil-140C₁₈, Nacalai Tesque, Kyoto, Japan) with 70–100 % MeOH to give six fractions. A portion (30.7 mg) of the fraction 2 (159 mg) that eluted with 70–80 % MeOH was purified by HPLC [YMC-Pack D-ODS-5 (20 $\times$ 250 mm), YMC Co., Kyoto, Japan, 80 % MeOH, 8 ml/min, detection at 205 nm] to arenarol (15.5 mg, t_R = 281 min) and isoarenarol (2.9 mg, t_R = 289 min). The arenarol structure was confirmed by NMR analysis and comparison with reported data (Schmiz et al. 1984).

Cell culture

B-16 mouse melanoma cells were cultivated in Eagle's MEM medium (Nissui, Tokyo, Japan) with 10 % FBS, 100 units/ml penicillin (Gibco, Grand Island, NY, USA), 100 μ g/ml streptomycin (Gibco), and 250 ng/ml amphotericin B (Gibco) at 37 °C in a 95 % air, 5 %

CO₂ atmosphere. GlcN (Sigma Aldrich, St. Louis, MO, USA)-induced amelanotic B16 melanoma cells were prepared by culturing B-16 melanotic melanoma cells for 7 days in the presence of GlcN at a concentration of 0.1 %. NHMs were maintained in Medium 254 with HMGS containing 0.2 % bovine pituitary extract (BPE), 0.5 % FBS, 3 ng/ml human basic fibroblast growth factor (hFGF-b), 5×10^{-7} M hydrocortisone, 5 μ g/ml insulin, 5 μ g/ml transferrin, 10 ng/ml phorbol 12-myristate 13-acetate (PMA) and 3 μ g/ml heparin at 37 °C in a 95 % air and 5 % CO₂ atmosphere.

Melanin contents

GlcN-induced amelanotic B16 melanoma cells were seeded at a density of 2×10^5 in 60-mm dishes. After overnight incubation, cells were cultured in the presence or absence of arenarol for 72 h. The cells were washed twice with phosphate-buffer saline (PBS) and harvested by treatment with 0.05 % trypsin/0.02 % EDTA. The harvested cells were centrifuged, and the pellet was dissolved by 1 N NaOH, followed by incubation at 60 °C for 2 h. The amount of melanin in the solution was determined by measuring the absorbance at 405 nm. Melanin contents were expressed as delta absorbance (405 nm)/ μ g protein.

Measurement of TYR activity

The enzymatic activity of TYR was measured using 3-methyl-2-benzothiazolinone hydrazine (MBTH) by a modification of the method of Winder and Harris (Winder and Harris 1991). TYR activity is expressed as delta absorbance (492 nm)/ μ g protein.

Cell viability assay

Cell viability assay was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MTT reagent was added at 3 h before the end of the culture. The supernatants were carefully removed, formazan crystals dissolved in dimethylsulfoxide (DMSO) and their absorbance measured at 570 nm.

Real-time RT-PCR

The expression of melanocyte-specific genes in NHMs was examined using real-time RT-PCR. One μ g of

total RNA from each sample was reverse-transcribed to cDNA using a reverse transcription system (Qiagen, Valencia, CA, USA). cDNAs were then synthesized using a Rever Tra Ace qPCR RT Kit (Toyobo, Tokyo, Japan) by reverse transcription of 1 µg total RNA using oligo dT and Moloney murine leukemia virus reverse transcriptase. Real-time RT-PCR with SYBR Green was performed using Power SYBR Green PCR (Applied Biosystem Japan, Tokyo, Japan) in an ABI Prism 7300 system (Applied Biosystem). The PCR conditions were 1 cycle of 50 °C for 2 min and 94 °C for 15 min, followed by 40 cycles of 94 °C for 15 s, 53 °C for 30 s and 74 °C for 1 min. The primer sequences used for the PCR amplifications were as follows: GAPDH: forward: 5'-GAAGGTGAAGGTCGGAGTCAAC-3', reverse: 5'-GTCCTTCCACGATACCAAAGTTG-3', TYR: forward: 5'-CCTCAAAGCATGCACAAT-3', reverse: 5'-GACGACCAGCAAGCTCACAAG-3', MITF: forward: 5'-TCCGTCTCTCACTGGATTGGTG-3', reverse: 5'-CGTGAATGTGTGTTTCATGCCTGG-3', TYRP-1: forward: 5'-TCATCTATTCCTGAATGGAACAGG-3', reverse: 5'-AATGAGTGCAACCAGTAACAAAGC-3', and DCT: forward: 5'-TCCGCTAGCCATGGGCTTG TGGGATGGGG-3', reverse: 5'-ACCGTCGACTGGTAGGCTTCCTCCGTGTAT-3'.

Western blotting

Cell lysates were separated on 10 % SDS-PAGE gels and were then transferred to PVDF membranes (Millipore Co., Billerica, MA, USA). The membranes were probed with antibodies against Raf-1, phospho-Raf-1s, MEK, phospho-MEK, ERK1/2, phospho-ERK1/2, CREB, phospho-CREB, MITF, TYR, TYRP-1, DCT and β -actin. After washing, blots were incubated with HRP-conjugated secondary antibodies. Finally, signals were detected by ECL or ECL prime (GE Healthcare, Piscataway, NJ, USA), and were visualized using an LAS-1000 Chemiluminescence Imaging system (Fujifilm, Tokyo, Japan).

Raf-1 kinase assay

Raf-1 kinase activity was determined by Raf-1 kinase assay kit (Millipore) consisting of 20 µl of magnesium chloride/APT cocktail and 0.84 µl (1 µg) of unactivated MEK and 1 µl (0.1 µg, truncated) activated

Raf-1. After incubation for 30 min at 30 °C, Raf-1 activity was evaluated by the level of MEK phosphorylation using western blot analysis with anti-phospho-MEK antibody.

Statistical analysis

All data are expressed as mean $\pm$ SD ($n = 3$) unless noted otherwise. For multiple comparisons, data were tested by one-way analysis of variance (ANOVA), and subsequently using the Tukey's or Dunnett multiple comparison test. P values less than 0.05 are considered to be statistically significant.

Results

Isolation of active substance from a methanol extract of the Okinawan marine sponge

Using the re-melanizing recovery process of GlcN-induced amelanotic B16 melanoma cells as a screen, we have found that a marine sponge extract has a potent anti-pigmenting effect and finally identified sesquiterpenoid (arenarol) as its major active compound according to the procedure described in the “[Materials and methods](#)” section (Fig. 1).

The inhibitory effect of arenarol on melanogenesis in B16 mouse melanoma cells

The addition of arenarol at concentrations of 0.3, 1.0 and 3.0 µM in the re-pigmenting recovery process of GlcN-induced amelanotic B16 cells elicited a marked inhibitory effect on TYR activity and melanin synthesis at 3 days post-treatment, which was not accompanied by any cytotoxic effect at any of the concentrations tested (Fig. 2).

Effect of arenarol on TYR activity and the viability of NHMs

We next examined the effects of arenarol on EDN1-stimulated TYR activity in NHMs. The addition of arenarol at concentrations of 0.3, 1.0 and 3.0 µM to NHMs 3 h prior to the EDN1 (10 nM) stimulation elicited a significant abrogating effect on the up-regulated TYR activity at concentrations of 1.0 and

Fig. 1 Isolation of active substances from a methanol extract of an Okinawan marine sponge

Fig. 2 The inhibitory effect of arenarol on melanogenesis in B16 melanoma cells. GlcN-treated amelanotic B16 mouse melanoma cells were re-cultured in the absence of GlcN with arenarol at the indicated concentrations for 3 days and were then subjected to the MTT assay to measure cellular toxicity. Cell lysates or cell pellets were used for the measurements of TYR

activity and melanin content, respectively. **a** photographs of cell pellets at day 3, **b** Melanin content at day 3, **c** Relative TYR activity at day 3, **d** Cell viability by MTT assay at day 3. Values are mean ± SD derived from 3 independent experiments. **p < 0.01 compared with arenarol-untreated control

3.0 μM after 72 h of culture (Fig. 3a). In contrast, treatment of cell lysates of NHMs cultured for 72 h after EDN1 stimulation with arenarol at the same concentrations did not affect the up-regulated TYR activity at the indicated concentrations (Fig. 3b). In these culture conditions with treatment of arenarol at the indicated concentrations, there was no reduction in the cell viability of NHMs (Fig. 3c).

Effect of arenarol on the expression of melanocyte-specific proteins in NHMs at transcriptional and translational levels

Real-time RT-PCR analysis revealed that the addition of arenarol at a concentration of 3.0 μM significantly abrogated the up-regulated gene expression of TYR, TYRP-1 and DCT at 12 h post-stimulation (Fig. 4A).

Fig. 3 Effect of arenarol on TYR activity and cell viability of NHMs. **a** TYR activity in cell lysates from NHMs which were treated with arenarol and cultured for 72 h with EDN1 stimulation. **b** TYR activity when arenarol was directly added to cell lysates from NHMs which were cultured for 72 h without arenarol treatment. **c** Cell viability by MTT assay at 72 h. Values are mean $\pm$ SD derived from 3 independent experiments. * $p < 0.05$, ** $p < 0.01$ compared with EDN1-stimulated and arenarol-untreated control

Consistently, western blot analysis demonstrated that the addition of arenarol significantly suppressed the increased protein expression of TYR, TYRP-1 and DCT at a concentration of 3.0 μM (Fig. 4B). Since the expression of genes encoding melanocyte-specific proteins are strictly regulated by the protein level of MITF (Bentley et al. 1994; Bertolotto et al. 1998; Sato-Jin et al. 2008), we next examined the effect of arenarol on the EDN-1-stimulated expression of MITF. Real-time RT-PCR analysis revealed that the addition of arenarol at a concentration of 3.0 μM significantly abrogated the up-regulated gene expression of MITF at 1 h post-stimulation (Fig. 4C-a). Consistently, western blotting analysis demonstrated that the addition of arenarol significantly suppressed the increased protein expression of MITF at a concentration of 3.0 μM at 6 h post-stimulation (Fig. 4C-b).

Effect of arenarol on EDN-1 signaling in NHMs

Since the sum of our findings suggests that arenarol interrupts an upstream pathway that leads to MITF gene expression, we next determined whether arenarol interrupts the EDN1-induced phosphorylation of Raf-1s (serine 338 or 259 and tyrosine 340/341), MEK, ERK, CREB and/or MITF in NHMs. When arenarol was added at the indicated concentrations 3 h prior to EDN1

treatment, the EDN1-stimulated phosphorylation of MEK, ERK, CREB and MITF but not Raf-1s was significantly suppressed at 15 min post-EDN1 treatment (Fig. 5a/b). Since CREB is phosphorylated by both RSK and PKA (Sato-Jin et al. 2008), we next determined whether arenarol interrupts CREB phosphorylation due to an activated cAMP/PKA linkage. When arenarol was added at the indicated concentrations 3 h prior to forskolin treatment, the forskolin-stimulated phosphorylation of CREB was not affected at 15 min post-forskolin treatment, while MEK phosphorylation remained unchanged at an undetectable level (Fig. 5c).

Effect of arenarol on Raf-1 kinase activity in vitro

We next asked whether arenarol has a potential to directly inhibit Raf-1 kinase activity, which results in the observed down-regulation of MEK phosphorylation. The incubation of arenarol for 3 h with the activated Raf-1 did not suppress MEK phosphorylation (Fig. 6a).

Effects of the protein phosphatase inhibitor okadaic acid on the abrogated phosphorylation of MEK by arenarol

To elucidate the mechanism(s) underlying the impairing effect of arenarol on the phosphorylation of MEK,

which may be an initial point for sequential phosphorylation linkages, we used the serine/threonine-protein phosphatase (PP) 2A inhibitor okadaic acid (Bialojan and Takai 1988; Hardie 1990) to examine its effect on the phosphorylation of MEK. Western blotting of these signaling molecules revealed that whereas arenarol attenuated the up-regulated phosphorylation of MEK by EDN1 stimulation, okadaic acid at the indicated concentrations did not reverse the down-regulated MEK phosphorylation to the EDN1-stimulated control levels (Fig. 6b).

Discussion

Using the re-pigmenting recovery process of GlcN-induced amelanotic B16 melanoma cells as a screen, we found that arenarol isolated from a marine sponge extract has a potent anti-pigmenting effect without any cytotoxicity, which is accompanied by the down-regulation of TYR activity in arenarol-treated B-16 melanoma cells. To elucidate its anti-melanogenic mode of action, there are at least 3 possibilities to consider; (1) arenarol may directly inhibit TYR activity, (2) arenarol may stimulate the proteolytic degradation of TYR, and/or (3) arenarol may attenuate the expression of TYR at the transcriptional and translational levels. In line with this approach, we used NHMs to elucidate those possible mechanisms. These studies showed that while arenarol at a concentration of 3 μ M does not have any cytotoxic effects on NHMs or any direct inhibitory effects on TYR activity in vitro, a 3 h pre-incubation with arenarol before the EDN1-stimulation abrogates the enhanced TYR activity in EDN1-treated NHMs at 72 h post-stimulation, the level of which does not exceed the non-stimulated basal level. This abrogating effect is accompanied by the significantly attenuated expression levels of TYR at the transcriptional and translational levels. Therefore, it is likely that arenarol attenuates the EDN1-stimulated melanogenesis by down-regulating levels of melanocyte-specific proteins, especially TYR, at the transcriptional level. Since the expression of these 3 melanocyte-specific genes tested are downstream targets of MITF and are regulated by the MITF protein level (Bentley et al. 1994; Bertolotto et al. 1998; Sato-Jin et al. 2008) which is known to be up-regulated at the

transcriptional and translational levels by EDN1 stimulation of NHMs (Sato-Jin et al. 2008), we asked whether the abrogating effects of arenarol are associated with changes in the gene and/or protein levels of MITF. Interestingly, MITF is markedly up-regulated in EDN1-treated NHMs, but is significantly suppressed by treatment with arenarol at both the transcriptional and translational levels. The concomitant abrogating effects on the up-regulated levels of these 3 melanocyte-specific proteins and their targeted transcription factor MITF by arenarol at the transcriptional and translational levels strongly suggests that the EDN1-triggered intracellular signaling is interrupted by arenarol upstream of the expression of MITF. This prompted us to examine the effects of arenarol on the EDN1-activated intracellular signaling pathway which leads to the increased expression of MITF.

In NHMs, EDN1 binding to its receptor EDNRB accelerates the hydrolysis of polyphosphoinositide by the action of phospholipase C γ , which produces IP3 and diacylglycerol which in turn mobilizes intracellular Ca²⁺⁺ and activates PKC, respectively (Yada et al. 1991). The direct phosphorylation of Raf-1 by PKC isoforms has been suggested to play an essential role in the activation mechanism of subsequent MAPK signaling pathways consisting of the Raf-1/MEK/ERK1/2/CREB-signaling cascades (Kolch et al. 1993; Schönwasser et al. 1998; Mason et al. 1999; Marais et al. 1998). The activation of PKC also increases levels of cyclic AMP and the subsequent activation of PKA results in the phosphorylation of CREB which is also induced by the action of RSK kinase following ERK activation (Imokawa et al. 2000). The activation of CREB through dual phosphorylation, both by PKA and by RSK activation, leads to the increased gene and protein expression of the melanocyte-master transcription factor, MITF (Sato-Jin et al. 2008). Since the EDN1-triggered intracellular signaling pathway includes the SCF-triggered MAPK signaling and α MSH-triggered cAMP/PKA intracellular signaling cascades (Imokawa et al. 2000), we first examined the effect of arenarol on EDN1-signaling by focusing on the phosphorylation of Raf-1s, MEK, ERK, MITF and CREB. Western blot analysis of the phosphorylation of intracellular signaling molecules in the MAPK pathway revealed that among the signaling intermediate proteins tested, the EDN1-stimulated

Fig. 4 Effect of arenarol on the expression of melanocyte-specific proteins in NHMs at the transcriptional and translational levels. **A** Transcriptional levels of TYR, TYRP-1 and DCT. **B** Translational levels of TYR, TYRP-1 and DCT. **C** Transcriptional and translational levels of MITF. NHMs were treated with arenarol at a concentration of 3.0 μ M and after 3 h of culture, EDN1 (10 nM) was added and NHMs were cultured for further 1 (only for MITF) or 12 h after which real-time RT-PCR was performed using primers for MITF (**C-a**) or TYR (**A-a**), TYRP-1 (**A-b**) and DCT (**A-c**). NHMs were treated with arenarol at the indicated concentrations and after 3 h of culture, EDN1 (10 nM) was added and the cells were cultured for further 6 (only for MITF) or 24 h after which western blot analysis was performed using antibodies to MITF (**C-b**), or TYR (**B-a, b**), TYRP-1 (**B-a, c**) and DCT (**B-a, d**). Representative immunoblots from 3 independent experiments are shown. Values are mean $\pm$ SD derived from 3 independent experiments. * $p < 0.05$, ** $p < 0.01$ compared with EDN1-stimulated and arenarol-untreated control

phosphorylation of MEK, ERK, MITF and CREB, but not of Raf-1s, is distinctly abrogated by the treatment with arenarol at 15 min post EDN-1-treatment. Since in EDN1-triggered NHMs, CREB is phosphorylated by dual signaling pathways consisting of the PKC/MAPK and cAMP/PKA pathways (Sato-Jin et al. 2008), we asked whether the abrogating effect of arenarol on the EDN1-induced phosphorylation of CREB is also mediated via interruption of the cAMP/PKA cascade. Western blotting analysis of CREB phosphorylation in the forskolin-activated cAMP/PKA pathway revealed that CREB phosphorylation stimulated by forskolin is not affected by arenarol. This suggests that the signaling site that is initially affected by arenarol occurs at the phosphorylation of MEK (MAPKK) due to Raf-1 (MAPKKK) kinase activity, followed by the suppressed phosphorylation of ERK (MAPK), which leads to the diminished phosphorylation of MITF and CREB.

As for the signaling mechanism involved in the abrogated phosphorylation of MEK by arenarol, there are at least 3 possibilities; (1) arenarol may directly inhibit Raf-1 activity which would also lead to the attenuation of MEK phosphorylation, (2) arenarol may stimulate protein phosphatases probably conjugated with MEK, which dephosphorylate the phosphorylated MEK, and/or (3) arenarol may interrupt Raf-1 activation which would result in the attenuated phosphorylation of MEK. As for the first possibility, an assay for Raf-1 activity revealed that arenarol at concentrations of 0.3, 1.0 and 3.0 μ M has no ability to

inhibit Raf-1 activity in vitro. Further, as for the second possibility, our inhibition study with the serine/threonine protein phosphatase inhibitor okadaic acid demonstrated that the abrogating effect of arenarol on the up-regulated phosphorylation of MEK is not reversed by pretreatment with okadaic acid. It is likely that the abrogating effect of arenarol on the EDN1-induced phosphorylation of MEK is not involved in a stimulatory effect of arenarol on the activity of serine/threonine protein phosphatase probably conjugated with MEK.

As for the third possibility, it should be noted that Raf-1 activation mechanisms are very complicated with essential requirements of Raf-1-Ras interaction (Marais et al. 1998) and multiple phosphorylation sites with different functions for Raf-1 activation (Abraham et al. 2000). Raf-1 serves as a convergent signaling molecule between tyrosine kinase and PKC signaling, and it is phosphorylated and activated by Ras GTP protein through Ras-Raf-1 interaction during tyrosine kinase/MAPK signaling. As for the detailed molecular mechanism of Raf-1 activation, although Raf-1 binds directly to the effector domain of activated GTP-bound Ras proteins, this interaction does not stimulate Raf-1 kinase activity (Moodie et al. 1993). Rather, the role of the Ras-Raf interaction is to recruit Raf-1 to the plasma membrane, where it can be activated by membrane lipids or other protein kinases (Stokoe et al. 1994; Leever et al. 1994). The phosphorylation of Raf-1 both on tyrosine and on serine/threonine residues is required for its enzymatic activity. On the other hand, during EDN1/PKC/MAPK signaling, Raf-1 is also phosphorylated at S499 or S497 by PKC for the Raf-1 activation (Schonwasser et al. 1998) for which the formation of Ras-GTP-Raf complexes are also essentially required (Marais et al. 1998). In this study, we used 3 antibodies to serine 259/338 and tyrosine 340/341 phospho-Raf-1 for the following reasons; Raf-1 is phosphorylated at serine 338 by Ras activation in the tyrosine kinase associated signaling linkage (Chong et al. 2003). Ser 259 of Raf-1 has been identified as an inhibitory phosphorylation site (Abraham et al. 2000) and is phosphorylated by PKC (Kolch et al. 1993), substantially reflecting a phosphorylation pattern similar to PKC-activated-Raf-1 with stimulatory phosphorylation sites such as serine 497 or 499 (Kolch et al. 1993; Carroll and May 1994; Barnard et al. 1998) to which antibodies are currently not

Fig. 5 Effect of arenarol on EDN1 signaling in NHMs.

a Western blotting of lysates of EDN1-stimulated NHMs, **b** Densitometric analysis of western blots of lysates of EDN1-stimulated NHMs. **c** Forskolin stimulated NHMs. NHMs were treated with 10 nM EDN1 (**a/b**) or 50 μ M forskolin (**c**) in the presence or absence of arenarol at the indicated concentrations. Lysates were harvested at 15 min post-treatment with EDN1 or forskolin and were subjected to western blotting using antibodies to both phospho- or non-phospho-Raf-1s, MEKs, ERKs, CREBs and MITF. Representative immunoblots from 3 independent experiments are shown. Values are mean $\pm$ SD derived from 3 independent experiments. * $p < 0.05$, ** $p < 0.01$ compared with EDN1-stimulated and arenarol-untreated control

commercially available. Based on these findings, our observation that EDN1-stimulated Ser phosphorylation of Raf-1 at 259 or 338 is not abrogated by arenarol in concert with the suppressed phosphorylation of subsequent MEK suggests the third possibility that Raf-1 activation is interrupted by arenarol via impaired phosphorylation mechanisms other than Ser 259 or 338 phosphorylation, which are known as

inhibitory or stimulatory phosphorylation, respectively, although further studies are required to reach a final conclusion about this.

In conclusion, the sum of these findings suggests that arenarol abrogates the EDN1-stimulated expression of melanocyte-specific proteins by interrupting MEK phosphorylation in an as yet unknown Raf-1 inactivation mechanism.

Fig. 6 Effect of arenarol on Raf-1 kinase activity in vitro and effect of okadaic acid on MEK phosphorylation. **a** Effect on Raf-1 kinase activity: in vitro Arenarol was pre-incubated for 3 h with activated Raf-1 and Raf-1 activity was measured as described in “Materials and methods”. **b** Effect of okadaic acid: NHMs were starved for 12 h and were then preincubated with arenarol and/or okadaic acid for 3 h at the indicated concentrations. MEK phosphorylation was evaluated as described in “Materials and methods”. Representative immunoblots from 3 independent experiments are shown

Acknowledgments We thank GCOE and Leading Program in Nagoya University and a scholarship of Mitsubishi UFJ Trust and Banking for their financial supports.

References

- Abdel-Malek Z, Swope VB, Suzuki I, Akcali C, Harriger MD, Boyce ST, Urabe K, Hearing VJ (1995) Mitogenic melanogenic stimulation of normal human melanocytes by melanotropic peptides. *Proc Natl Acad Sci USA* 92:1789–1793
- Abraham D, Podar K, Pacher M, Kubicek M, Welzel N, Hemmings BA, Dilworth SM, Mischak H, Kolch W, Baccarini M (2000) Raf-1-associated protein phosphatase 2A as a positive regulator of kinase activation. *J Biol Chem* 275:22300–22304
- Ando H, Funasaka Y, Oka M, Ohashi A, Furumura M, Matsunaga J, Matsunaga N, Hearing VJ, Ichihashi M (1999) Possible involvement of proteolytic degradation of tyrosinase in the regulatory effect of fatty acids on melanogenesis. *J Lipid Res* 40:1312–1316
- Barnard D, Diaz B, Clawson D, Marshall M (1998) Oncogenes, growth factors and phorbol esters regulate Raf-1 through common mechanisms. *Oncogene* 17:1539–1547
- Bentley NJ, Eisen T, Goding CR (1994) Melanocyte-specific expression of the human tyrosinase promoter: activation by the microphthalmia gene product and role of the initiator. *Mol Cell Biol* 14:7996–8006
- Bertolotto C, Buscà R, Abbe P, Bille K, Aberdam E, Ortonne JP, Ballotti R (1998) Different cis-acting elements are involved in the regulation of TRP1 and TRP2 promoter activities by cyclic AMP: pivotal role of M boxes (GTCATGTGCT) and of microphthalmia. *Mol Cell Biol* 18:694–702
- Bialojan C, Takai A (1988) Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. Specificity and kinetics. *Biochem J* 256:283–290
- Carroll MP, May WS (1994) Protein kinase C-mediated serine phosphorylation directly activates Raf-1 in murine hematopoietic cells. *J Biol Chem* 269:1249–1256
- Chong H, Vikis HG, Guan KL (2003) Mechanisms of regulating the Raf kinase family. *Cell Signal* 15:463–469
- Chung KW, Park YJ, Choi YJ, Park MH, Ha YM, Uehara Y, Yoon JH, Chun P, Moon HR, Chung HY (2012) Evaluation of in vitro and in vivo anti-melanogenic activity of a newly synthesized strong tyrosinase inhibitor (E)-3-(2,4 dihydroxybenzylidene)pyrrolidine-2,5-dione (3-DBP). *Biochim Biophys Acta* 1820:962–969
- Hachiya A, Kobayashi A, Ohuchi A, Takema Y, Imokawa G (2001) The paracrine role of stem cell factor/c-kit signaling in the activation of human melanocyte in ultraviolet B-induced pigmentation. *J Invest Dermatol* 116:578–586
- Hachiya A, Kobayashi A, Yoshida Y, Kitahara T, Takema Y, Imokawa G (2004) Biphasic expression of two paracrine melanogenic cytokines, stem cell factor and endothelin-1, in ultraviolet B-induced human melanogenesis. *Am J Pathol* 65:2099–2109
- Hardie DG (1990) Roles of protein kinases and phosphatases in signal transduction. *Symp Soc Exp Biol* 44:241–255
- Hattori H, Kawashima M, Ichikawa Y, Imokawa G (2004) The epidermal stem cell factor is over-expressed in lentigo senilis: implication for the mechanism of hyperpigmentation. *J Invest Dermatol* 122:1256–1265
- Imokawa G, Mishima Y (1982) Loss of melanogenic properties in tyrosinases induced by glycosylation inhibitors within malignant melanoma cells. *Cancer Res* 42:1994–2002
- Imokawa G, Mishima Y (1984) Functional analysis of tyrosinase isozymes of cultured melanoma cells during the recovery period following interrupted melanogenesis induced by glycosylation inhibitors. *J Invest Dermatol* 83:196–201
- Imokawa G, Yada Y, Miyagishi M (1992) Endothelins secreted from human keratinocytes are intrinsic mitogens for human melanocytes. *J Biol Chem* 267:24675–24680
- Imokawa G, Miyagishi G, Yada Y (1995) Endothelin-1 as a new melanogen: coordinated expression of its gene and the tyrosinase gene in UVB-exposed human epidermis. *J Invest Dermatol* 105:32–37
- Imokawa G, Kobayashi T, Miyagishi M (2000) Intracellular signaling mechanisms leading to synergistic effects of endothelin-1 and stem cell factor on proliferation of cultured human melanocytes: cross-talk via trans-activation of the tyrosine kinase c-kit receptor. *J Biol Chem* 275:33321–33328
- Kadono S, Manaka I, Kawashima M, Kobayashi T, Imokawa G (2001) The role of the epidermal endothelin cascade in the hyperpigmentation mechanism of lentigo senilis. *J Invest Dermatol* 116:571–577
- Kolch W, Heidecker G, Kochs G, Hummel R, Vahidi H, Mischak H, Finkenzeller G, Marmé D, Rapp UR (1993) Protein kinase C alpha activates RAF-1 by direct phosphorylation. *Nature* 364:249–252
- Komiyama K, Takamatsu S, Takahashi Y, Shinose M, Hayashi M, Tanaka H, Iwai Y, Omura S, Imokawa G (1993) New inhibitors of melanogenesis, OH-3984 K1 and K2, I. taxonomy, fermentation, isolation and biological characteristics. *J Antibiot* 46:1520–1525
- Leevers SJ, Paterson HF, Marshall CJ (1994) Requirement for Ras in Raf activation is overcome by targeting Raf to the plasma membrane. *Nature* 369:411–414

- Marais R, Light Y, Mason C, Paterson H, Olson MF, Marshall CJ (1998) Requirement of Ras-GTP-Raf complexes for activation of Raf-1 by protein kinase C. *Science* 280:109–112
- Mason CS, Springer CJ, Cooper RG, Superti-Furga G, Marshall CJ, Marais R (1999) Serine and tyrosine phosphorylations cooperate in Raf-1, but not B-Raf activation. *EMBO J* 18:2137–2148
- Moodie SA, Willumsen BM, Weber MJ, Wolfman A (1993) Complexes of Ras.GTP with Raf-1 and mitogen-activated protein kinase kinase. *Science* 260:1658–1661
- Paine C, Sharlow E, Liebel F, Eisinger M, Shapiro S, Seiberg M (2001) An alternative approach to depigmentation by soybean extracts via inhibition of the PAR-2 pathway. *J Invest Dermatol* 116:587–595
- Sato-Jin K, Nishimura EK, Akasaka E, Huber W, Nakano H, Miller A, Du J, Wu M, Hanada K, Sawamura D, Fisher DE, Imokawa G (2008) Epistatic connections between MITF and endothelin signaling in Waardenburg syndrome and other pigmentary disorders. *FASEB J* 22:1155–1168
- Schmiz FJ, Lakshmi V, Powell DR, Helm DVD (1984) Arenarol and arenarone: sesquiterpenoids with rearranged drimane skeletons from the marine sponge *Dysidea arenaria*. *J Org Chem* 49:241–244
- Schönwasser DC, Marais RM, Marshall CJ, Parker PJ (1998) Activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway by conventional, novel, and atypical protein kinase C isotypes. *Mol Cell Biol* 18:790–798
- Stokoe D, Macdonald SG, Cadwallader K, Symons M, Hancock JF (1994) Activation of Raf as a result of recruitment to the plasma membrane. *Science* 264:1463–1467
- Suzuki I, Cone RD, Im S, Nordlund J, Abdel-Malek ZA (1996) Binding of melanotropic hormones to the melanocortin receptor MC1R on human melanocytes stimulates proliferation and melanogenesis. *Endocrinology* 137:1627–1633
- Winder AJ, Harris H (1991) New assays for the tyrosine hydroxylase and dopa oxidase activities of tyrosinase. *Eur J Biochem* 198:317–326
- Yada Y, Higuchi K, Imokawa G (1991) Effects of endothelins on signal transduction and proliferation in human melanocytes. *J Biol Chem* 266:18352–18357
- Yoo HD, Leung D, Sanghara J, Daley D, Soest R, Andersen RJ (2003) Isoarenarol, a new protein kinase inhibitor from the marine sponge *Dysidea arenaria*. *Pharm Biol* 41:223–225