Substance P Stimulates Endothelin 1 Secretion via Endothelin-Converting Enzyme 1 and Promotes Melanogenesis in Human Melanocytes

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Substance P (SP) is a well-known neuropeptide implicated in the wound-healing process. The wound occasionally causes a pigmented scar. In the present study, we examined whether increased levels of SP affected melanogenesis. When human melanocytes were treated with SP, the melanin content increased and the pigmentation process accelerated in a dose-dependent manner. In addition to melanogenesis-related genes, the expression of neurokinin 1 receptor, endothelin 1 (EDN1), and EDN receptor type B (EDNRB) also increased at both the messenger RNA and protein levels. Interestingly, secreted EDN1 was observed in the melanocyte culture medium, and this phenomenon was significantly enhanced by SP treatment. Through knockdown experiments using small interfering RNAs (siRNAs), we confirmed that endothelin-converting enzyme 1 (ECE1), EDN1, and EDNRB were involved in SP-induced pigmentation and found that EDN1 secretion was affected by ECE1 and EDN1 siRNAs, but not by EDNRB siRNA. These findings indicate that ECE1 is essential for EDN1 secretion in melanocytes and that EDNRB functions downstream of secreted EDN1 to increase the cAMP levels and activate the melanogenesis-related phosphorylation cascade. This study provides *in vitro* evidence for a melanogenic function of SP in the skin and suggests that the SP-related signal is a potent target for regulating stress- or wound-induced pigmentation.

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INTRODUCTION

Substance P (SP) is one of the most abundant neuropeptides in the central nervous system and is implicated in a variety of physiological and psychological processes, including mental stress regulation and wound healing (O'connor *et al.*, 2004; Ebner and Singewald, 2006). SP belongs to the tachykinin family, is composed of 11 amino acids, and binds to at least three types of G protein–coupled receptors (the neurokinin receptors NK1R, NK2R, and NK3R), among which NK1R shows the highest affinity for SP (De Swert and Joos, 2006). Activation of the SP–NKR signaling pathway mediates various biological processes, such as pain (Summer *et al.*, 2007; Wei *et al.*, 2009), stress (Ebner and Singewald, 2006), anxiety (Ebner *et al.*, 2008), vasodilation (Lembeck and Holzer, 1979; Carthew *et al.*, 2012), wound healing, and inflammation (Wei *et al.*, 2009). As a physiological regulator or stress mediator of the human body, SP controls blood vessel relaxation and plasma exudation from post-capillary venules, leading to interstitial edema (Lembeck and Holzer, 1979; Carthew *et al.*, 2012).

When skin is stressed, SP is released from sensory nerves present in the upper dermal nerve plexus; these sensory nerves also innervate the epidermis (Hökfelt *et al.*, 1979; Hartschuh *et al.*, 1983). The released SP initiates skin inflammation via induction of vasodilation, plasma extravasation, and accumulation of immune factors (Katsanos *et al.*, 2008), as well as uncomfortable skin sensations such as numbness, itching (Andoh *et al.*, 1998), sensitivity, and tingling (Summer *et al.*, 2007; Wei *et al.*, 2009). SP is also involved in stress-induced hair loss (Peters *et al.*, 2007; Liu *et al.*, 2013). Among the representative neuropeptides that are released under stress conditions, calcitonin gene-related peptide is known to induce skin pigmentation (Toyoda *et al.*, 1999); however, the effect of SP on melanogenesis in humans is not known.

Melanin produced by melanocytes is responsible for the protection of skin cells against cancers and potential damage caused by UV radiation and environmental challenges (Costin and Hearing, 2007; Lin and Fisher, 2007). Despite its beneficial effects, dysregulation of melanin synthesis causes unexpected hyperpigmentation disorders, such as melasma

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Abbreviations: ECE1, endothelin-converting enzyme 1; EDN1, endothelin 1; HEMn, human epidermal melanocyte derived from neonate; KaMC, keratinocyte-adapted melanocytes isolated from keratinocyte culture; αMSH, alpha-melanocyte stimulating hormone; MITF, microphthalmia-associated transcription factor; mRNA, messenger RNA; PKA, protein kinase A; siRNA, small interfering RNA; SP, substance P

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and lentigo. Area of scarring, a natural result of the wound-healing process that is initiated by the SP signal cascade (Harrison and Geppetti, 2001), is also a major region of hyperpigmentation (Chadwick *et al.*, 2012). Melanocyte- or dermis-derived stem cells are potentially involved in pigmentation in the scarred area (Zabierowski *et al.*, 2011); however, the underlying mechanism remains unknown.

In the present study, we examined the effect of SP on melanogenesis in human melanocytes and in a reconstituted human skin model. We observed that SP accelerated melanogenesis and increased melanin production in a dose-dependent manner. We verified the involvement of the NK1R–ECE1–EDN1–EDNRB pathway in SP-induced pigmentation using small interfering RNAs (siRNAs). Furthermore, we detected a considerable increase in endothelin 1 (EDN1) secretion after SP treatment and verified that endothelin-converting enzyme 1 (ECE1) was an essential factor for this process. Finally, we examined the cAMP levels and the downstream phosphorylation cascade that is activated during SP-induced melanogenesis.

RESULTS

SP increases pigmentation in human melanocytes

To examine whether SP in the skin affects melanogenesis, we treated primary human epidermal melanocytes derived from neonates (HEMns) and keratinocyte-adapted melanocytes isolated from keratinocyte cultures (KaMCs) (Cho *et al.*, 2014) with various concentrations of SP. Over 5 nM SP affected the viability of both cell types (Supplementary Figure S1a online); therefore, we examined pigmentation in the presence of 1 or 2 nM SP, which did not affect viability or cause DNA damage (Supplementary Figure S1 online). SP treatment induced pigmentation and increased melanin content in both cell types in a dose-dependent manner (Figure 1a and b). This phenomenon was also observed in primary human epidermal melanocytes derived from adults (HEMas) (Supplementary Figure S2 online). We examined the effects of SP over extended periods of time by treating the cells with SP every other day for 14 days and found that SP-treated cells showed an increase in pigmentation at an earlier time point in a dose-dependent manner compared with the non-treated control (Figure 1c). The melanin levels in cells treated with 2 nM SP became saturated and reached a plateau at ~day 5; however, in the non-treated cells, the melanin levels reached a plateau at ~day 14, and thereafter no additional differences in melanin content were observed between SP-treated and non-treated cells. To estimate the relative effect of SP on melanogenesis, we compared it with alpha-melanocyte stimulating hormone (αMSH), a representative pigmentary neuropeptide. We treated KaMCs with 2 nM SP or different concentrations of αMSH (100, 500 nM) for 14 days and compared the melanin content produced. Interestingly, compared with the plateau in the melanin content around day 5 in response to SP, αMSH reached a plateau at around day 10, although a much higher concentration of αMSH (500 nM) was used (Supplementary Figure S3a online). A lower concentration of αMSH (100 nM) resulted in a marginal increase in melanin levels over 2 weeks, suggesting that, compared with αMSH, SP had a more potent effect on *in vitro* melanogenesis.

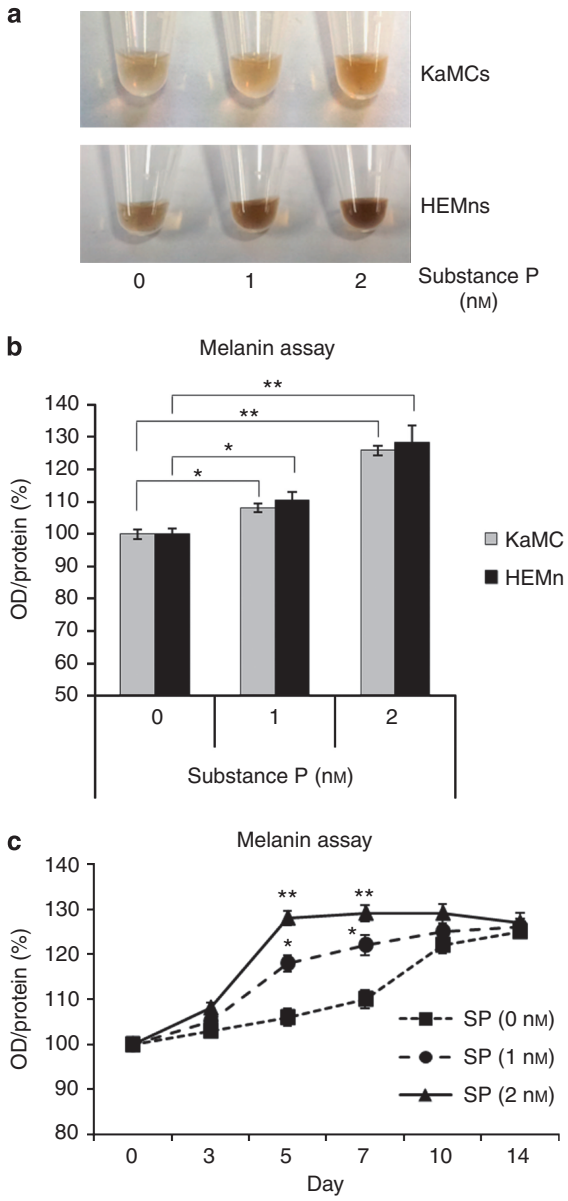

Figure 1. Substance P (SP) increases pigmentation in human melanocytes. (**a**) Primary human epidermal melanocytes derived from neonates (HEMns) and keratinocyte-adapted melanocytes (KaMCs) were treated with 1 or 2 nM substance P for 5 days. The cell pellets were dissolved in 1 N NaOH solution, and representative images were obtained. (**b**) The melanin content was determined by measuring the absorbance at 490 nm. The melanin level was normalized to the protein input. The data are presented as mean ± SD ($n = 5$; $^*P < 0.05$, $^{**}P < 0.01$). OD, optical density. (**c**) The melanin content was analyzed at 0, 3, 5, 7, 10, and 14 days post treatment of SP in HEMns. The data are presented as mean ± SD ($n = 5$; $^*P < 0.05$, $^{**}P < 0.01$).

SP stimulates the expression of pigmentation-related genes

We examined the messenger RNA (mRNA) and protein expression levels of melanogenesis-related genes by real-time

quantitative PCR and western blot analysis after SP treatment. As anticipated, the mRNA expression of microphthalmia-associated transcription factor (MITF), TRP1, TRP2, TYR, MART1, and KIT significantly increased when the cells were treated with 1 or 2 nM SP (Figure 2a), and the protein levels of MITF, TRP1, TRP2, and TYR accordingly increased in both KaMCs and HEMns (Figure 2b). These results suggest that SP stimulated the expression of melanogenesis-related genes, and similar effects were observed after treating melanocytes with a high concentration of αMSH (Supplementary Figure S3b and S3c online).

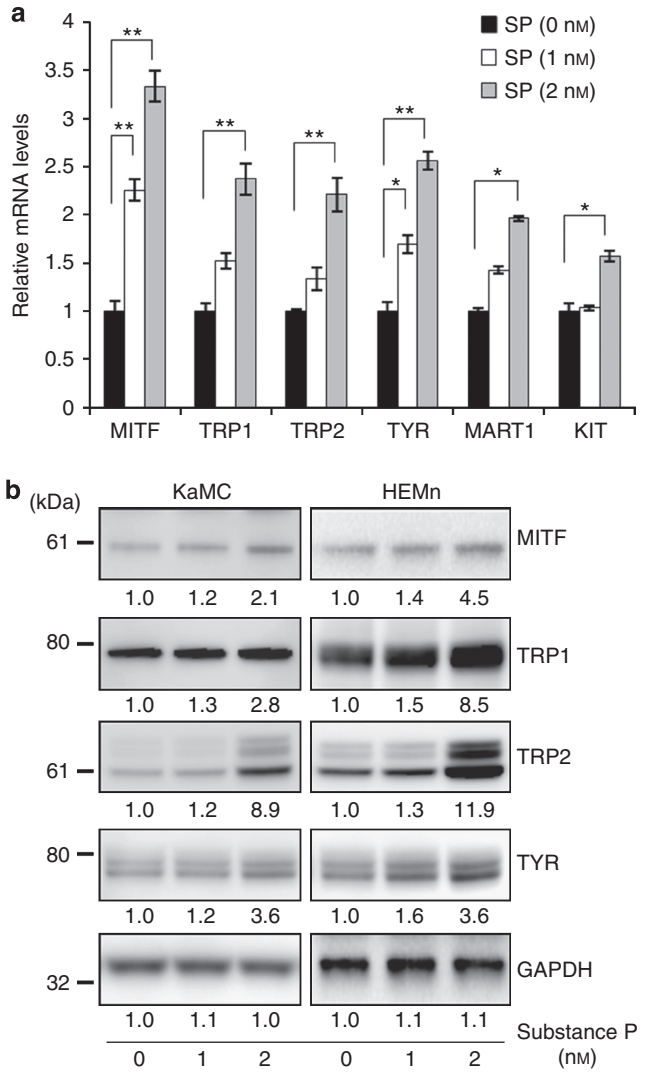

Figure 2. Substance P (SP) increases the expression of pigmentation-related genes. The expression levels of messenger RNAs (mRNAs) (**a**) were determined by real-time quantitative PCR at 48 hours in keratinocyte-adapted melanocytes (KaMCs), and the protein levels (**b**) were analyzed by western blot analysis at day 5 in KaMCs and human epidermal melanocytes derived from neonates (HEMns) after treatment with 1 or 2 nM SP. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA and protein levels were used for the normalization of each mRNA and as a loading control for the protein. The data are presented as mean ± SD ($n=3$; $^{*}P<0.05$, $^{**}P<0.01$).

SP upregulates the expression of NK1R, EDN1, and EDNRB in addition to EDN1 secretion

SP and its receptor NK1R are endocytosed after the formation of the SP–NK1R complex, and ECE1 may be involved in SP degradation and induce NK1R recycling following endocytosis of the SP–NK1R complex (Roosterman *et al.*, 2007; Pelayo *et al.*, 2011). ECE1 also has a role in cleaving Big-EDN to generate an active, secretory EDN1 (Khimji and Rockey, 2011). Thus, we examined the expression of NK1R, ECE1, and EDNs to determine whether they functioned as downstream signaling molecules for SP. NK1R and EDN1 mRNA and protein were expressed in KaMCs, and the levels prominently increased after SP treatment in a dose-dependent manner (Figure 3a and b, NK1R, EDN1). By contrast, the mRNA and protein levels of ECE1, EDN2, and EDN3 were not significantly altered with SP treatment (Figure 3a and b, ECE1, EDN2, EDN3). Similar SP effects were observed in HEMns (Supplementary Figure S4a and S4b online). Interestingly, using an ELISA analysis, we observed that EDN1 was secreted into the culture media of both KaMCs and HEMns, and this secretion was significantly enhanced by SP treatment (Figure 3c). Initially, we measured the secreted EDN1 at day 5 when the melanin level reached a plateau after SP treatment, but the secretion had already occurred as early as 72 hours, when the mRNA levels of EDN1 and melanogenesis-related genes were still elevated (Supplementary Figure S4c and S4d online). Considering that EDN1 is not secreted without cleavage, these data imply that ECE1 is involved in the secretion of EDN1. Among the EDN receptors, the protein expression of EDNRA was relatively high but was not affected by SP treatment (Figure 3b; Supplementary Figure S4b online, EDNRA). However, the expression of EDN receptor type B (EDNRB) protein, which is primarily involved in melanogenesis, was increased in response to SP treatment (Figure 3b; Supplementary Figure S4b online, EDNRB). These data suggest that the NK1R–ECE1–EDN1–EDNRB cascade is potentially involved in SP-induced pigmentation.

The NK1R–ECE1–EDN1–EDNRB signaling cascade is involved in SP-induced pigmentation

To verify whether the NK1R–ECE1–EDN1–EDNRB signaling cascade mediates SP-induced pigmentation, we performed an siRNA assay against each component. SP treatment increased the melanin content compared with the non-treated control (Figure 4a and b, first two lanes). However, when each siRNA against ECE1, EDN1, or EDNRB was applied to the melanocytes, a marked reduction in melanin content was observed even in the presence of SP, which is in contrast to that observed in cells treated with a scrambled control siRNA (Figure 4a and b, ECE1, EDN1, EDNRB siRNAs). The EDNRA siRNA also reduced the melanin content, but not to the level observed for the other siRNAs (Figure 4a and b, EDNRA siRNA). We confirmed the reduction of each protein after siRNA treatment by western blot analysis using specific antibodies (Figure 4c, ECE1, EDN1, EDNRA, and EDNRB). When ECE1, EDN1, and EDNRB were downregulated by the siRNAs, the levels of the TYR and TRP2 proteins were notably decreased in the presence of SP compared with the scrambled

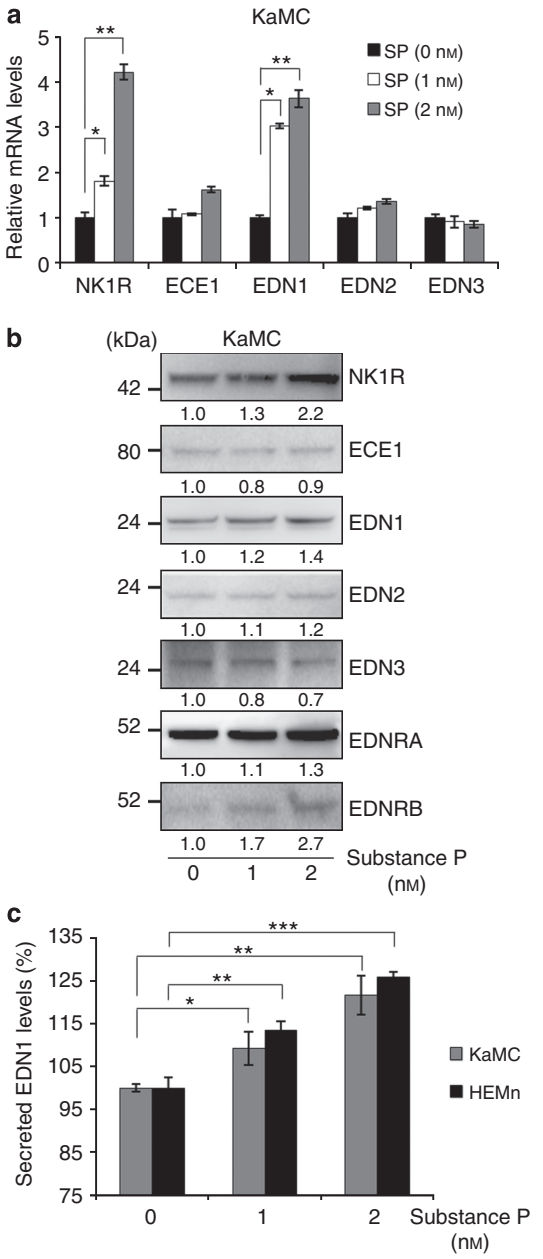

Figure 3. Substance P (SP) upregulates the expression of NK1R, endothelin (EDN)1, and EDN receptor type B (EDNRB) and EDN1 secretion. (**a**) The messenger RNA (mRNA) expression of each gene was determined by real-time quantitative PCR analysis at 48 hours after treatment with 1 or 2 nM SP in keratinocyte-adapted melanocytes (KaMCs). (**b**) The protein expression of each gene was analyzed by western blot analysis using the specific antibody for each protein at day 5 after SP treatment. (**c**) The amount of secreted EDN1 in the supernatant of cells treated with 1 or 2 nM SP for 5 days was measured by ELISA. The data are presented as mean ± SD ($n=5$; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$). HEMns, human epidermal melanocytes derived from neonates.

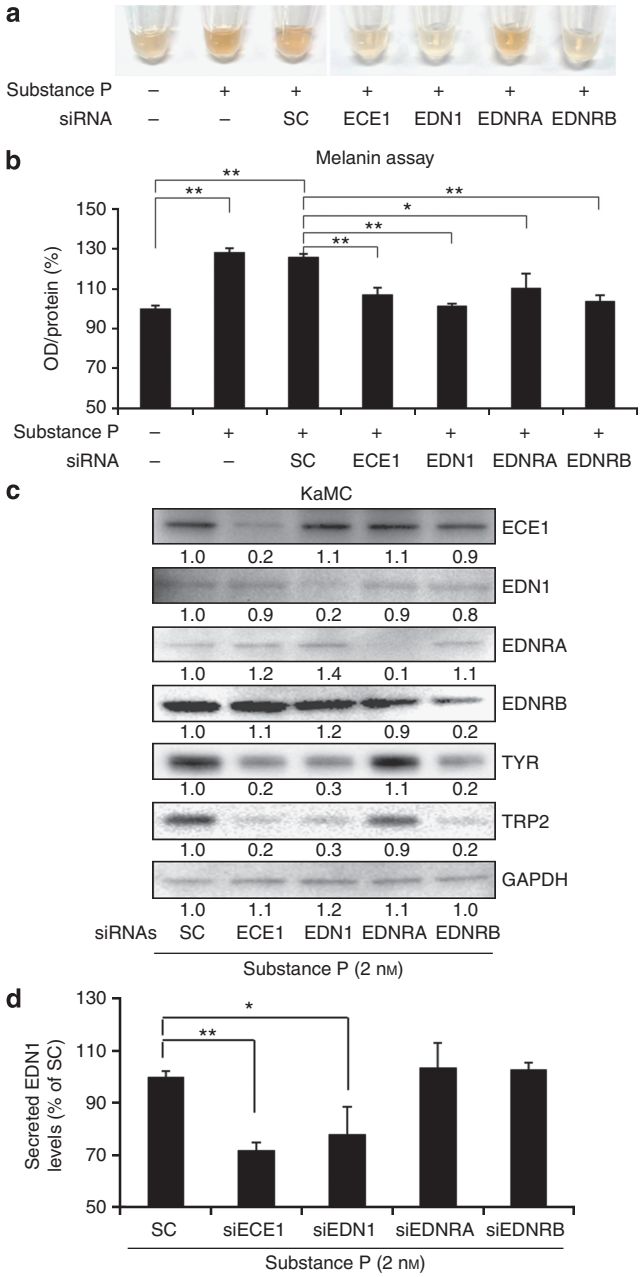

Figure 4. The NK1R–ECE1–EDN1–EDNRB signaling cascade is involved in substance P (SP)-induced pigmentation. (**a**) The small interfering RNA (siRNA) against each gene was introduced into keratinocyte-adapted melanocytes (KaMCs) every other day for 5 days in the presence of 2 nM SP. The cell pellets were dissolved in 1 N NaOH, and representative images were obtained. SC, scrambled siRNA control. (**b**) The melanin content was determined by measuring the absorbance at 490 nm. The total melanin level was normalized to protein input. The data are presented as mean ± SD ($n=3$; $^{*}P<0.05$, $^{**}P<0.01$). (**c**) In total, 25 μg of each protein was subjected to western blot analysis using specific antibodies after treatment of KaMCs with each siRNA. The protein expression levels of TYR and TRP2 were monitored after the introduction of each siRNA. (**d**) The amount of secreted EDN1 in the supernatant of KaMCs after treatment with each siRNA in the presence of SP was measured by ELISA at day 5. The data are presented as mean ± SD ($n=5$; $^{*}P<0.05$, $^{**}P<0.01$). ECE1, endothelin-converting enzyme 1; EDN1, endothelin 1; EDNRB, EDN receptor type B; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; OD, optical density.

control or EDNRA siRNA-treated cells (Figure 4c, TYR, TRP2), indicating that ECE1, EDN1, and EDNRB, but not EDNRA, are involved in SP-induced pigmentation. We also examined whether the secretion of EDN1 induced by SP treatment was affected by ECE1, EDN1, EDNRA, and EDNRB siRNAs. When

siRNAs against ECE1 or EDN1 were introduced, the level of secreted EDN1 was significantly reduced, but siRNAs against EDNRA and EDNRB did not affect EDN1 secretion (Figure 4d). This finding suggests that ECE1 is an upstream signaling molecule in this pathway and is responsible for the secretion of EDN1, but that EDNRB is a downstream effector of secreted EDN1 and the EDN1–EDNRB signaling cascade can be initiated upon binding of secreted EDN1 to EDNRB. To further verify the participation of NK1R and ECE1 in SP-induced pigmentation, we treated the cells with specific antagonists of NK1R and ECE1, RP-67580 (Pelayo *et al.*, 2011) and CGS35066 (Trapani *et al.*, 2000), respectively, and found that both antagonists significantly decreased melanin content, tyrosinase activity, and EDN1 secretion induced by SP treatment (Supplementary Figure S5 online). These data strongly suggest that NK1R and ECE1 are specifically involved in SP-induced pigmentation by affecting EDN1 secretion.

SP increases intracellular cAMP levels and activates melanogenesis-related phosphorylation signaling

After the EDN1–EDNRB signaling pathway is activated by the binding of EDN1 to EDNRB, the intracellular levels of cAMP increase and the downstream signaling cascade is activated to regulate MITF expression (Buscà and Ballotti, 2000). In the present study, we confirmed that ECE1 was essential for EDN1 secretion and that EDNRB was downstream of secreted EDN1 (Figure 4d). To verify that EDN1–EDNRB signaling was activated after SP treatment, we examined the cAMP levels and phosphorylation status of melanogenesis-related kinases. In both KaMCs and HEMns, the cAMP levels were significantly increased in response to SP treatment for 48 hours (Figure 5a). Considering that the cAMP levels usually increased within minutes of treatment, we evaluated a time course response over 48 hours. The levels of cAMP began to increase gradually and showed significant differences among the groups at ~24 hours, which were sustained thereafter (Supplementary Figure S6 online). We confirmed that phospho-protein kinase A (PKA), phospho-p38 mitogen-activated protein kinase (MAPK), and phospho-extracellular signal–regulated kinase (ERK)1/2 levels increased following SP treatment; however, the levels of phospho-p70^{S6K} decreased (Figure 5b). The unique expression pattern of each phospho-protein during melanogenesis is well known. For e.g., increased levels of cAMP result in the phosphorylation and activation of MITF via inhibition of p70^{S6K} (Cass and Meinkoth, 1998) and PKA phosphorylation in melanocytes (Bertolotto *et al.*, 1998). These data indicate that SP induces the activation of EDN1–EDNRB and its downstream melanogenesis-related signaling in human melanocytes.

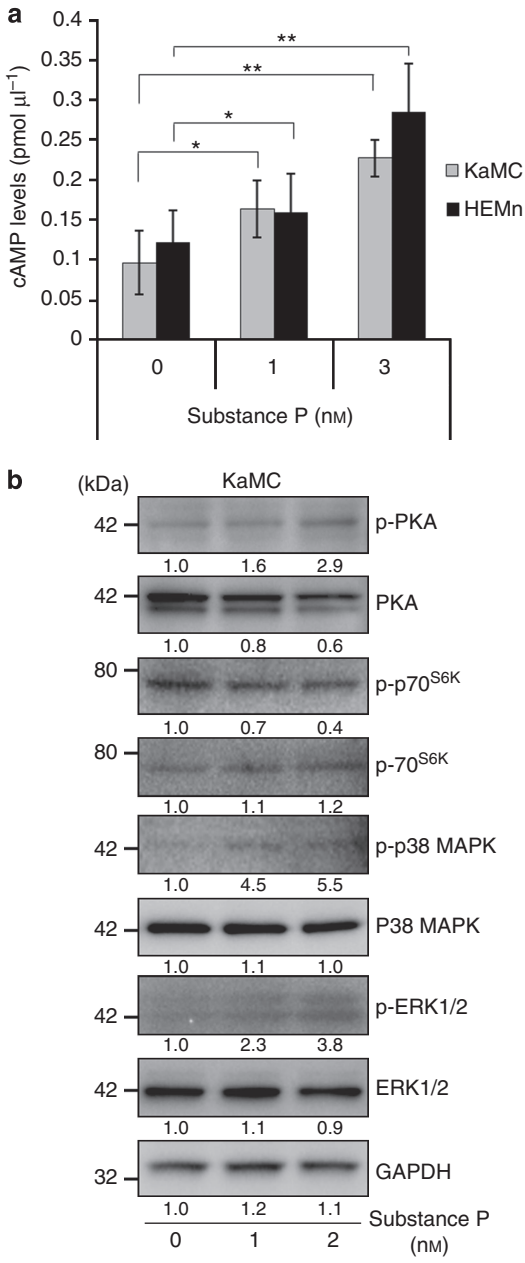

Figure 5. Substance P increases intracellular cAMP levels and activates melanogenesis-related phosphorylation signaling. (**a**) Keratinocyte-adapted melanocytes (KaMCs) and human epidermal melanocytes derived from neonates (HEMns) were treated with 1 or 2 nM SP for 48 hours and dissolved in 0.1 mM HCl. The cAMP levels were determined using the cAMP assay kit according to the manufacturer's instructions. The data are presented as mean ± SD ($n = 6$; *$P < 0.05$, **$P < 0.01$). (**b**) In total, 25 μg of protein from KaMCs treated with 1 or 2 nM SP was subjected to western blot analysis using the specific antibody against each phosphorylated form or total protein. ERK, extracellular signal–regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MAPK, mitogen-activated protein kinase; PKA, protein kinase A.

SP increases the melanin content in a reconstituted human skin model

To address the contribution of SP to the induction of human skin pigmentation, we treated a reconstituted human skin model containing melanocytes (Neoderm-ME) with different concentrations of SP and measured the melanin content. In response to continuous SP treatment for 3 weeks, the degree of pigmentation was strengthened and the total melanin content was significantly increased in 2 or 5 nM SP-treated wells compared with non-treated controls (Figure 6). These results

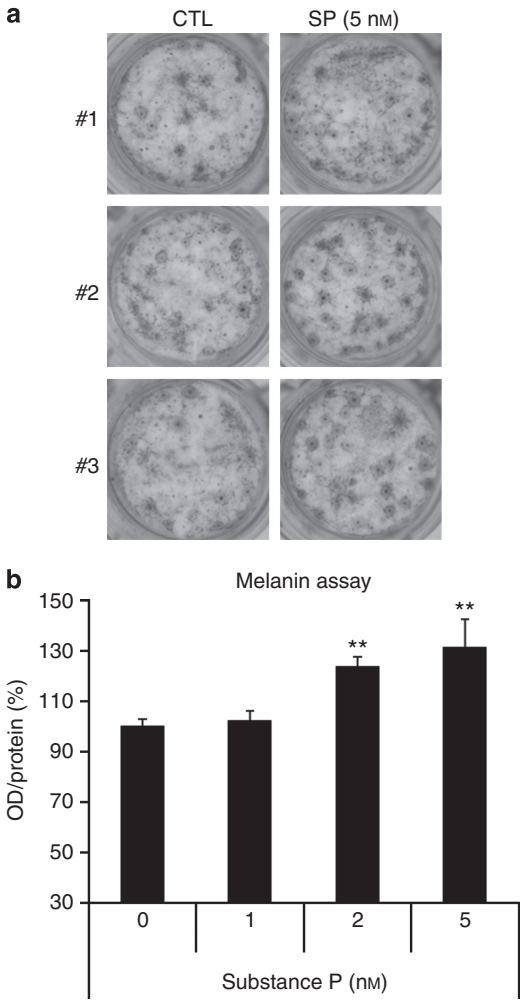

Figure 6. Substance P (SP) increases the melanin content in a reconstituted human skin model. The Neoderm-ME was treated with 0, 1, 2, or 5 nM SP for 3 weeks by replenishing the medium every other day. (**a**) The representative images were obtained from 5 nM SP-treated wells at day 21 post treatment. CTL, non-treated control. (**b**) The melanin content was determined by measuring the absorbance at 490 nm after dissolving the Neoderm-ME in 1 N NaOH solution. The melanin level was normalized to the total protein. The data are presented as mean ± SD ($n = 3$; $^{**}P < 0.01$). OD, optical density.

suggest that SP can contribute to melanogenesis in an *in vivo* environment in which melanocytes are surrounded by keratinocytes.

DISCUSSION

Many patients notice that scar regions are occasionally pigmented after the healing process; however, the mechanism regulating this pigmentation process remains unknown. SP is released from sensory nerve fibers by mentally and/or physically unbalanced conditions, such as pain, anxiety, and stress. SP also has a role as a regulatory factor in the wound-healing process and in the pro-inflammatory response. In the present study, we verified that SP, as a melanogenic factor, induces pigmentation by increasing EDN1 secretion via the NK1R–ECE1 pathway, resulting in the activation of the EDN1–EDNRB signaling cascade in human melanocytes and in a reconstituted human skin model.

Recently, it was reported that SP reduces pigmentation by inhibiting melanogenesis-related genes and proteins in the murine melanoma cell line B16-F10 (Ping *et al.*, 2012). However, when we treated human melanocytic cells with 1 or 2 nM SP, a concentration that is within the physiologically relevant range in human skin, which is ~1.3 nM in uninjured normal skin and 2.6 nM in hypertrophic scars (Scott *et al.*, 2005), the melanin content prominently increased in all tested human cells, such as HEMns, KaMCs, and human epidermal melanocytes derived from adults, in a dose-dependent manner (Figure 1; Supplementary Figure S2 online). Furthermore, we determined that SP treatment accelerated the pigmentation process until the melanin content reached a plateau rather than continuously increasing the pigment levels (Figure 1c). In addition, SP treatment resulted in a remarkable elevation of the mRNA and protein levels of melanogenesis-related genes within 5 days post treatment (Figure 2a and b). These results strongly suggest that the melanocytic cells originating from humans or mice have considerably different responses to SP and demonstrate opposite effects. Although the reason for this difference between humans and mice remains to be investigated, the different endogenous SP levels according to cell origins and types may be one possible reason for the inconsistent effects of SP on pigmentation.

NK1R has a high affinity for tachykinin family members, such as SP, hemokinins, and endokinins A and B (Muñoz *et al.*, 2010), and NK1R expression has been shown to be increased by SP treatment (Ping *et al.*, 2012). In agreement with a previous report, we found that NK1R is expressed in human melanocytes, and its mRNA and protein levels are elevated in response to SP treatment (Figure 3a and b). ECE1 was previously reported to be a regulator of SP–NK1R stability in cells overexpressing ECE1–GFP (green fluorescent protein) and myenteric neurons of the mouse intestine (Roosterman *et al.*, 2007; Pelayo *et al.*, 2011). When SP binds to NK1R, SP–NK1R-β-arrestin forms a signalosome and is endocytosed with ECE1 on the cell surface, generating an early endosome in which ECE1 disrupts the SP–NK1R complex potentially by degrading SP and inducing NK1R recycling to the plasma membrane (Roosterman *et al.*, 2007; Pelayo *et al.*, 2011). In addition to the role of ECE1 as a regulator of the endocytic signaling of SP–NK1R, ECE1 is a known key enzyme that mediates melanogenesis by catalyzing the specific cleavage of inactive Big-EDN1 to produce an active EDN1 in keratinocytes and endothelial cells (Yanagisawa *et al.*, 1988; Kido *et al.*, 1997), as demonstrated by the increased level of secreted EDN1 in several hyperpigmented lesions (Manaka *et al.*, 2001). ECE1 regulates the endocytic signaling and trafficking of SP1–NK1R and activates EDN1 via direct cleavage; therefore, we initially hypothesized that SP–NK1R-dependent endocytosed ECE1 interacts with and cleaves the inactive Big-EDN1 residing within vesicles in the secretory pathway in human melanocytes. We verified that EDN1 secretion and pigmentation after SP treatment were significantly reduced by ECE1 knockdown (Figure 4d), suggesting a role for ECE1 as a key mediator of SP-induced pigmentation by

regulating EDN1 secretion. In addition to facilitating an interaction between ECE1 and Big-EDN1 in response to SP-induced endocytosis, the increased EDN1 expression might also contribute in part to the elevated EDN1 secretion as long as it is cleaved by ECE1, which is a necessary step for EDN1 secretion.

In the skin, EDNs are usually released from keratinocytes and bind to EDNRs on melanocytes to relay the melanogenesis signaling cascade (Lahav 2005). Although we used a monoculture system without endothelial cells or keratinocytes, we successfully detected increased mRNA and protein expression of EDN1 and its secreted form in melanocytes after SP treatment. EDN2 and EDN3 were still expressed in melanocytes, but their mRNA and protein expression did not change after SP treatment (Figure 3a and b). These data indicate that the melanocyte itself produces active EDNs responsible for melanogenesis and that EDN1 is the primary EDN involved in SP-induced pigmentation.

The secreted, active EDN1 binds to the receptors EDNRA and EDNRB, which are required for the migration of melanoblasts during embryonic development (Imokawa *et al.*, 2000) and are expressed in melanocytes (Hachiya *et al.*, 2002). On the basis of the results obtained by western blot analysis and siRNA treatment (Figure 3b; Figure 4), EDNRB, but not EDNRA, protein levels were found to be increased after SP treatment and were involved in SP-induced pigmentation. After EDN1 binds to EDNRB, which occurred in an autocrine manner in our study, the complex initiates a well-known signaling pathway involved in pigmentation via cAMP (Buscà and Ballotti, 2000; Le Pape *et al.*, 2008). Furthermore, a phosphorylation cascade is activated that includes PKA (Yun *et al.*, 2011), p38 MAPK (Tachibana 2001), and ERK1/2 (Yanase *et al.*, 2001), which are considered to be phosphorylated, or $p70^{S6K}$, which is dephosphorylated, during melanogenesis (Ohguchi *et al.*, 2005). In accordance with these reports, we confirmed that the levels of cAMP increased and phosphorylation of melanogenesis-related kinases occurred (Figure 5).

To our knowledge it was previously unreported that SP, a pro-melanogenic factor, directly induces pigmentation by stimulating the specific signaling cascades. SP is released under stress conditions and accumulates around wounds; this finding may explain why after the healing process the scar becomes pigmented and skin becomes pigmented under physically or psychologically stressed conditions. SP and its related signaling pathways may be a potent target for depigmentation under stress or wound conditions in which SP naturally accumulates.

MATERIALS AND METHODS

Cell culture

Primary HEMns were purchased from Life Technologies (Carlsbad, CA), and KaMCs (Cho *et al.*, 2014) were developed and kindly provided by Dr Eun-Gyung Cho (Bioscience Research Division, R&D Unit, AmorePacific Corporation, Yongin, Gyeonggi-do, Korea) who is an author in this study. The AmorePacific institutional review board agreed to use human primary cells without its approval as far as cells were commercially available and thus purchased from a company. Both cell types were cultured in a humidified incubator containing 5% CO_2 in Medium 254 (M254; Life Technologies) supplemented with human melanocyte growth supplement (Life Technologies). The medium was replaced every other day for 7 days. A reconstituted human skin model, Neoderm-ME, was purchased and maintained according to the manufacturer's instructions (Tego Science, Seoul, Korea).

Melanin assay

The cells were treated with 1 or 2 nM SP (Bachem Fechemikalien, Budendorf, Switzerland) in the presence or absence of various concentrations of the NK1R antagonist RP-67580 or the ECE1 inhibitor CGS35066 or in the presence of each siRNA for 5 days. The Neoderm-ME was continuously treated with 0, 1, 2, or 5 mM SP for 3 weeks. The cell pellets and Neoderm-ME were dissolved in 1 N sodium hydroxide (Sigma-Aldrich, St Louis, MO) at 58 °C for 15 minutes by vortexing periodically using a Thermomixer (Eppendorf, Hamburg, Germany). The melanin content was determined by measuring absorbance at 490 nm using the Synergy H2 microplate reader (BioTek, Winooski, VT) and was normalized to the protein input.

Real-time quantitative PCR

Total RNA was extracted using TRIzol reagent (Life Technologies) according to the manufacturer's instructions, and 1 μg of total RNA was utilized to synthesize the complementary DNAs using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Pittsburgh, PA). Approximately 1 μg of complementary DNA sample and each TaqMan probe (Life Technologies) were diluted into a reaction mixture of Quantitect Probe PCR Kit (Qiagen, Valencia, CA), and PCR was performed using the 7500 Fast real-time PCR system (Life Technologies). Each TaqMan probe is listed in Supplementary Table 1 online, and all data are presented as a fold change relative to the glyceraldehyde-3-phosphate dehydrogenase control and were acquired from three independent experiments.

Western blot analysis

The proteins were resolved using SDS-PAGE, transferred onto membranes, and probed with the following antibodies: (a) anti-MITF, anti-NK1R, anti-EDN1, anti-EDN3, anti-p38 MAPK, anti-phospho-p38 MAPK, anti-p70S6K, and anti-phospho-p70S6K purchased from Thermo Scientific; (b) anti-TRP1, anti-TRP2, anti-GAPDH, anti-PKAα, and anti-phospho-PKAα/β/γ purchased from Santa Cruz Biotechnology (Dallas, TX); (c) anti-EDNRA and anti-EDNRB purchased from Abcam (Cambridge, MA); (d) anti-ERK1/2 and anti-phospho-ERK1/2 purchased from R&D systems (Minneapolis, MN); and (e) anti-Tyrosinase purchased from Upstate Biotechnology (Lake Placid, NY).

ELISA assay for secreted endothelin 1

Each cell was treated with 1 or 2 nM SP and incubated for 5 days. The culture medium was collected and centrifuged at 13,000 r.p.m. for 15 minutes to remove any debris. The secreted EDN1 was measured according to the manufacturer's instructions (Enzo Life Sciences, Farmingdale, NY).

cAMP assay

The cells were treated with 1 or 2 nM SP for 2 days and harvested with 0.1 mM hydrochloric acid (Sigma-Aldrich) after washing with

phosphate-buffered saline two times. After centrifugation at 600*g* at room temperature, the cAMP level in the supernatants was measured using the cAMP assay kit according to the manufacturer's instructions (BioVision, Milpitas, CA).

siRNA assay

The cells in 60-mm dishes were treated with 50 nM of each SMARTpool ON-TARGETplus siRNA (Dharmacon, Pittsburgh, PA) using Lipofectamine RNAi MAX reagent according to the manufacturer's instructions (Life Technologies). The siRNAs used were as follows: tachykinin receptor 1 (#L-005733), EDN1 (#L-016692), EDNRA (#L-005485), EDNRB (#L-003657), non-targeting siRNA #1 (#D-001810).

Statistical analysis

All data are presented as mean ± SD. A two-tailed Student's *t*-test was used to analyze differences between the two groups, and the threshold for statistical significance was set at 0.05 ($*P<0.05$).

CONFLICT OF INTEREST

The authors state no conflict of interest.

Author contributions

PJP and E-GC conceived and designed the experiments. PJP performed the experiments. PJP, TRL, and E-GC analyzed the data. PJP, TRL, and E-GC wrote the paper.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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