Inhibition Mechanism of Endothelin-1-induced Ca^{2+} Mobilization of Antimelanogenic Ingredient: 1,2-*O*-Diferulylglycerol

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Abstract

Endothelins secreted from keratinocytes are intrinsic madiators for human melanocytes in UVB-induced pigmentation. Antimelanogenic ingredient, 1,2-*O*-diferulylglycerol(SM709) isolated from bamboo extract inhibited the melanin synthesis of B16F10 melanoma cells by 62% . To understand the cellular mechanism of antimelanogenic activity of SM709 in human melanocytes, the effects of SM709 on the ET-1-induced Ca^{2+} mobilization were investigated. ET-1 receptors in human melanocytes were characterized by using specific antagonist and found that ET-1 increased intracellular Ca^{2+} by activating ET-B receptor. SM709 completely blocked the ET-1-induced intracellular Ca^{2+} increase and its inhibitory effect showed dose- and time-dependent manners. To investigate the role of SM709 on intracellular Ca^{2+} store, when the Ca^{2+} store was partially depleted by thapsigargin; a specific inhibitor of ER-type Ca^{2+}-ATPase, caffeine-induced Ca^{2+} mobilization did not changed in the presence or absence of SM709, suggesting that SM709 has no effect on the Ca^{2+} store. It is known that LPA receptor and P_2 receptor are linked to $InsP_3$ second messenger system. When these receptors in melanocytes were activated by LPA and ATP, the intracellular Ca^{2+} signaling was observed even in the presence of SM709. From the above results, it can be suggested that SM709 has an antimelanogenic activity by antagonizing the ET-B receptor, resulting in subsequent intracellular Ca^{2+} signaling, in UV induced pigmentation.

Introduction

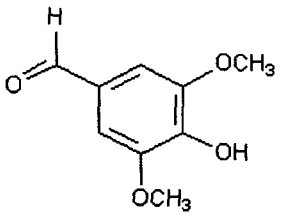
UVB radiation is the main physiological stimulus for human skin pigmentation. UV-exposed skin produces more melanocytes with an increase in melanin production. Melanin biosynthesis, melanocyte proliferation and differentiation appear to be affected by many intrinsic and extrinsic factors.[1,2] During 1990s, among keratinocyte-secreted paracrine factors, prostaglandins PGE2, α-melanocyte stimulating hormone(α-MSH), adrenocorticotropic hormone(ACTH), endothelins(ETs) and nitric oxide(NO) were studied for their effects in

melanin biosynthesis.[1-4] ET-1 was first described as an endothelium-derived factor with potent vasoconstrictive effects and it is now known to be synthesized by numerous cell types and to act, for the most part, as a paracrine regulator, of many target cells. Human epidermal keratinocytes in culture were found to be synthesize ET-1, particularly after treatment with UVRs.[5] Imokawa et al. reported that human keratinocytes produce and secrete ETs, which can be strong mitogens as well as melanogens for human melanocytes in UVB-induced pigmentation.[3] Binding of ETs to their receptors(ET-A, ET-B) stimulates phospholipase C(PLC) via the ET-receptor-coupled G protein and generates inositol 1,4,5-tris-phosphate(IP_3) and 1,2-diacylglycerol(DAG). These molecules, in turn, induce an increase in intracellular Ca^{2+} and activation of protein kinase C(PKC), respectively, and eventually lead to a variety of biological events, such as contraction, cell proliferation and melanin synthesis etc.[6] In our previous study, we reported that one of antimelanogenic ingredients, 1,2-*O*-diferuloylglycerol(SM709) isolated from bamboo inhibited tyrosine hydroxylase and DOPA oxidase activities by 18 and 60%, respectively. It also inhibited the melanin biosynthesis by 62% in B16F10 melanoma cells. In the present study, we investigated the effect of SM709 on the ET-1-induced signal transduction to further clarify its cellular mechanism of antimelanogenic activity in cultured human melanocytes.

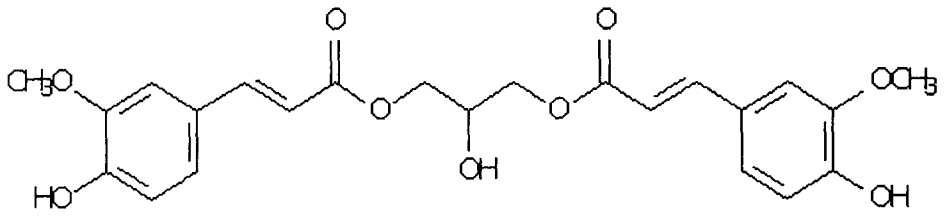
Materials and methods

Chemicals

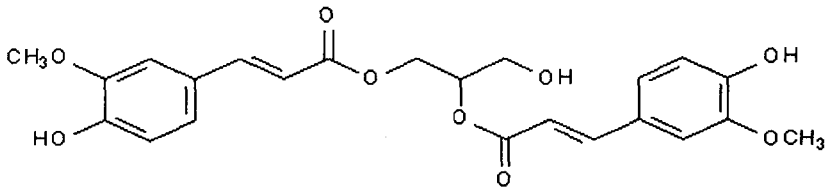
ET-1, ethylene glycol-bis(β-amino-ethyl-ether)-*N,N,N',N'*-tetraacetic acid(EGTA), thapsigargin, caffeine, LPA(1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphate; 18:1-LPA) were purchased from Sigma(St. Louis, Mo., USA). BQ-123(cyclo[-D-Try-D-Asp-Pro-D-Val-Leu-]), BQ-788(*N*-[*N*-{*N*-(2,6-dimethyl-1-piperidinyl)carbonyl]-4-methyl-L-leucyl)}-1-(methoxy-carbonyl) tryptophyl]-D-norleucine monosodium), ATP were purchased from RBI(Natick, Mass., USA). Fluro-3/AM was purchased from Molecular probes(Eugene, Ore., USA), and melanocyte growth media(MGM) was purchased from Clonetics(San Diego, USA). Fetal bovine serum and antibiotics were purchased from Gibico BRL(Gaithersburg, Md., USA). 4-hydroxy-3,5-di-methoxyphenyl aldehyde(SM707), 1,3-*O*-diferulylglycerol(SM708) and 1,2-di-*O*-feruloylglycerol (SM709) were isolated from bamboo(*Phyllostachys bambusoides*, part used; stem) and used in this experiment.



4-hydroxy-3,5-di-methoxyphenyl aldehyde(SM707)



1,3-*O*-diferulylglycerol(SM708)



1,2-*O*-diferuloylglycerol(SM709)

Cell culture

Human melanocytes were isolated from neonatal foreskins and maintained in MGM media supplemented with 4% fetal calf serum(FCS), 5 nM TPA, 0.6ng/ml bFGF, 1μg/ml transferrin and antibiotics 1at 37 °C under a 5% CO_2 atmosphere.

Measurement of Ca^{2+} by confocal microscopy imaging

For conforcal imaging the Ca^{2+}-sensitive dye fluo-3 was used in intact cells as described by Khirough et al.[7] Cells were incubated for 40-60 min at 37°C with 4 μM Fluo-3/AM (Molecular Probes, Eugene, Oregone) added to culture medium, and then washed three times before the experiments. Fluorescence images were obtained by the 488 nm light bandpass filter(confocal laser scanning microscope, LSM 510, Carl Zeiss Co.) of the Ar-Kr laser and the signals were analysed by LSM 5 image browser. Ca^{2+} transients were measured in terms of fractional amplitude($\Delta F/F_0$; where F_0 is the baseline fluorescence level, and ΔF is the rise over the baseline).

Results

Effects of SM709 on ET-1-induced intracellular Ca^{2+} increase

The effects of the isolated compounds(SM707, SM708, SM709) on ET-1-induced Ca^{2+} increase were observed. ET-1 of 10 nM induced a transient Ca^{2+} increase as shown in Fig.1A and the peak reached maximum in about 30 sec and thereafter it showed fast return to an initial Ca^{2+} level within 5 min. However, the steady-state level didn't return to the same initial Ca^{2+} level. ET-1-induced Ca^{2+} increase(Fig.1B) after removing extracellular Ca^{2+} showed similar pattern to that of Fig.1A. However, the peak height and following the steady-state level was a little lower in Fig 1A. From these results, we can suggest that ET-1-induced intracellular Ca^{2+} increase was affected by both extra income and intra secretion from the store of Ca^{2+} in human melanocytes. Anti-melanogenic compounds(SM707, 708, 709) isolated from bamboo was investigated on ET-1-induced Ca^{2+} increase. Among 3 compounds, SM709 completely blocked the ET-1-induced effect(Fig. 2) and the inhibitory effect was dependent on the concentration and on the preincubation time(Fig. 3); however SM707 and SM708 showed no effect. Accordingly, we can suggest that SM709 affect the receptor protein of ET-1 and Ca^{2+} channel on the cell and store membrane.

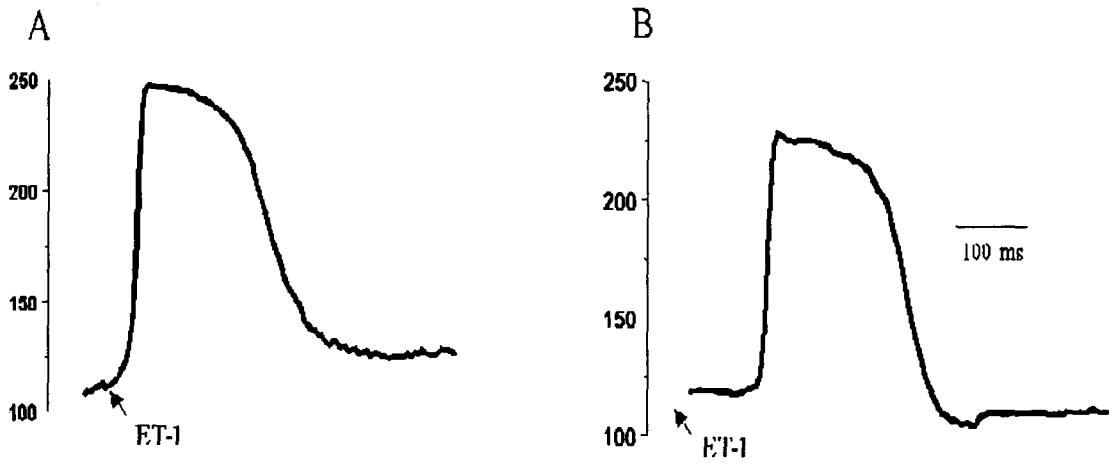


Fig.1. ET-1-induced intracellular Ca^{2+} increase in single melanocyte. A: Typical intracellular Ca^{2+} response obtained with endothelin-1. Melanocyte was stimulated with 10 nM ET-1 and a transient increase in intracellular Ca^{2+} was recorded. B: Intracellular Ca^{2+} response in the absence of extracellular Ca^{2+}. Extracellular Ca^{2+} was removed by the chelation with 2 mM EGTA and 10 nM ET-1 was added to record intracellular Ca^{2+} concentration.

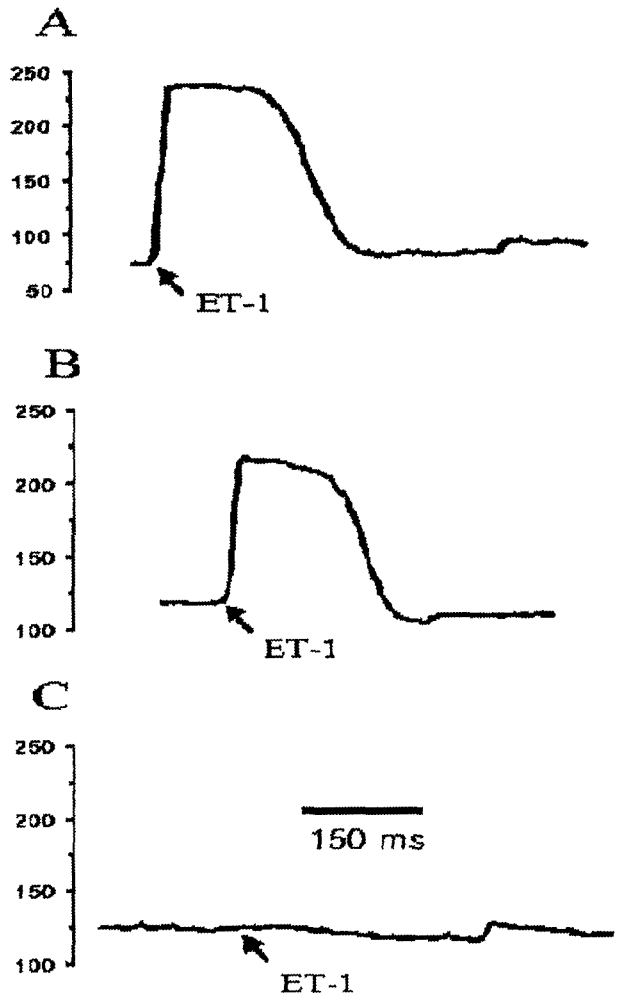


Fig. 2. Effects of the isolated compounds from bamboo on ET-1-induced intracellular Ca^{2+} increase. Melanocytes were incubated for 30 min with one of the isolated compounds, SM707(A), SM708(B), and SM709(C). Concentrations treated were 100 μM.

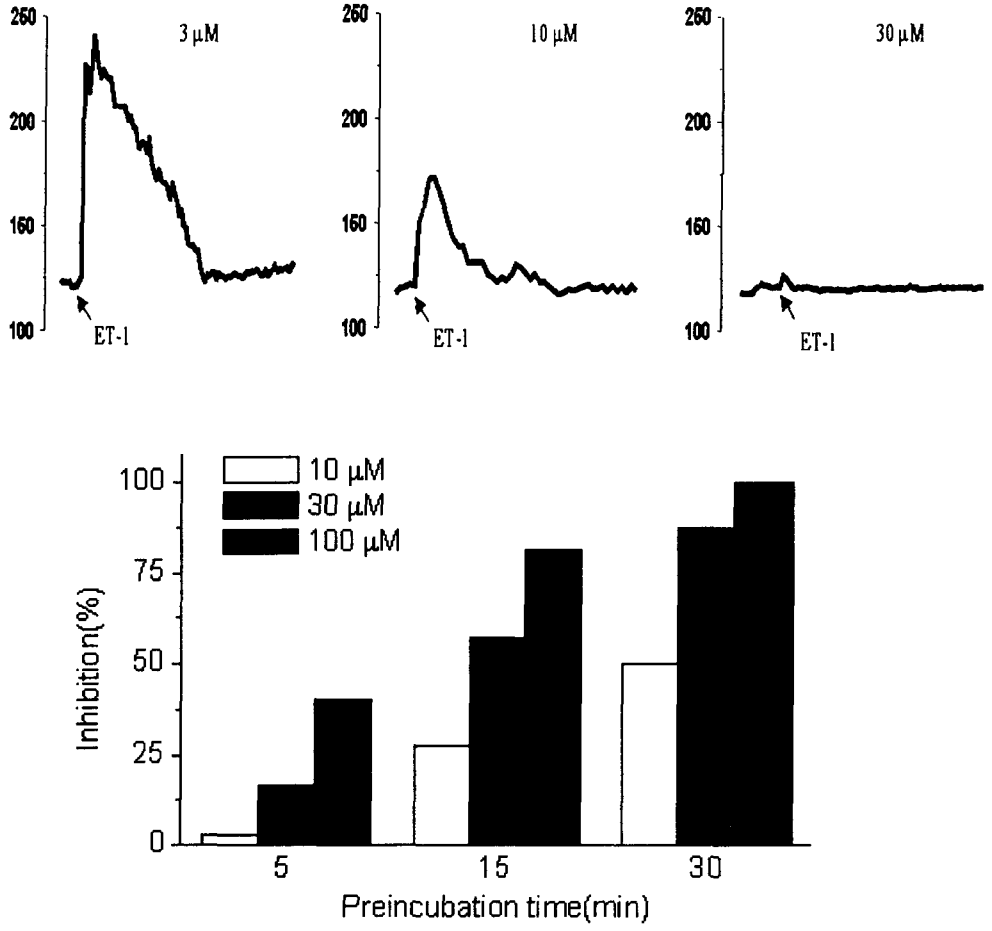


Fig. 3. Dose and time dependence of SM709 effect on intracellular Ca^{2+} mobilization. SM709 was treated at three different concentrations, 10, 20, and 30 μM(Upper panel). ET-1-induced Ca^{2+} increases were measured after incubations for 5, 15, and 30 min(Lower panel). The data represent average values obtained from 8-15 cells.

ET-1 receptor subtype in melanocyte

To characterize ET-1 receptor subtype in melanocyte, we measured the effect of specfic antagonist BQ-123 for ET-A and BQ-788 for ET-B. Treatment of 100 nM BQ-123, ET-A subtype antagonist didn't affect intracellular Ca^{2+} increase in melanocyte(Fig. 4A). However, BQ-788, ET-B subtype antagonist completely inhibited intracellular Ca^{2+} increase(Fig. 4B). These results suggest that a component of the ET-1-induced Ca^{2+} increase is mediated by the ET-B receptor in cultured human melanocytes.

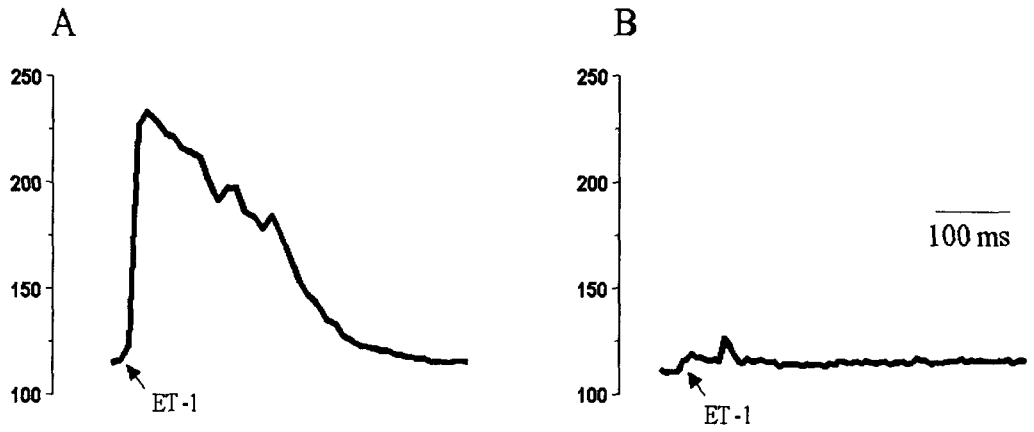


Fig. 4. Identification of ET-1 receptor by using endothelin receptor antagonists. Melanocytes were pretreated with BQ-123(A) or BQ-788(B), specific antagonist of ET-A or ET-B receptor, respectively. ET-1 was treated at 10 nM and intracellular Ca^{2+} concentration was monitored. The concentrations of antagonists added to melanocytes were 100 nM.

Effect of SM-709 on Ca^{2+} signaling pathway induced by ET-1

To elucidate the action mechanism of SM709 in the inhibition of Ca^{2+} increase, we examined where SM709 blocked Ca^{2+} signaling pathway induced by ET-1. First of all, we examined the effect of SM709 on intracellular Ca^{2+} release from the Ca^{2+} store of melanocyte. It was known that thapsigargin discharges intracellular Ca^{2+} stores by specfic inhibition of the endoplasmic reticulum Ca^{2+}-ATPase. Treatment of 10 μM thapsigargin induced transient intracellular Ca^{2+} increase and this effect didn't reduced by 100 μM SM709 in the pretreatment cell for 30 min(Fig. 5A). The effect by caffeine was same as that of thapsigargin in the pretreatment of SM709. These results suggest that SM709 has no effect on the store. Accordingly, as the next step, we investigated that whether SM709 can inhibit ET-1 induced Ca^{2+} mobilization in the former process of $InsP_3$ production or not.(refer to Fig. 8. ET-1-mediated Ca^{2+} signaling in melanocytes) It is known that LPA and ATP activate LPA receptor and P_2-type purine receprtor, respectively.[8,9] They also activate $InsP_3$ second messenger system the same as through ET receptors. Treatment of 100 μM SM709 didn't affect Ca^{2+} increase induced by LPA (Fig. 6)and ATP(Fig. 7). From the above results, as SM709 didn't affect the store and $InsP_3$ second messenger system, we strongly suggest that SM709 blocks the ET-1 effect by antagonizing the ET-B receptor.

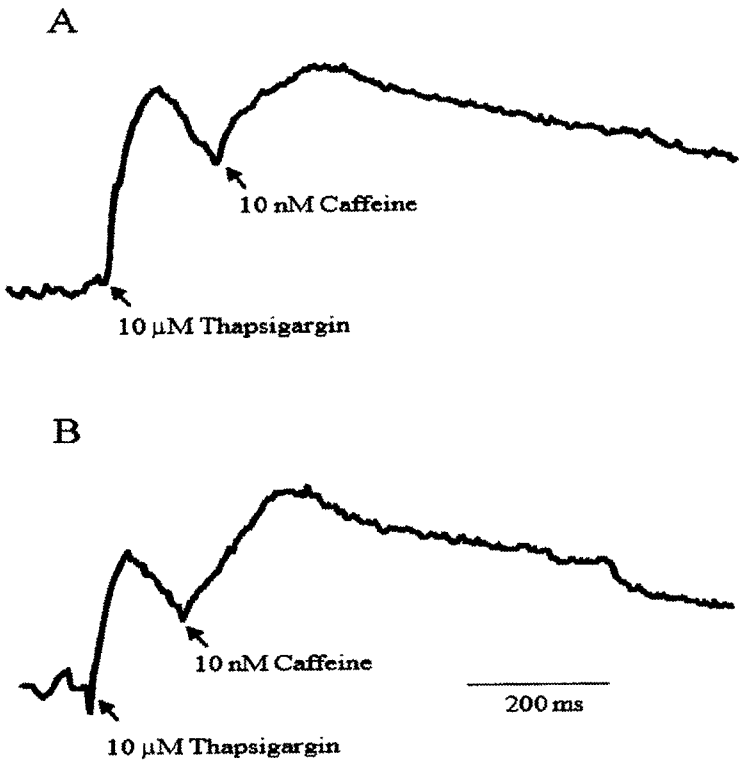


Fig. 5. Effect of SM709 on intracellular Ca^{2+} release from the Ca^{2+} stores of melanocytes. A: Intracellular Ca^{2+} release was measured after the depletion of Ca^{2+} stores. Intracellular Ca^{2+}-ATPase was inhibited by 10 μM thapsigargin and Ca^{2+} release was inhibited by 10 nM caffeine. B: Intracellular Ca^{2+} release in the presence of SM709. Melanocytes were pre-treated with 100 μM SM709 and the Ca^{2+} release was inhibited by 10 nM caffeine after the depletion of Ca^{2+} stores.

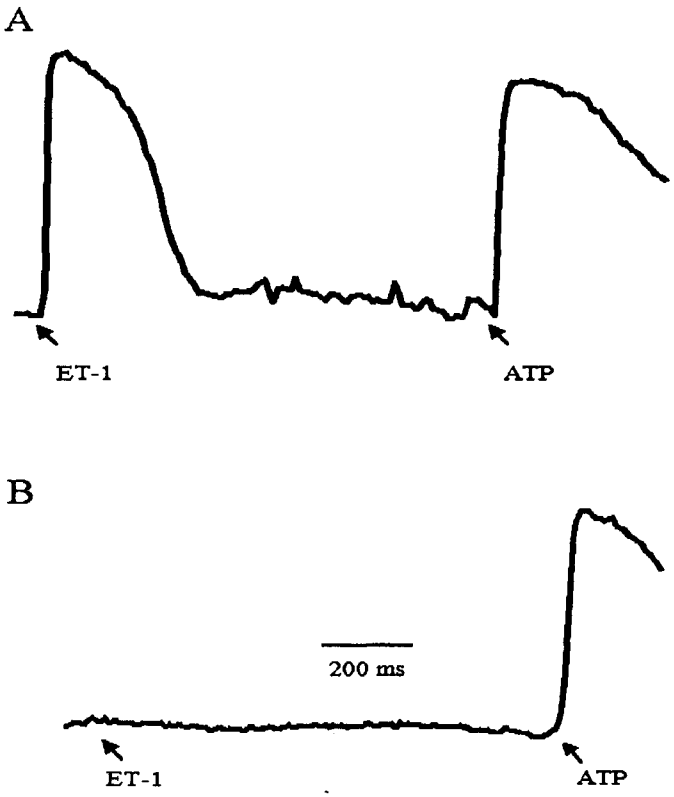


Fig. 6. Effect of SM709 on ATP-induced intracellular Ca^{2+} increase. A: ET-1- and ATP-induced Ca^{2+} increases. Intracellular Ca^{2+} increase was initiated by ET-1. A subsequent addition of 1 mM ATP also increased cytosolic Ca^{2+}. B: Effects of ET-1 and ATP on cytosolic Ca^{2+} increases in a melanocyte pre-treated with 100 μM SM709.

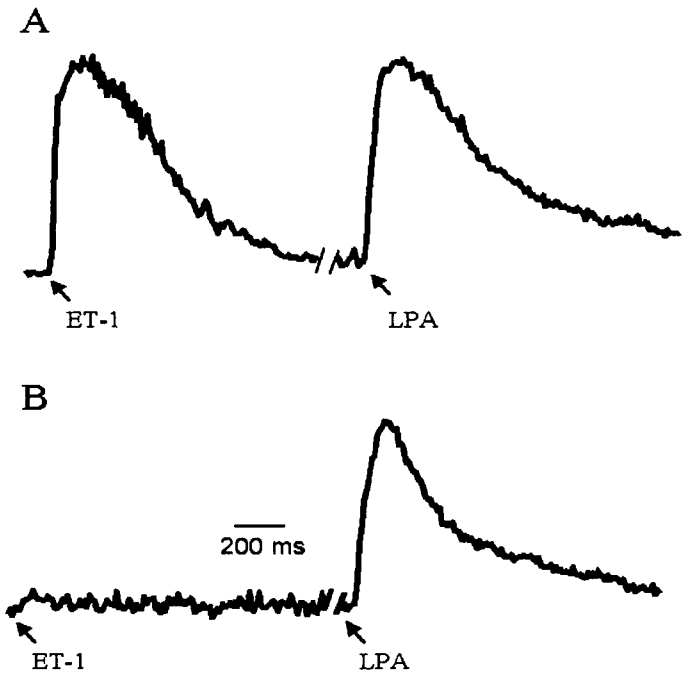


Fig. 7. Effect of SM709 on LPA-induced intracellular Ca^{2+} increase. (A) ET-1- and LPA-induced Ca^{2+} increases. Intracellular Ca^{2+} increase was initiated by ET-1. A subsequent addition of 0.2 μM LPA also increased cytosolic Ca^{2+}. (B) Effects of ET-1 and LPA on cytosolic Ca^{2+} increases in a melanocyte pre-treated with 100 μM SM709.

Discussion

UV light is one of the major factor for human skin pigmentation. UV light can directly act on melanocytes to induce melanin biosynthesis. During 1990s, paracrine factors secreted from keratinocytes which influence melanin biosynthesis, PGE2, α-MSH, ACTH, ET-1 and NO etc. were reported and their roles were studied.[1-6] ETs which produced and secreted by keratinocytes plays an essential role in the maintenance of melanocyte proliferation and UV induced hyperpigmentation. Yada et al reported that human melanocyte proliferation and differentation could be stimulated by endothelin derivatives via a receptor-mediated signal transduction pathway.[10] In addition, Imokawa et al. reportd ETs can be strong mitogens as well as melanogens for human melanocytes in UV-induced pigmentation and the inhibitory effect on ET-1 induced Ca^{2+} mobilization can be an antimelanogenic effect on UV-induced

pigmentation.[3,11] In our previuos study, SM709 showed potent antimelanogenic activities; that is, approximately 60% inhibition activity on DOPA oxidase and 62% inhibition percentage on melanin biosynthesis in B16F10 melanoma cell at the concentration of 10 μM. Accordingly, we tried to investigate another physiological activity and the action mechanism of SM709 in cellular level. In this study, SM709 blocked ET-1 induced Ca^{2+} mobilization completely and its inhibitory effect was dependent both on the concentration and on the time. We also confirmed that ET-1 interacted with the ET-B receptor subtype to induce a Ca^{2+} increase in human melanocytes. The result is same as that of Kang et al.[12], but different from that of Imokawa et al.[11] The reason for the different expression of ET-1 receptor substype was still unknown. A schematic ET-1-induced Ca^{2+} signaling pathway in melanocyte has shown in Fig. 8. When we investigated which pathway did SM709 block in ET-1-induced Ca^{2+} signaling pathway, the inhibitory effect of SM709 was not related to the blocking of Ca^{2+} store as shown in Fig. 5A and 5B. Accordingly, we believed that the effect of SM709 is presumably believed to work on the earlier part of signal transduction pathway than the Ca^{2+} store. It is known that endothelial cells have LPA receptor and P_2 receptors which are linked to $InsP_3$ escond messenger system.[8] Accordingly, the effect of SM709 was compared to those of lysophophatidic acid(LPA) and P_2 receptors since their drug receptors share the second messenger system with the ET-1 receptor. However, when these receptors were activated by LPA and ATP, the intracellular Ca^{2+} signaling was observed even in the presence of SM709. From the above results, we strongly suggest that SM709 blocks the ET-1 effect by antagonizing the ET-B receptor in human melanocytes and it acts as a useful whitening ingredient by inhibiting the ET-B receptor, resulting in subsquent intracellular Ca^{2+} signaling in UVB induced pigmentation.

Conclusion

In this study, we investigated the effect of SM709 on ET-1-induced Ca^{2+} mobilization in human melanocyte. SM 709 of 100μM completely blocked the ET-1-induced Ca^{2+} mobilization by ET_B but not by ET_A receptor subtype in dose- and time-dependent manners. We also observed that SM709 didn't related to the blocking of the subsequent intracellualr Ca^{2+} signaling. In conclusion, SM709 is a specific inhibitor of ET-1-induced Ca^{2+} signaling through ET-B receptor in cultured human melanocytes, which in turn suggests that it might be a useful whitening ingredient in UVB induced pigmentation.

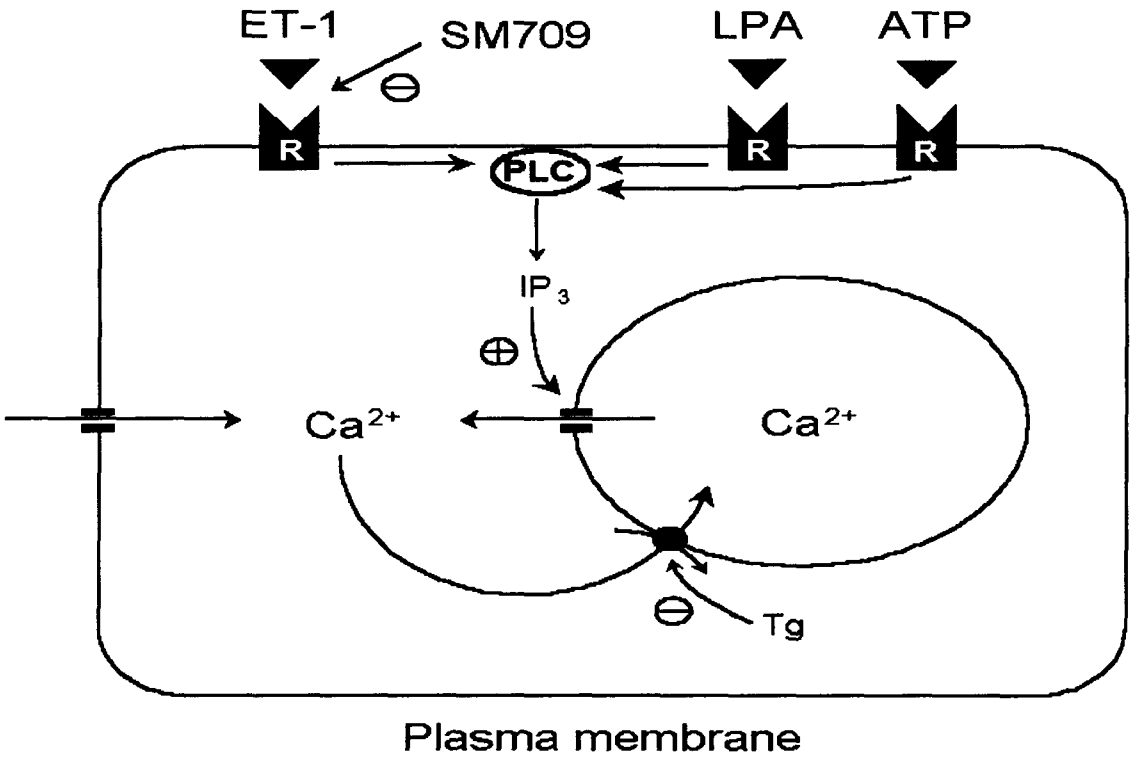


Fig. 8. ET-1-mediated Ca^{2+} signaling in melanocytes.

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