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SM709, Ingredient of Antimelanogenic Bamboo Extract, Blocks Endothelin-1-induced $[Ca^{2+}]_i$ Increase in Human Melanocytes

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Endothelins secreted from keratinocytes are intrinsic mitogens and melanogens of human melanocytes in UVB-induced hyperpigmentation. To elucidate the cellular mechanism of antimelanogenic activity of bamboo extract, the effects of three ingredients of bamboo extract on endothelin 1 (ET-1)-induced Ca^{2+} mobilization were investigated in cultured human melanocytes. ET-1 receptors in human melanocytes were characterized by using specific antagonist, and ET-1 was found to increase intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) by activating ET-B receptor. SM709 (1,2-*O*-diferulyl-glycerol), an ingredient of bamboo extract, inhibited ET-1-induced $[Ca^{2+}]_i$ increase in a concentration- and time-dependent manner, although another ingredients SM707 and SM708 had no effect on ET-1-induced $[Ca^{2+}]_i$ increase in human melanocytes. SM709 (100 μM), however, did not affect $[Ca^{2+}]_i$ increase induced by thapsigargin and caffeine, suggesting that SM709 has no effect on the Ca^{2+} store in melanocytes. Furthermore, SM709 did not affect $[Ca^{2+}]_i$ increase induced by LPA or ATP, known as G protein-mediated PLC activators like ET-1. Taken together, it is suggested that SM709 antagonizes ET-1-induced transmembrane signaling through ET-B receptor, which maybe a possible underlying mechanism of antimelanogenic activity of bamboo extract in human melanocytes.

Key Words: Bamboo extract, Endothelin 1, ET-B receptor, Melanocytes

INTRODUCTION

Melanocytes, a type of epidermal cells, synthesize a cell-specific product melanin, and melanin transferred to epidermal keratinocytes functions as a basal skin color and protects the skin against ultraviolet (UV) irradiation, the main physiological stimulus for human skin pigmentation. Melanogenesis and melanocyte proliferation are affected by various intrinsic and extrinsic factors (Tsuboi et al, 1994; Dissanayake & Mason, 1998; Berking et al, 2001; Hachiya et al, 2001). The finding of paracrine linkage of endothelins (ETs) between keratinocytes and melanocytes showed that ETs are intrinsic mediators for human melanocytes in several epidermal hyperpigmentation (Yada et al, 1991; Imokawa et al, 1992). Epidermal keratinocytes have been known to produce and secrete ETs (Imokawa et al, 1992; Yohn et al, 1993), and defects of ET receptor have been documented in inherited pigmentary disease such as Hirschsprung disease (Puffenberger et al, 1994; Amiel et al, 1996) and Shah-Waardenberg syndrom (Edery et al, 1996). ET-1 has now been considered to be important physiological ligand as a unique intrinsic mitogen and melanogen for human melanocytes (Imokawa et al, 1992; Imokawa et al, 1996).

ET-1 increases tyrosinase activity, and elevates tyrosinase and tyrosinase-related protein-1 mRNA expression levels in cultured human melanocytes (Imokawa et al, 1995). Ingredients purified from the extract of inner bark of bamboo (*Phyllostachys reticulata*) were reported to inhibit tyrosinase and melanin biosynthesis in cultured human melanocytes and B16 mouse melanoma cells (Cho et al, 1999). However, the cellular mechanism(s) of the antimelanogenic effects of the bamboo extracts have not yet been known. ET-1 is a powerful melanogen at the concentration which increases intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in melanocytes (Imokawa et al, 1995). It is, therefore, tempting to investigate whether the ingredient(s) of bamboo extract affect the signaling pathway of ET-1 in human melanocytes. In the present study, we demonstrated that SM709 (1,2-*O*-diferulyl- glycerol), an ingredient of bamboo extract, inhibited the ET-1-induced $[Ca^{2+}]_i$ increase in human melanocytes.

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ABBREVIATIONS: ET-1, endothelin 1; ETs, endothelins; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; SM707, 4-hydroxy-3,5-di-methoxyphenyl aldehyde; SM708, 1,3-*O*-diferulylglycerol; SM709, 1,2-*O*-diferuloylglycerol.

METHODS

Cell culture

Human melanocytes were isolated from neonatal foreskins according to the methods described previously (Eisenger & Marko, 1982). Cells were maintained in Medium 154 supplemented with human melanocyte growth supplement (HMGS Kit, Cascade Biologics, USA) at 37°C under 5% CO_2. The medium was changed every 3 days. For experiment, melanocytes were seeded at a density of 1×10^4 cells/coverslip on 18-mm-diameter glass coverslips (Superior, Germany) in a 12-well plate, containing serum-free MGM medium and used on 2 days after plating.

Measurement of $[Ca^{2+}]_i$

For confocal imaging, the Ca^{2+}-sensitive dye fluo-3 was used in intact cells as described by Khirough et al. (1997). Cells were incubated for 40~60 min at 37°C with 4 μM Fluo-3/AM (Molecular Probes, USA) added to culture medium, and then washed three times before the experiments. Fluo-3 emission was induced by the Ar-Kr laser of the confocal laser-scanning microscope (Carl Zeiss LSM 410, Germany) with the 488 nm band filter and detected by a 515 nm longpass emission filter. All images from the scanning were processed to analyze changes of $[Ca^{2+}]_i$ in a single cell level. The signal transients were expressed as fractional amplitude, $\Delta F/Fo$, where Fo is the baseline fluorescence level and ΔF is the rise over the baseline.

Drugs

Pure preparations containing single components of bamboo extracts (SM707, 4-hydroxy-3,5-di-methoxyphenyl aldehyde; SM708, 1,3-*O*-diferulylglycerol; SM709, 1,2-*O*-diferuloylglycerol) were kindly donated by Aekyung Industrial Corporation (Daejeon 305-345, Korea). Fig. 1 shows the chemical structure of three ingredients of the bamboo extract. ET-1 ATP and LPA were purchased from Sigma Co. (USA), and BQ123 and BQ 788 were from American peptide company (USA) and RBI (USA), respectively.

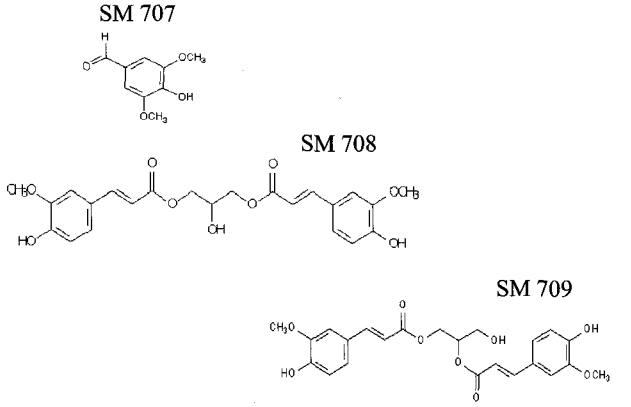

Fig. 1. Chemical structures of compounds isolated from antimelanogenic bamboo extracts and used in the study.

RESULTS

Changes in $[Ca^{2+}]_i$ were induced by ET-1 treatment in single, cultured human melanocytes, and Fig. 2 shows typical $[Ca^{2+}]_i$ increase induced by ET-1. $[Ca^{2+}]_i$ increased rapidly within 30 second after single melanocytes were stimulated with 10 nM ET-1. $[Ca^{2+}]_i$, thereafter, decreased within 5 min to minimum levels which were usually higher than the stable resting $[Ca^{2+}]_i$ levels before the ET-1 stimulation (Fig. 2A). ET-1-induced $[Ca^{2+}]_i$ increase was observed more than 80% of the cells tested at each control experiment. The second ET-1 stimulation given within 30 min after the first stimulation did not further increase $[Ca^{2+}]_i$ (data not shown), suggesting homologous desensitization. We next investigated the effect of three ingredients of antimelanogenic bamboo extract (see Fig. 1 for their chemical structures) on ET-1-induced $[Ca^{2+}]_i$ increase in human melanocytes. Although there are similarities in chemical structure between SM 709 and SM708 or in potency of tyrosin kinase inhibition between SM709 and SM707 (Cho et al, 1999), only SM709 effectively inhibited ET-1-induced $[Ca^{2+}]_i$ increase in human melanocytes. 10 nM ET-1-induced $[Ca^{2+}]_i$ increase was completely inhibited by 30 min of preincubation with 100 μM SM709, but not by 100 μM SM708 or SM707 even after 60 min of preincubation (Fig. 2B, C and D).

As shown in Fig. 3, SM709 (10~100 μM) inhibited ET-1-induced $[Ca^{2+}]_i$ increase in human melanocytes in a concentration- and time-dependent manner. After 30 min of preincubation with 100 μM SM709, ET-1-induced $[Ca^{2+}]_i$ increase was completely inhibited. However, after 30 min of preincubation with 10 and 30 μM SM709, ET-1 still increased $[Ca^{2+}]_i$ in some melanocytes, although the maximum ratio of ET-1-induced $[Ca^{2+}]_i$ increase was lower than that of control cells: The increase was inhibited by 50.1± 10.7 and 87.5±5.7% of tested melanocytes, respectively (Fig. 3). Furthermore, SM709 inhibition of ET-1-induced $[Ca^{2+}]_i$ increase was time-dependent: After 5, 15 and 30 min of preincubation with 30 μM SM709, ET-1-induced $[Ca^{2+}]_i$ increase was inhibited by 27.7±3.7, 57.1±9.7 and 81.3± 4.5% of tested melanocytes, respectively (Fig. 3).

The ET-1-induced $[Ca^{2+}]_i$ increase has earlier been attributed to mobilization of Ca^{2+} from inositol 1,4,5-trisphosphate (IP_3)-sensitive intracellular Ca^{2+} stores (Kang et al, 1998). Therefore, using thapsigargin, which has been known to release Ca^{2+} from IP_3-sensitive intracellular Ca^{2+} stores by inhibiting Ca^{2+}-ATPase of the endoplasmic reticulum (Thastrup et al, 1990), we investigated whether SM709 depleted IP_3-sensitive intracellular Ca^{2+} stores. Cells were treated with 500 nM thapsigargin with or without 30 min of preincubation with 100 μM SM709, followed by stimulation with caffeine. The addition of thapsigargin induced a marked increase in $[Ca^{2+}]_i$, and subsequent addition of caffeine caused further $[Ca^{2+}]_i$ increase in melanocytes (Fig. 4A). Preincubation of melanocytes with 100 μM SM709 did not affect neither thapsigargin- nor caffeine-induced $[Ca^{2+}]_i$ increase (Fig. 4B).

The ET-1-induced $[Ca^{2+}]_i$ increase could be due to phospholipase C (PLC) activation via G-protein-coupled ET receptors (Sakurai et al, 1990; Kang et al, 1998). To further elucidate inhibitory mechanism on ET-1-induced $[Ca^{2+}]_i$ increase, we investigated the effects of SM709 on $[Ca^{2+}]_i$ increase induced by lysophosphatidic acid (LPA)(Pietruck et al, 1997; Noh et al, 1998) and ATP (Murthy & Makhlouf, 1998; Liu et al, 2000), a known different G protein-coupled

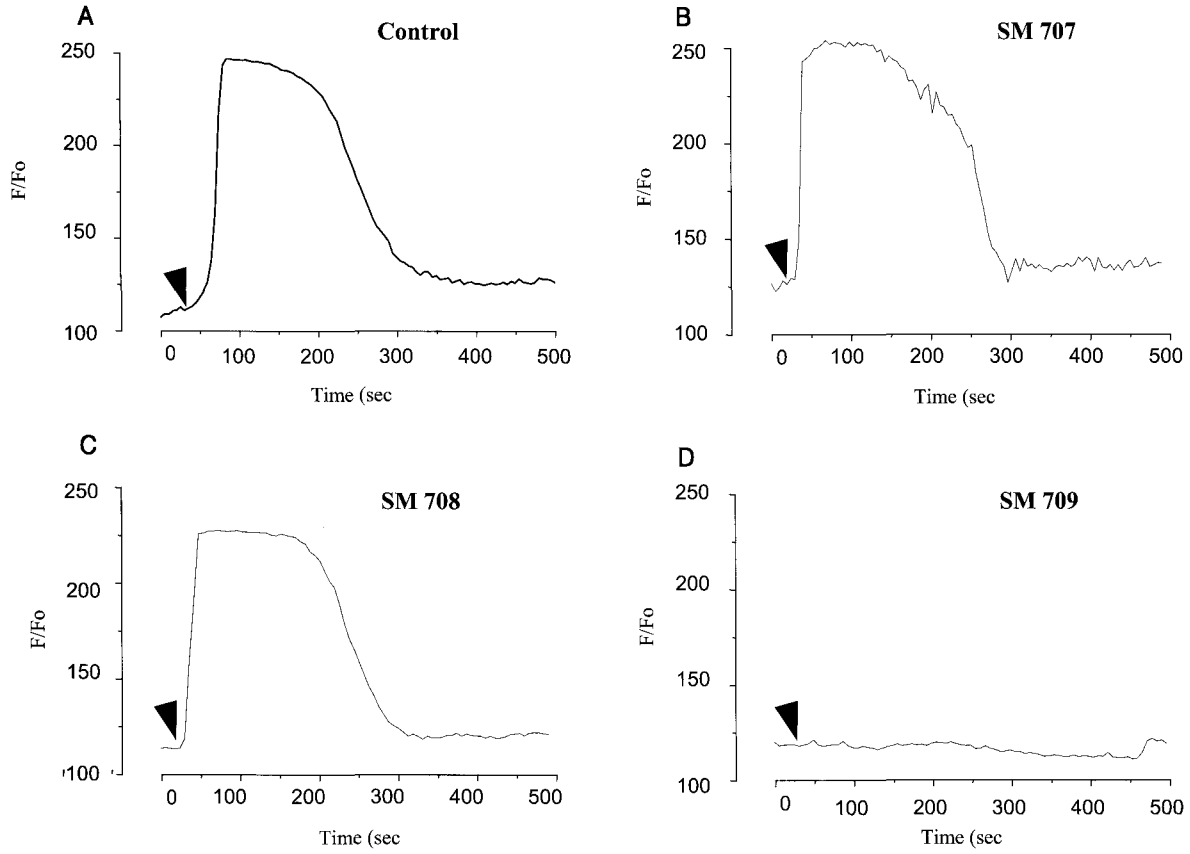

Fig. 2. Effects of three ingredients isolated from antimelanogenic bamboo extract on ET-1-induced $[Ca^{2+}]_i$ increase. Melanocytes were incubated with one of the isolated compounds, 100 μM SM707 (A), SM708 (B), and SM709 (C) for 30 min. The arrows indicate the treatment of ET-1.

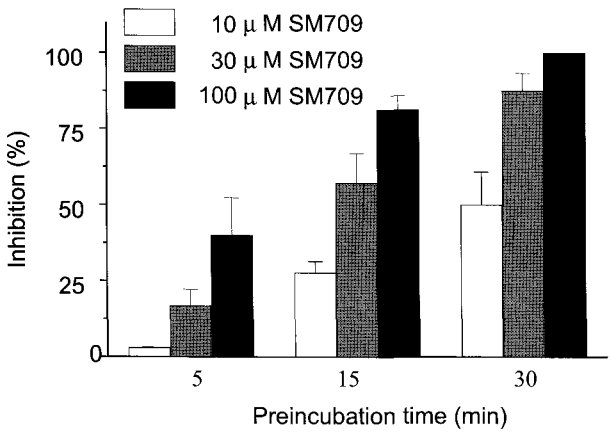

Fig. 3. Dose- and time-dependence of SM709 inhibition on ET-1-induced $[Ca^{2+}]_i$ increase in human melanocytes. ET-1-induced $[Ca^{2+}]_i$ increases were measured at 5, 15, and 30 min after pretreatment with one of three different concentrations of SM709 (10, 30, and 100 μM). The data expressed as the percent inhibition of mean fluorescent intensities observed in SM709 preincubated cells over those in control cells. Each data were obtained from 3~5 independent experiments.

receptor agonists. ATP (1 mM) and LPA (0.2 μM) did induce $[Ca^{2+}]_i$ increase in human melanocytes even after ET-1 stimulation (Fig. 5A and 5C). ATP- and LPA-induced $[Ca^{2+}]_i$ increases were still observed after 30 min of preincubation with 100 μM SM709, although ET-1-induced $[Ca^{2+}]_i$ increases were completely blocked in the same cells (Fig. 5B and 5D). These results may suggest selective effect of SM709 on ET receptors.

ET receptor subtype, which is involved in the ET-1 induced $[Ca^{2+}]_i$ increase in human melanocytes, has been a matter of debate. Therefore, we finally investigated the receptor subtype by using BQ-123, a specific ET-A receptor antagonist (Ihara et al, 1992), and BQ-788, a specific ET-B antagonist (Ishikawa et al, 1994). As shown in Fig. 6A, 100 nM BQ-123 did not affect the ET-1 (10 nM)-induced $[Ca^{2+}]_i$ increase. However, 100 nM BQ-788 completely inhibited the ET-1 (10 nM)-induced $[Ca^{2+}]_i$ increase (Fig. 2B), suggesting that the ET-1-induced $[Ca^{2+}]_i$ increase is mediated by ET-B receptor in the present cultured human melanocytes.

DISCUSSION

In the present study, SM709, an ingredient of antimelanogenic bamboo extract inhibited ET-1-induced $[Ca^{2+}]_i$ increase in human melanocytes in a concentration- and

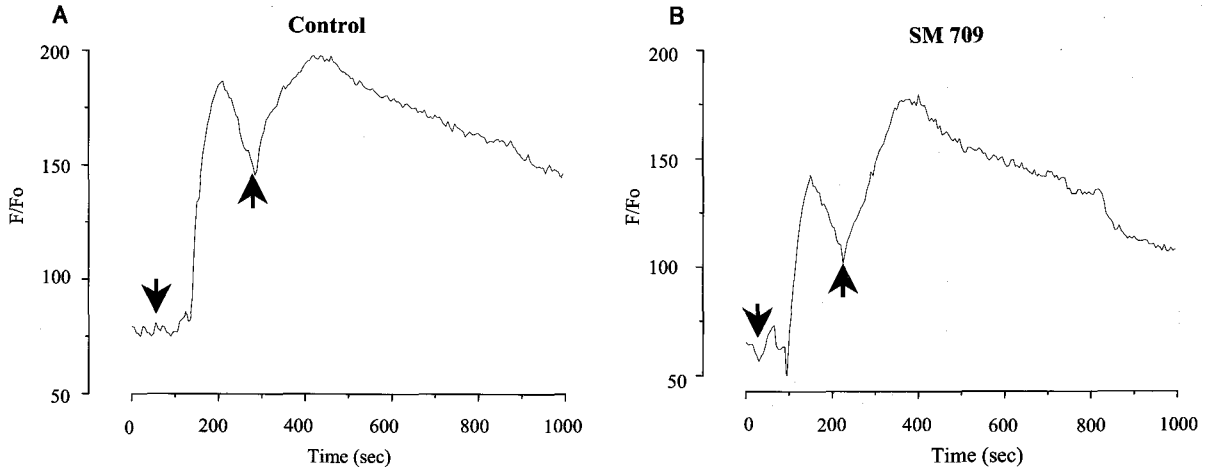

Fig. 4. Effect of SM709 on intracellular Ca^{2+} release from Ca^{2+} stores in melanocytes. $[Ca^{2+}]_i$ changes were monitored after the addition of 500 nM thapsigargin and 1 mM caffeine in control (A) and 100 μM SM709 (30 min) pretreated melanocytes (B). The first and second arrows indicate the time of addition of thapsigargin and caffeine, respectively, at each panel.

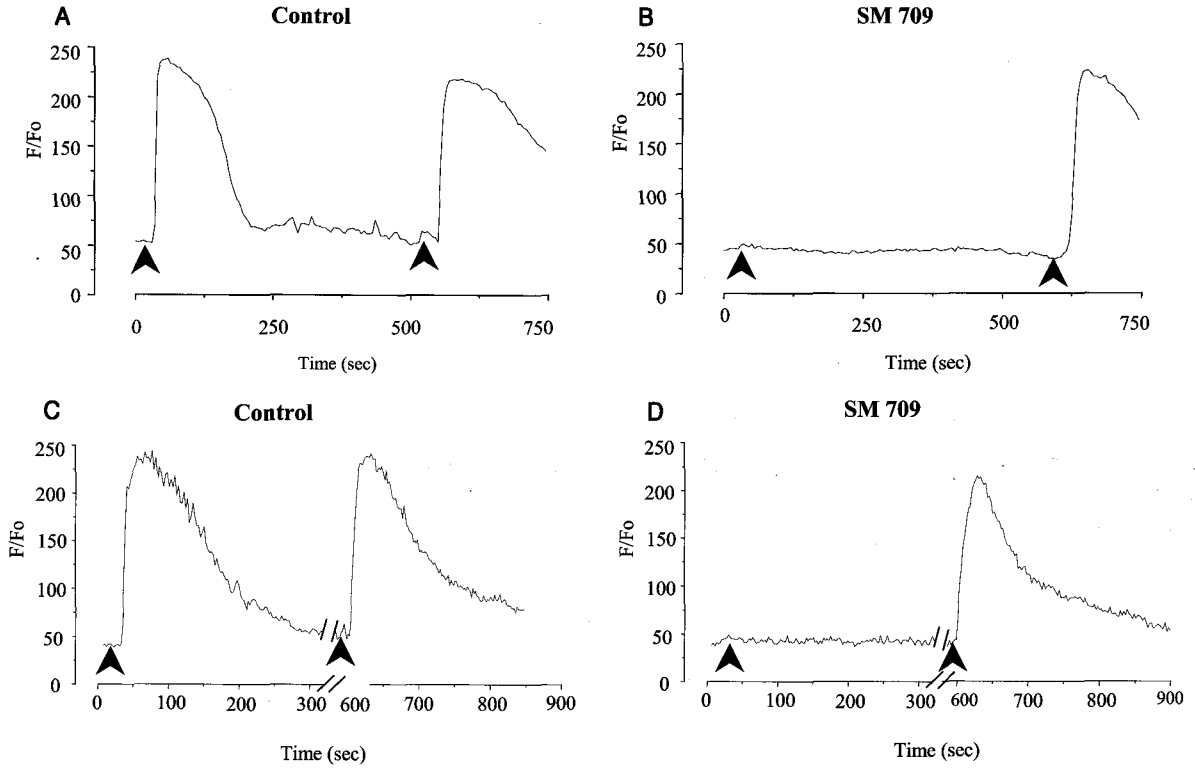

Fig. 5. Effect of SM709 on ATP- and LPA-induced $[Ca^{2+}]_i$ increase. ATP (1 mM) - and LPA (0.2 μM)-induced $[Ca^{2+}]_i$ increases following ET-1 treatment in control cells (A and C, respectively). Effects of pretreatment (30 min) of 100 μM SM709 on ATP- and LPA-induced $[Ca^{2+}]_i$ increases following ET-1 treatment (B and D, respectively). The first arrow at each panel indicates the time of ET-1 treatment. The second arrows indicate the ATP (A and B, respectively) or LPA (C and D, respectively) treatment

time-dependent manner. However, SM709 did not affect $[Ca^{2+}]_i$ increase induced by ATP and LPA, which activate PLC through G-protein-coupled receptor, as much as ET-1-induced $[Ca^{2+}]_i$ increase, indicating no mobilization of Ca^{2+} from IP_3-sensitive intracellular Ca^{2+} stores. These findings suggest that SM709 is an active component of bamboo extract and a specific inhibitor against transmembrane signaling of ET-1 in melanocytes.

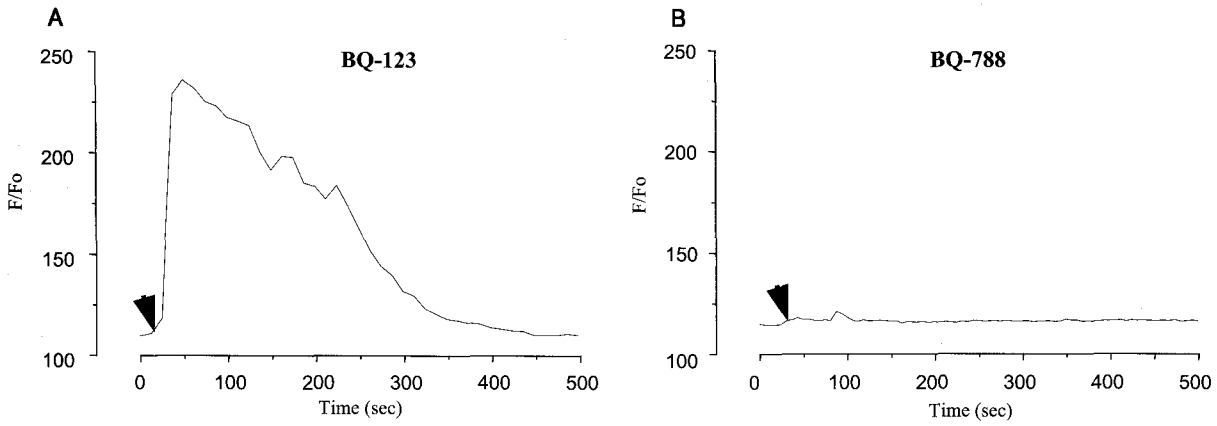

Fig. 6. Effects of endothelin receptor antagonists on ET-1-induced $[Ca^{2+}]_i$ increase in human melanocytes. Intracellular Ca^{2+} response was obtained with 10 nM ET-1 in the presence of BQ-123 specific antagonist of ET-A receptors (A) and BQ-788 ET-B receptor (B). The concentration of antagonists added to melanocytes was 100 nM. The arrows indicate the ET-1 treatment.

ETs are now regarded as an important physiological ligand that induces signaling in melanocytes. Keratinocyte-derived ET-1 has been known as an intrinsic stimulatory growth factor for human melanocytes in UV melanogenesis (Imokawa et al, 1992). ET-1 produces a transient $[Ca^{2+}]_i$ increase in melanocytes through binding to G-protein coupled ET receptors (Imokawa et al, 1992; Kang et al, 1998). However, the types of receptor involved in transmembrane signaling of ET-1 in melanocytes have been a matter of debate in human melanocytes (Imokawa et al, 1996). reported that the ET-1-induced $[Ca^{2+}]_i$ increase was completely inhibited by 50 nM BQ-123, an ET-A receptor antagonist. In contrast, Kang et al. showed inhibition of the $[Ca^{2+}]_i$ increase by BQ-788, an ET-B receptor antagonist, and no inhibition of the ET-1-induced $[Ca^{2+}]_i$ increase up to 30 μM BQ-123. This is consistent with our present results, which showed that ET-1-induced $[Ca^{2+}]_i$ increase was inhibited by 100 nM BQ-788 but not by BQ-123 (100 nM). The expression of ET-B receptors in human melanocytes has been reported (Tada et al, 1998) and defects in the gene coding the ET-B receptor result in inherited pigmentary disorders in humans (Bynash et al, 1994). These observations support our finding that ET-1 induced $[Ca^{2+}]_i$ increase through ET-B receptor in human melanocytes.

The ET-1-induced $[Ca^{2+}]_i$ increase could be produced by phospholipase C (PLC) activation via G-protein-coupled ET receptors, and due to mobilization of Ca^{2+} from inositol 1,4,5-trisphosphate-sensitive intracellular Ca^{2+} stores (Kang et al, 1998). LPA evoked a transient rise in $[Ca^{2+}]_i$, concomitant with formation of IP_3 in various cells (Pietruck et al, 1997; Noh et al, 1998). In the present study, SM709 did not affect LPA-induced $[Ca^{2+}]_i$ increase via Ca^{2+} release from IP_3-sensitive intracellular Ca^{2+} stores. Even after pretreatment with SM709, thapsigargin and LPA induced $[Ca^{2+}]_i$ increase. These results suggested that SM709 did not affect the Ca^{2+} mobilization by ET-1 from IP_3 -sensitive intracellular Ca^{2+} stores in melanocytes. ATP, also known to activate PLC via G protein-linked receptors, increased $[Ca^{2+}]_i$ in the presence of SM709 (Fig. 5). Therefore, our results in the present study suggest that SM709 could block the transmembrane signaling of ET-1 through a specific inhibition(s) against ET-B receptor in human melanocytes.

The important role of $[Ca^{2+}]_i$ was suggested in modulating the responses of melanocytes to melanogenic stimuli rather than in melanogenesis of human melanocytes (Casberg et al, 1995). ET-1 plays a central role in UVB -induced pigmentation (Yada et al, 1991; Imokawa et al, 1992). ET-1 secreted by UVB from surrounding keratinocytes couid activate melanocytes, resulting in proliferation and melanogenesis. ET-1 is a powerful melanogen at the concentration which induces $[Ca^{2+}]_i$ increase in melanocytes (Imokawa et al, 1995). Taken together, our results that SM709 inhibits transmembrane calcium signaling of ET-1 may suggest that it could prevent UVB-induced pigmentation.

In the present study, we demonstrated that SM709 inhibited ET-1-induced $[Ca^{2+}]_i$ increase in human melanocytes. This could most likely be a possible cellular mechanism of antimelanogenic activity of the bamboo extract. However, there are several limitations in our study. We were unable to obtain information on the exact target molecule (s) of the drug binding, and an extensive study of SM709 binding with ET receptors seems to be required to address this question. Furthermore, it would be greatly helpful in understanding possible role of $[Ca^{2+}]_i$ and developing effective skin-lightening agents, if the effect of SM709 on UVB-induced melanogenesis in melanocytes was clearly elucidated.

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