Prostaglandin E$_2$ Enhances Neurotrophin-4 Production via EP3 Receptor in Human Keratinocytes

Naoko Kanda, Satsuki Koike, and Shinichi Watanabe

Department of Dermatology, Teikyo University, School of Medicine, Tokyo, Japan

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ABSTRACT

Atopic dermatitis is characterized by increased skin innervation. The expression of neurotrophin-4 is enhanced in the epidermal keratinocytes of lesions with atopic dermatitis and may be related to hyperinnervation in these lesions. Prostaglandin E$_2$ (PGE$_2$) levels are increased in lesions with atopic dermatitis; thus, PGE$_2$ may be involved in the development of this disease. We examined the in vitro effects of PGE$_2$ on neurotrophin-4 production in human keratinocytes. PGE$_2$ and EP1/EP3 agonist sulprostone increased neurotrophin-4 secretion and mRNA levels without altering its mRNA stability. Antisense Sp1 oligodeoxynucleotide and Sp1 inhibitor mithramycin A suppressed PGE$_2$ and sulprostone-induced neurotrophin-4 expression, indicating the requirement for Sp1 for expression. PGE$_2$ or sulprostone markedly enhanced the phosphorylation, DNA binding, and transcriptional activity of Sp1 and modestly increased Sp1 mRNA and protein levels. PGE$_2$ or sulprostone induced the membrane translocation of protein kinase C$_\alpha$ and the phosphorylation of extracellular signal-regulated kinase (ERK). PGE$_2$-induced increases in neurotrophin-4 expression, Sp1 transcriptional and DNA-binding activity, Sp1 mRNA and protein levels, and ERK phosphorylation were suppressed by antisense EP3 oligodeoxynucleotide, inhibitors of phosphatidylinositol-specific phospholipase C, conventional protein kinase C, and mitogen-activated protein kinase/ERK kinase 1 (MEK1). These results suggest that PGE$_2$ enhances neurotrophin-4 production by activating Sp1 via the EP3/phosphatidylinositol-specific phospholipase C/protein kinase C$_\alpha$/MEK1/ERK pathway. PGE$_2$ may promote innervation in skin lesions with atopic dermatitis via the induction of neurotrophin-4.

Chronic inflammatory skin lesions such as prurigo in atopic dermatitis are characterized clinically by intense pruritus and histologically by increased nerve fibers (Sugiura et al., 1997; Grewe et al., 2000). In particular, free sensory nerve endings that act as receptors of pruritic sensation are frequently increased at the dermoepidermal junction of lesions with atopic dermatitis (Sugiura et al., 1997). The hyperinnervation in these skin lesions may be related to the locally enhanced production of neurotrophic factors (Grewe et al., 2000). It has recently been reported that the expression of a neurotrophic factor, neurotrophin-4 (NT-4), is markedly enhanced in the epidermal keratinocytes of prurigo lesions with atopic dermatitis (Grewe et al., 2000). NT-4 binds high-affinity tyrosine kinase receptor trkB and low-affinity p75 neurotrophin receptor (p75NTR) on neural cells (Barbacid, 1994). Another tyrosine kinase receptor, trkA, also serves as NT-4 receptor with lower efficiency (Barbacid, 1994). The activation of trkB stimulates phosphatidylinositol 3-kinase or extracellular signal-regulated kinase (ERK) (Segal and Greenberg, 1996), whereas the activation of p75NTR stimulates nuclear factor $\kappa$B in neurons (Carter et al., 1996). The activation of trkB alone or together with p75NTR by NT-4 can thus promote the survival and differentiation of neurons (Segal and Greenberg, 1996), which may induce sensory nerve sprouting in atopic prurigo lesions. trkB or p75NTR is expressed on several immune cells, such as eosinophils, mast cells, macrophages, and T cells (Nassenstein et al., 2003). NT-4 promotes survival and CD69 expression in allergen-stimulated endobronchial eosinophils in allergic asthma (Nassenstein et al., 2003). In lesions with atopic dermatitis, keratinocyte-derived NT-4 may modulate the survival or...
function of immune cells and may serve as a substrate for the immune and the nervous systems.

A lipid mediator, prostaglandin E₂ (PGE₂), is produced by a variety of cells in the skin, such as mast cells, macrophages, dendritic cells, and keratinocytes (Kanda et al., 2004). PGE₂ may play either a pro- or an anti-inflammatory role in the skin that is dependent on the target cell type or activation status; the intradermal injection of PGE₂ induces local vaso-dilation and wheals (Greaves and Camp, 1988). On the other hand, PGE₂ enhances the production of immunosuppressive type 2 cytokines, such as interleukin-4 or interleukin-10, and thus suppresses the induction of delayed-type hypersensitivity in the skin (Shreedhar et al., 1998). PGE₂ levels are elevated in atopic skin lesions (Reilly et al., 2000). Thus, it is anticipated that PGE₂ regulates NT-4 production by keratinocytes in lesions with atopic dermatitis. However, the effects of PGE₂ on NT-4 production have not been precisely examined.

Prostaglandin E₂ binds four different G-protein-coupled receptors, EP1 to EP4, on the cell surface (Negishi et al., 1995). Keratinocytes express all four receptors with much higher levels of EP2 and EP3 than those of EP1 and EP4 (Kanda et al., 2004). The activation of EP1 or EP3 on keratinocytes induces intracellular Ca²⁺ signal via the activation of phosphatidylinositol-specific phospholipase C (PI-PLC), whereas the activation of EP2 or EP4 generates a cyclic AMP signal (Kanda et al., 2004). Because the activation of PI-PLC generates diacylglycerol that stimulates protein kinase C (PKC) (Negishi et al., 1990), PGE₂ stimulates PKC via EP1 or EP3 in rat growth zone chondrocytes (Sylvia et al., 2001) or bovine adrenal chromaffin cells (Negishi et al., 1990). PGE₂ also activates ERK that is dependent on or independent of PKC via EP1 or EP3 in human lung cancer A549 cells (Yano et al., 2002) and rat mesangial cells (Suganami et al., 2001).

In this study, we investigated the in vitro effects of PGE₂ on NT-4 production by human keratinocytes. We found that PGE₂ has a stimulatory effect and further analyzed the mechanism of the effects.

Materials and Methods

Reagents. PGE₂, prostaglandin E₁ (1-OH PGE₁), and sulprostone were purchased from Cayman Chemical (Ann Arbor, MI). H-89, cycloheximide, mithramycin A, curcumin, and actinomycin D were purchased from Sigma Chemical Co. (St. Louis, MO). U73122, PD98059, Go 6976, and rottlerin were obtained from Calbiochem (La Jolla, CA).

Culture of Keratinocytes. Human neonatal foreskin keratinocytes were cultured in a serum-free KGM medium (Cambrex Bio Science Walkersville, Walkersville, MD) consisting of keratinocyte basal medium (KBM) supplemented with 0.5 μg/ml hydrocortisone, 5 ng/ml epidermal growth factor, 5 μg/ml insulin, and 0.5% bovine pituitary extract. Cells in the third passage were used.

NT-4 Secretion. Keratinocytes (5 × 10⁴/well) were seeded in triplicate into 24-well plates in 0.4 ml of KGM, adhered overnight, washed, and incubated with KBM including 1 μM indomethacin for 24 h. The cells were washed and treated with the indicated concentrations of PGE₂, sulprostone, or 1-OH PGE₁ in KBM, including indomethacin, for the indicated periods. The culture supernatants were assayed for NT-4 by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN). In some experiments, the keratinocytes were preincubated with signal inhibitors for 30 min before the addition of PGE₂, sulprostone, or 1-OH PGE₁.

Reverse Transcription-Polymerase Chain Reaction. The keratinocytes were incubated as above for the indicated periods, and then the total cellular RNA was extracted and reverse-transcribed to produce cDNA (Kanda and Watanabe, 2003). The cDNA was ther-mocycled for polymerase chain reaction (PCR) as described previously (Kanda and Watanabe, 2003; Pang et al., 2003). The primers for amplification and the sizes of the respective PCR products were as follows: NT-4, 5'-CTGTGCCATGTCCTGCT-3' and 5'-GGCAT-GAGCTC-3' for 209 bp; Sp1, 5'-ACAGTGGACTTGCTAC-3' and 5'-GCTGTTTTCTCGCTTATG-3' for 370 bp; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-GCAGGGGAGGC- CAAAAGG-3' and 5'-TGGCAGCCCGGCTCAAAG-3' for 566 bp (Küst et al., 2002; Kanda and Watanabe, 2003; Pang et al., 2003). PCR was performed by one denaturing cycle at 95°C for 3 min, 30 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s, and a final extension at 72°C for 3 min. PCR products were analyzed by electrophoresis, and densitometric analysis was performed using ATTO lane analyzer version 3 (ATTO Corporation, Osaka, Japan). NT-4 or Sp1 mRNA levels were normalized to those of GAPDH.

mRNA Stability Analysis. The keratinocytes were treated with PGE₂, sulprostone, or 1-OH PGE₁ for 8 h. RNA synthesis was blocked by actinomycin D (5 μg/ml), and RNA was isolated 0, 1, 2, 4, 8, 16, and 32 h later. Reverse transcription (RT)-PCR was performed as described above, and the decay of mRNA was determined from the band density ratios of NT-4/GAPDH as described previously (Kanda et al., 2004).

Plasmid and Transfection. Transfected Sp1 reporter vector (Luc Sp1) containing Sp1 enhancer elements (5'-ATTCCATGGCGGCGGCGGAGGCGGAG-3', consensus sequence underlined) in front of TATA box upstream of firefly luciferase reporter was purchased from Panomics (Redwood City, CA). Transient transfection was performed with FuGENE 6 (Roche Diagnostics, Indianapolis, IN) as described previously (Kanda et al., 2004). The keratinocytes were plated in 35-mm dishes and grown to approximately 60% confluence. Luc Sp1 (1 μg) and 0.2 μg of herpes simplex virus thymidine kinase promoter-linked Renilla luciferase vector (pHRL-TK) (Promega, Madison, WI) mixed with 3 μl of FuGENE 6 were added to the keratinocytes. After 24 h, the cells were washed and incubated in KBM containing indomethacin for 24 h and then treated with PGE₂, sulprostone, or 1-OH PGE₁. After 24 h, the firefly and Renilla luciferase activities of the cell extracts were quantified using the dual luciferase assay system (Promega). Sp1 transcriptional activity was expressed as a ratio of firefly:Renilla luciferase activity.

Electrophoretic Mobility Shift Assay. Electrophoretic mobility shift assay (EMSA) was performed using an EMSA kit (Panomics) by incubating a biotin-labeled probe with a nuclear extract. The probe used was double-stranded oligonucleotide with consensus Sp1 sequence (5'-ATTCCATGGCGGCGGCGGAG-3'). The keratinocytes were pretreated with signal inhibitors for 30 min and then treated with PGE₂, sulprostone, or 1-OH PGE₁ for 2 h. Nuclear extracts were obtained from the keratinocytes using a nuclear extraction kit (Panomics) according to the manufacturer's instructions. For the gel shift assays, 5 μg of nuclear extracts were incubated with 10 ng of the labeled probe at room temperature for 30 min. Protein-DNA complexes were separated by 6% polyacrylamide gel and electrically transferred to a Biodyne B membrane (Pall Corporation, East Hills, NY) for chemiluminescence band detection. In the antibody supershift experiments, nuclear extracts were preincubated with rabbit polyclonal anti-Sp1 or Sp3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min before the addition of the probe.

Treatment with Antisense Oligodeoxynucleotide. Antisense oligonucleotides against Sp1, Sp3, EP₁, or EP₃ proteins were synthesized as phosphorothioate-modified oligonucleotides and were high-pressure liquid chromatography-purified as described previously (Hata et al., 1998; Su et al., 2004). The oligonucleotides were Sp1 (5’-ATATTAGGGCAGCATTCCACTCCAGG-3’), Sp3 (5’-AGTACCGAGG- CCTGGAATCTGGACT-3’), EP1 (5’-GCAAGGGGCTCATGTCAGG-
3'), EP3 (5'-GTCTCCCTCATGTTGGGC-3'), and control-scrambled (5'-AGTACAGGACTGAGTTGCTACT-3'). The keratinocytes were transfected with a final volume of 0.2 μM of the indicated oligonucleotides premixed with FuGENE 6 in KGM for 24 h. The medium was aspirated, and the cells were cultured with KGM including indomethacin (1 μM) for 24 h and then treated with PGE2, sulprostone, or 1-OH PGE1. In some experiments, these antisense oligonucleotides were transfected together with Luc Sp1 and pHL-TK.

**Western Blotting.** The phosphorylation of ERK was analyzed by Western blotting. The keratinocytes were pretreated with signal inhibitors for 30 min and then treated with PGE2, sulprostone, or 1-OH PGE1 for 10 min. The cells were lysed, and equal amounts of whole-cell lysates (20 μg/lane) were resolved by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was blocked and incubated with anti-phospho-ERK1/2 or anti-ERK1/2 antibody (Santa Cruz Biotechnology) followed by peroxidase-conjugated secondary antibodies (Bio-Rad, Hercules, CA). The blots were developed using an enhanced chemiluminescence kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK), and band intensity was measured by densitometric analysis.

The amount and phosphorylation status of Sp1 protein were also determined by Western blotting. The keratinocytes were treated with PGE2, sulprostone, or 1-OH PGE1 for 2 h. The nuclear extracts were separated and electrophoretically resolved and then transferred to membranes. The membranes were incubated with anti-Sp1 or anti-Oct-1 antibody (Santa Cruz Biotechnology) and then with secondary antibodies and developed as described above.

The subcellular localization of PKC isoforms was examined as described previously (Kanda and Watanabe, 2004). Nuclei and debris were removed from the whole-cell lysates by centrifugation (500g, 5 min), and this postnuclear fraction was centrifuged (10,500g, 90 min). The supernatant was saved as the cytosolic fraction. The pellet was homogenized in the same buffer, except that it contained 0.1% Triton X-100. The samples were mixed continuously for 1 h at 4°C and then centrifuged as described above. This supernatant was saved as the membrane fraction. Twenty micrograms of proteins of the cytosolic or membrane fractions were subjected to SDS-polyacrylamide gel electrophoresis and transferred as above, and the blots were incubated with anti-PKCα, βI, βII, γ, δ, ε, η, or ζ antibodies (Santa Cruz Biotechnology) followed by secondary antibodies and developed as above. The antisense oligonucleotide-induced reduction in the respective protein levels was examined by Western blotting using the whole-cell lysates and anti-EP1 and EP3 antibodies (Cayman Chemical), anti-Sp1, Sp3, or GAPDH antibody (Santa Cruz Biotechnology), and secondary antibodies as described above.

**Measurement of PGE2 Release.** The keratinocytes were incubated with KBM in the presence or absence of 1 μM indomethacin for 48 h. The supernatant PGE2 amount was measured by enzyme-linked immunosorbent assay.

**Statistical Analyses.** Statistical evaluation of the results was performed by one-way analysis of variance using Dunnet’s multiple comparison test for Figs. 2B, 3B, 4A, 7A, and 7F. The results were considered significant at a value of P < 0.05.

**Results**

**Enhancement of NT-4 Secretion and mRNA Expression by PGE2 or Sulprostone.** Constitutive PGE2 release from the keratinocytes was 4.21 ± 0.48 pmol/10^6 cells (mean ± S.E.M., n = 4). Cyclooxygenase inhibitor indomethacin (1 μM) reduced PGE2 release to an undetectable level (<0.36 pmol/10^6 cells) but did not reduce cell viability (>95% viable) or alter EP1, 2, 3, and 4 expression in the keratinocytes as determined by flow cytometry (data not shown). To avoid the influence of endogenous PGE2 from the keratinocytes, further experiments were performed in the presence of indomethacin.

The keratinocytes constitutively secreted small amounts of NT-4 for 48 h (mean ± S.E.M. 172.0 ± 22.4 pg/10^6 cells,
n = 4), and the secretion was dose-dependently increased by PGE2 or EP1/EP3 agonist sulprostone, whereas the secretion volume was not altered by EP2/EP4 agonist 1-OH PGE1 (Fig. 1A), indicating that EP1 or EP3 is involved in PGE2-induced NT-4 secretion. PGE2 and sulprostone (10^{-6} M) increased NT-4 secretion 9.9- and 10.3-fold compared with the controls, respectively. PGE2 or sulprostone-induced increases in NT-4 secretion were abolished by cycloheximide (Fig. 1A), indicating the requirement of de novo protein synthesis. A significant increase in NT-4 secretion compared with the controls was observed beginning 8 h after PGE2 or sulprostone stimulation, increasing and continuing up to 48 h (Fig. 1B).

To identify the PGE2 receptor isoforms involved in NT-4 secretion, we studied whether antisense oligonucleotide against EP1 or EP3 suppresses PGE2 or sulprostone-induced increases in NT-4 secretion. Antisense oligonucleotide against EP1 or EP3 selectively suppressed the expression of the respective receptor protein (Fig. 2A). Antisense EP3 suppressed PGE2 or sulprostone-induced NT-4 secretion, but antisense EP1 or control-scrambled oligonucleotide did not (Fig. 2B), indicating that EP3 but not EP1 is involved in PGE2 or sulprostone-induced NT-4 secretion. We next examined whether PGE2 or sulprostone increases NT-4 mRNA levels in the keratinocytes. At 8 h of incubation, PGE2 and sulprostone increased NT-4 mRNA levels 6.9- and 7.6-fold compared with the controls, respectively, whereas the levels were not altered by 1-OH PGE1 (Fig. 2C). Antisense EP3 blocked PGE2, but antisense EP1 did not block PGE2 or sulprostone-induced increases in NT-4 mRNA level, which paralleled the results for NT-4 secretion (Fig. 2B). These results suggest that PGE2 or sulprostone increases NT-4 production at the pretranslational level via EP3.

We next examined whether PGE2 or sulprostone increased the stability of NT-4 mRNA. The estimated half-life of NT-4 mRNA was mean ± S.E.M. (n = 4) 8.3 ± 0.9, 8.0 ± 0.9, 8.2 ± 0.8, and 8.2 ± 0.9 h in the control, PGE2, sulprostone, and 1-OH PGE1 (each 10^{-6} M treated keratinocytes), respectively, and there were no significant differences in the comparison of either of the two groups (P > 0.05 by one-way analysis of variance using Scheffe’s multiple comparison test). Thus, PGE2 or sulprostone did not increase the stability of NT-4 mRNA, indicating that these agents enhance NT-4 production at the transcriptional level.
Involvement of Sp1 in PGE₂ or Sulprostone-Induced NT-4 Expression. Although human NT-4 promoter has not yet been completely identified, rat NT-4 transcription can be controlled by two promoters, a proximal promoter containing several Sp1-binding sites and a distal promoter containing two activator protein-1 (AP-1) sites (Salin et al., 1997). Thus, we analyzed the involvement of transcription factors Sp1 or AP-1 in PGE₂-induced NT-4 expression using specific inhibitors and antisense oligonucleotides. Treatment with antisense oligonucleotide against Sp1 or Sp3 selectively reduced the levels of phosphorylated (105 kDa) and nonphosphorylated (90 kDa) Sp1 and levels of large (100 kDa) and small (60 kDa) Sp3 isoforms, respectively (data not shown). Mithramycin (10⁻⁶ M) or antisense Sp1 suppressed PGE₂-induced increases in NT-4 mRNA expression (Fig. 3A) and protein secretion (Fig. 3B), whereas AP-1 inhibitor curcumin, control-scrambled oligonucleotide, or antisense Sp3 did not. Similar results were obtained when sulprostone was used instead of PGE₂ (data not shown). These results suggest that Sp1 is responsible, whereas AP-1 may be dispensable for PGE₂ or sulprostone-induced NT-4 expression. We then analyzed whether PGE₂ or sulprostone enhances the transcriptional activity or DNA binding of Sp1.

Effects of PGE₂ or Sulprostone on the Transcriptional Activity, DNA Binding, Phosphorylation, and mRNA or Protein Levels of Sp1. PGE₂ or sulprostone enhances the transcriptional activity and DNA binding of Sp1. The keratinocytes were transiently transfected with luciferase vector linked to GC-rich Sp1 binding sequences in front of TATA box (Luc Sp1). PGE₂ or sulprostone increased Sp1-dependent transcriptional activity, whereas 1-OH PGE₁ did not (Fig. 4A). Mithramycin A or antisense Sp1 suppressed PGE₂-induced increases in Sp1 transcriptional activity, whereas antisense Sp3 or control oligonucleotide did not, supporting the existence of Sp1-specific activity. PGE₂ (Fig. 4A) or sulprostone-induced increases (data not shown) in Sp1 activity were suppressed by antisense EP3 but not by antisense EP1. These results suggest that PGE₂ or sulprostone increases Sp1-dependent transcriptional activity via EP3. EMSA was then performed using nuclear extracts and an oligonucleotide probe containing consensus GC-rich Sp1 binding sequences. At 2 h of incubation, PGE₂ (Fig. 4B, lane 2) or sulprostone (lane 3) increased the amount of DNA-protein complex with the Sp1 probe, whereas 1-OH PGE₁ did not alter the amount (lane 4). PGE₂ (Fig. 4B) or sulprostone-induced increases (data not shown) in the complex were suppressed by antisense EP3 (lane 7) but not by antisense EP1 (lane 6). The DNA-protein complex induced by PGE₂ was completely abolished by mithramycin A (lane 8), and anti-Sp1 antibody supershifted the complexes (lanes 9 and 11) but anti-Sp3 antibody did not (lanes 10 and 12), indicating the presence of Sp1 in the complexes. These results suggest that PGE₂ or sulprostone enhances Sp1 binding to DNA via EP3.

We then investigated whether PGE₂ or sulprostone-induced Sp1 binding and transcriptional activity was due to increased Sp1 protein expression and/or post-translational modification. PGE₂ or sulprostone markedly increased the ratio between phosphorylated and nonphosphorylated Sp1 19.0- or 24.5-fold, respectively, compared with the controls (Fig. 5A), whereas 1-OH-PGE₁ was much less potent at a 1.8-fold increase. PGE₂ (Fig. 5A) or sulprostone-induced Sp1 phosphorylation (data not shown) was suppressed by antisense EP3 but not by antisense EP1. The total protein level of Sp1 in the nuclear extract was slightly increased by 84 and 55% after PGE₂ and sulprostone treatment, respectively (Fig. 5A). In parallel to protein levels, PGE₂ and sulprostone modestly increased Sp1 mRNA levels by 222 and 169%, respectively (Fig. 5B). The PGE₂-induced increases in Sp1 protein (Fig. 5A) and mRNA levels (Fig. 5B) were suppressed by antisense EP3 but not by antisense EP1. These results suggest that PGE₂ or sulprostone markedly enhances Sp1 phosphorylation and modestly increases mRNA and protein levels via EP3, and these effects may lead to the enhancement of Sp1 binding and transcriptional activity.

Involvement of the PI-PLC-PKCα-MEK1-ERK Pathway in PGE₂-Induced Sp1 Activation and NT-4 Production. It has been reported that the activation of EP1 or EP3 by PGE₂ is linked to the stimulation of PI-PLC, PKC, or ERK (Negishi et al., 1990, 1995; Yano et al., 2002), whereas the activation of EP2 or EP4 induces a cyclic AMP signal that stimulates protein kinase A (PKA) (Negishi et al., 1995; Kanda et al., 2004). The transcriptional activity of Sp1 is also stimulated by PKC, ERK, or PKA (Black et al., 2001).

To know the signaling pathway(s) involved in PGE₂ or the sulprostone-induced activation of Sp1, we examined whether specific signaling enzyme inhibitors suppress the effects of these agents. PGE₂-induced increases in Sp1 transcriptional activity (Fig. 6A), DNA binding (Fig. 6B), phosphorylation levels (Fig. 6C), and in protein (Fig. 6C) or mRNA levels (Fig. 3).
6D) were completely suppressed by PI-PLC inhibitor U73122, conventional PKC inhibitor Gö6976, and PD98059, which suppresses ERK by inhibiting MEK1 to phosphorylate ERK1/2. On the other hand, novel PKC inhibitor rottlerin or PKA inhibitor H-89 did not block the effects of PGE2 on Sp1 levels and activities. Similar results were obtained when sulprostone was used instead of PGE2 (data not shown). These results suggest that PI-PLC, conventional PKC, and MEK1 are responsible for PGE2 or the sulprostone-induced activation of Sp1.

Because PGE2-induced NT-4 expression seemed to be mediated by Sp1, we analyzed whether signal inhibitors suppressing the PGE2-induced activation of Sp1 similarly suppressed PGE2-induced NT-4 expression. In parallel to the results for Sp1, PGE2-induced increases in NT-4 mRNA levels (Fig. 6E) and protein secretion (Fig. 6F) were completely suppressed by U73122, Gö6976, and PD98059, whereas these were not altered by rottlerin or H-89. Similar results were obtained when sulprostone was used instead of PGE2 (data not shown). These results suggest that PI-PLC, conventional PKC, and MEK1 are responsible for PGE2 or the sulprostone-induced expression of NT-4 as well as the activation of Sp1 by these agents.

MEK1 activates ERK via dual phosphorylation on threonine and tyrosine residues (Kanda and Watanabe, 2004). Thus, we examined the phosphorylation status of ERK after treatment with PGE2 or sulprostone. At 10 min, PGE2 or sulprostone enhanced the dual phosphorylation of ERK1 (44 kDa) and ERK2 (42 kDa), whereas 1-OH PGE1 did not (Fig. 7). The total ERK1/2 levels were not altered by these agents. PGE2 (Fig. 7) or sulprostone-induced ERK1/2 phosphorylation (data not shown) was suppressed by antisense EP3, U73122, Gö6976, and PD98059 while not suppressed by antisense EP1, rottlerin, or H-89. The results suggest that PGE2-induced ERK activation is mediated via EP3 and requires PI-PLC, conventional PKC, and MEK1 activities. Human keratinocytes express conventional PKCα, novel PKCδ, ε, and η, and atypical PKCζ (Reynolds et al., 1994). The translocation of PKC from cytosol to membrane has been commonly accepted as an index of PKC activation (Reynolds et al., 1994). Thus, we examined whether conventional PKCα is translocated from cytosol to membrane by PGE2 or sulprostone. PGE2 or sulprostone induced the membrane translocation of PKCα from cytosol (Fig. 8). The membrane translocation of PKCα by PGE2 was suppressed by U73122 and antisense EP3 but not by antisense EP1 (Fig. 8). Other conventional PKC isoforms (PKCβ1, βII, or γ) were not detected in the cytosol or membrane fractions from the keratinocytes (data not shown). The membrane translocation of PKCδ, ε, η, and ζ was not induced by PGE2 or sulprostone (data not shown). The results suggest that PKCα is selectively activated by PGE2 or sulprostone via EP3 and PI-PLC.
Discussion

In this study, PGE2 enhanced NT-4 production in keratinocytes via EP3 receptor. There have been no reports on the function of EP3 in neurotrophin production. Our findings thus suggest a novel key role of EP3 in NT-4 induction. EP3 may mediate NT-4 production in other cell types than keratinocytes and may play a neurotrophic role in tissues outside the skin. The stimulation of EP3 may also potentiate the production of other neurotrophins such as nerve growth factor or NT-3. We should further examine whether the activation of EP3 induces the production of these neurotrophins in human keratinocytes.

The EP3-mediated transcription of NT-4 was dependent on the activity of Sp1. Human NT-4 promoter has not been completely characterized; however, it may have several Sp1-binding sites because antisense Sp1 suppressed NT-4 expression. Further studies should identify the Sp1 elements responsible for human NT-4 transcription.

The EP3-mediated transcriptional activity and DNA binding of Sp1 (Fig. 4, A and B) positively correlated with its phosphorylation level (Fig. 5A), indicating that phosphorylation may potentiate the transcriptional activity of Sp1. The phosphorylation of Sp1 may dissociate transcriptional repressor(s) from Sp1, such as Sp1-I and p74, which inhibits the DNA binding and transcriptional activity of Sp1, respectively (Chen et al., 1994; Murata et al., 1994), or from Sp1-bound promoter, such as histone deacetylase 1 (Choi et al., 2002). Alternatively, the phosphorylation of Sp1 may recruit transcriptional coactivators such as p300 or enhance its interaction with basal transcriptional machinery such as dTAF1110 (Gill et al., 1994).

Fig. 5. Enhancement of Sp1 phosphorylation and increases in Sp1 protein or mRNA levels induced by PGE2 or sulprostone. The keratinocytes were transfected with antisense oligonucleotide (AS) against Sp1, Sp3, or control-scrambled oligonucleotide (Con) (each 0.2 μM) and then incubated with PGE2, sulprostone (Sul), or 1-OH PGE1 (1-OH) (each 10^-6 M) for 2 h. A, the nuclear extracts were blotted with anti-Sp1 or Oct-1 antibody. The band intensity ratio of phosphorylated Sp1 (pSp1)/nonphosphorylated Sp1 was corrected to that in the control (set as 1.0). The band intensity ratio of total Sp1 (phosphorylated and nonphosphorylated Sp1)/Oct-1 was corrected to that in the control. B, RNA was subjected to RT-PCR, and Sp1 mRNA levels were normalized to those of GAPDH and are shown as a -fold induction. The results shown are representative of four separate experiments.

Fig. 6. Suppression of PGE2-induced Sp1 activation or NT-4 expression by inhibitors of PI-PLC, conventional PKC, and MEK1. A, the keratinocytes were transfected with Luc Sp1 and phRL-TK and were preincubated with U73122 (U; 10^-6 M), Go 6976 (Go; 10^-8 M), rottlerin (Rott; 10^-6 M), PD98059 (PD; 10^-5 M), or H-89 (10^-7 M) and then incubated with PGE2 (10^-6 M). After 24 h, luciferase activity was analyzed. B, C, D, E, and F, keratinocytes without transfection were preincubated with inhibitors and then incubated with PGE2 as above. At 2 h, the nuclear extracts were subjected to EMSA using an Sp1 probe (B) or blotted with anti-Sp1 or Oct-1 antibody (C). The band intensity ratio of phosphorylated Sp1 (pSp1)/nonphosphorylated Sp1 was corrected to that in the control (set as 1.0). The band intensity ratio of total Sp1 (phosphorylated and nonphosphorylated Sp1)/Oct-1 was corrected to that in the control. B, C, D, and E, RNA was subjected to RT-PCR, and Sp1 mRNA levels were normalized to those of GAPDH and are shown as a -fold induction. The results shown are representative of four separate experiments. The data in A and F are mean ± S.E.M. of four separate experiments. †, P < 0.05, significantly different from the control values; ‡, P < 0.05, significantly different from values with PGE2 alone.
The stimulation of EP3 by PGE$_2$ activated ERK (Fig. 7), and this kinase was essential for the phosphorylation and transcriptional activity of Sp1 (Fig. 6). It has also been reported that the activation of ERK leads to the enhancement of the phosphorylation and DNA binding of Sp1 in human gastric adenocarcinoma cells (Merchant et al., 1999). Because Sp1 contains six putative ERK phosphorylation sites (Merchant et al., 1999), ERK may directly phosphorylate and activate Sp1 in PGE$_2$-stimulated keratinocytes. Alternatively, kinase(s) downstream of ERK may phosphorylate Sp1 in these cells. Further studies should identify direct Sp1 kinase induced by PGE$_2$ and phosphorylation sites on Sp1. In parallel to the enhanced phosphorylation of Sp1 (Fig. 5A), the protein and mRNA levels of Sp1 were increased by PGE$_2$ (Fig. 5, A and B), although the magnitude of the increased expression (approximately 2- or 3-fold compared with the controls) was much lower compared with that of phosphorylation (approximately 20-fold). It is possible that the increased expression of Sp1 may just result from the enhanced phosphorylation of Sp1, because Sp1 promoter itself contains several Sp1-binding elements and is positively regulated by its own gene product, Sp1 protein (Nicolas et al., 2001).

The activation of ERK via EP3 depended on the activity of PI-PLC and conventional PKC in the keratinocytes (Fig. 7). EP3 is linked to PI-PLC, and PI-PLC generates diacylglycerol, which activates conventional and novel PKCs (Negishi et al., 1990). The stimulation of EP3 by PGE$_2$ led to the activation of conventional PKC$_\alpha$ dependently on PI-PLC in human keratinocytes (Fig. 8). PKC$_\alpha$ was also activated by PGE$_2$ via PI-PLC in rat osteoblasts in vitro (Tang et al., 2005). On the other hand, in the medullary thick ascending limb of rat kidney, novel PKC$_\delta$ was activated by PGE$_2$ in vitro only in the presence of arginine vasopressin, whereas PKC$_\alpha$ was not (Aristimuno and Good, 1997). PGE$_2$ in vivo activated novel PKC$_\delta$s in rat colonic mucosa (Conte et al., 2004). Thus, PKC isoforms activated by PGE$_2$ may vary according to cell type, species, or experimental conditions and may reflect the relative expression, activity, or intracellular compartmentalization of individual PKC isoforms. PKC$_\alpha$ phosphorylates and activates c-Raf (Kolch et al., 1993), and the activated c-Raf may further phosphorylate and activate MEK1 that catalyzes the phosphorylation of ERK. Thus, the activation of EP3 may trigger the signaling cascade of PI-PLC-PKC-c-Raf-MEK1-ERK, resulting in the activation of Sp1. EP1 is also linked to the PI-PLC-PKC pathway in rat growth-zone chondrocytes (Sylvia et al., 2001) or linked to the activation of ERK in rat kidney mesangial cells (Suganami et al., 2001). However, antisense EP1 did not suppress the PGE$_2$-induced activation of PKC$_\alpha$ (Fig. 8) or ERK (Fig. 7) in human keratinocytes, indicating EP1-independent effects. This is possibly because the expression level and/or PGE$_2$ affinity of EP1 are much lower than those of EP3 in human keratinocytes (Kanda et al., 2004).

EP2/EP4 agonist 1-OH PGE$_1$ slightly increased the phosphorylation, mRNA, or protein levels of Sp1 (Fig. 5, A and B); however, it did not significantly increase the DNA binding and transcriptional activity of Sp1 (Fig. 4, A and B) or expression of NT-4 (Figs. 1 and 2). EP2 or EP4 is linked to PKA (Kanda et al., 2004), and Sp1 can be phosphorylated and activated by PKA (Black et al., 2001). Thus, PKA activated by 1-OH PGE$_1$ via EP2 or EP4 may phosphorylate Sp1. However, the phosphorylation level may be less than the threshold for driving the Sp1-dependent transcription of NT-4. The kinase(s) activating Sp1 may vary according to cell type or stimuli and reflect the relative activities of the signaling molecules in the target cells.

Our present findings indicate that PGE$_2$ in vivo promotes skin innervation via the induction of NT-4, especially in lesions with atopic dermatitis associated with elevated PGE$_2$ levels. The level of PGE$_2$ released from keratinocytes in vitro (4.21 pmol/10$^6$ cells, corresponding to approximately 0.52 nM) was less than the threshold (10 nM) for NT-4 induction (Fig. 1A) and may not enhance NT-4 production per se. However, in skin lesions with atopic dermatitis, more than the threshold levels of PGE$_2$ might be released from neighboring cells such as macrophages or mast cells infiltrating the lesions (Imayama et al., 1995; Kiekens et al., 2001). In atopic skin lesions, the amounts of proinflammatory cytokines, tumor necrosis factor-$\alpha$, interleukin-1, or interferon-$\gamma$ may be increased and this may promote PGE$_2$ release from the
neighboring cells by inducing the expression of cyclooxygenase-2 (Takayama et al., 2002). Thus, in skin lesions with atopic dermatitis, PGE₂ from cells neighboring keratinocytes may act on EP3 on keratinocytes and promote the synthesis and secretion of NT-4. The secreted NT-4 may support the survival and sprouting of nerve fibers, especially sensory C-fibers, and thus sustain a pruritic sensation. In addition, the secreted NT-4 may modulate skin inflammation by regulating the survival or activity of macrophages, mast cells, eosinophils, or T cells containing trkB or p75NTR. PGE₂ in skin lesions with atopic dermatitis thus may contribute to cross-talk between the immune and nervous systems by the induction of NT-4. We should further examine whether the topical addition of PGE₂ enhances NT-4 expression in the keratinocytes of skin lesions with atopic dermatitis and whether antiinse oligonucleotide against EP3 blocks the effects of PGE₂. EP3 may be a novel therapeutic target for atopic dermatitis.

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References


Address correspondence to: Dr. Naoko Kanda, Department of Dermatology, Teikyo University School of Medicine, 11-1, Kaga-2, Ibaraki-Ku, Tokyo 173-8605, Japan. E-mail: nmk@med.teikyo-u.ac.jp.