Contribution of Extracellular Signal-Regulated Kinase to UTP-Induced Interleukin-6 Biosynthesis in HaCaT Keratinocytes

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Abstract. UTP causes interleukin (IL)-6 production via mRNA expression through P2Y₂/P2Y₄ receptors in human HaCaT keratinocytes. In the present study, we analyzed the mechanism of UTP-induced IL-6 production in these cells. UTP, an agonist of P2Y₂/P2Y₄ receptors, induced phosphorylation of extracellular signal-regulated kinase (ERK) in a concentration- and time-dependent manner. PD98059, a MEK (mitogen-activated protein kinase kinase) inhibitor, and BAPTA-AM [O,O'-bis(2-aminophenyl)ethyleneglycol-N,N,N',N'-tetraacetic acid, tetraacetoxy-methyl ester], an intracellular Ca²⁺ chelator, reduced UTP-induced ERK phosphorylation and IL-6 mRNA expression. 2-APB [(2-aminoethoxy)diphenylborane], an inositol 1,4,5-trisphosphate (IP₃)-receptor antagonist, inhibited UTP-induced IL-6 mRNA expression; and the action of A23187, a Ca²⁺ ionophore, resembled the action of UTP. In contrast, protein kinase C (PKC) downregulation and pertussis toxin did not affect UTP-induced IL-6 mRNA expression, suggesting that PKC and Gi are not involved in the UTP-induced IL-6 production. However, AG1478, an epidermal growth factor (EGF)-receptor inhibitor, partially decreased UTP-induced ERK phosphorylation and IL-6 expression. These results suggest that UTP-induced IL-6 production is in part mediated via phosphorylation of ERK through Gq/11/IP₃/[Ca²⁺]i and transactivation of the EGF receptor.

Keywords: interleukin-6, extracellular signal-regulated kinase (ERK), HaCaT, Ca²⁺ elevation, epidermal growth factor (EGF) receptor

Introduction

Skin is the largest organ in the body, and it has various roles, including preventing the body from drying, protection from heat, and preventing the invasion of bacteria, and protection from other physical stimulations. The skin consists of the epidermis, dermis, and hypodermis. Keratinocytes in the epidermis and fibrobrasts in the dermis are known to produce various kinds of cytokines, such as interleukins and interferons (1, 2), showing that skin is an important organ as a site of immunoreaction.

Recently, it has been shown that keratinocytes release ATP in response to physical stimuli (3), as well as in response to various kinds of cells (4). Although ATP is known as an energy donor in the cells, it also acts as an extracellular signaling molecule for a cell-to-cell communication (5). ATP released from cells to the extracellular space binds to P2 receptors on cell membranes (6). P2 receptors are classified into P2X (P2X₁,₂,₃,₄,₅,₆,₇) and P2Y (P2Y₁,₂,₄,₆,₁₁,₁₂,₁₃,₁₄) receptors (7). P2X receptors are ligand-gated cation channels, stimulation of which results in increase of intracellular free Ca²⁺ concentrations ([Ca²⁺]i) (8). Recently, it was found that the P2X₂/₃ heterodimer is expressed in sense neuron terminal and mediates the signal of pain (9). P2Y receptors are G-protein coupled receptors (10). Among the P2Y receptors, P2Y₁,₂,₄,₆,₁₁ are linked to Gq/11, activation of which stimulates phospholipase C (PLC), resulting in the
production of two second messengers, inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (11). IP₃ releases Ca²⁺ from endoplasmic reticulum, whereas diacylglycerol activates protein kinase C (PKC) in the presence of Ca²⁺ and phosphatidylserine (11).

It is well known that interleukin-6 (IL-6) is involved in the immunoreaction (12). Recently, it is expected that IL-6 has a role in protecting peripheral nerve cells because IL-6 knockout mice show a significant delay in wound healing and neuropathy and/or delay in regeneration of the neuroaxis (13).

It has been shown that extracellular signal regulated kinase (ERK) signaling is involved in cytokine production including IL-6 (14). ERK is well-characterized as one of the mitogen-activated protein kinases (MAPKs) and is primarily associated with the regulation of proliferation, anti-apoptosis, differentiation, and gene expression (15, 16).

Recently, it has been shown that P2 receptors are expressed in keratinocytes (17, 18), but little is known about their physiological significance. During studies to clarify the role of ATP released from keratinocytes, we found that ATP produced IL-6 through P2Y receptors in HaCaT keratinocytes (19). Although IL-6 production has been widely investigated in various kinds of cells (20), the mechanism of IL-6 production via P2Y₂/P2Y₄ receptor in keratinocytes remains unclear. In the present study, we investigated the intracellular signaling pathway for IL-6 production via P2Y₂/P2Y₄ receptor in HaCaT keratinocytes.

Materials and Methods

Cell culture

Human HaCaT keratinocytes, which were given to us by Dr. N.E. Fusenig (German Cancer Research Center, Heidelberg, Germany), were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (50 units/ml) and streptomycin (50 µg/ml) in a humidified atmosphere of 5% CO₂/95% air at 37°C (19).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from agonist-stimulated or quiescent cells by the TRI REAGENT (Sigma-Aldrich Co., St. Louis, MO, USA), according to the manufacturer’s protocol. A first stranded cDNA primed by Oligo(dT) primer (Promega Co., Madison, WI, USA) was prepared from total RNA (1 µg) by ReverTraAce (Toyobo Co., Ltd., Tokyo), and it was diluted with water by 5 times to use as a template for the PCR analysis. Primers for human IL-6, designed based on the published cDNA sequences, are as follows: sense, 5’-AGAGTATGAGGAAACAAGCC-3’ and antisense, 5’-TACATTGCGGAAGGCCT-3’. β-Actin mRNA was determined as a positive control. Primers for human β-actin were also designed based on the published cDNA sequences and were as follows: sense, 5’-AGGGAATCGTGCTGACAT-3’ and antisense, 5’-TCCTGCTTGTAGCACC-3’. PCR was carried out in a 10 µl solution containing 10 × PCR buffer (1 µl), RT template (1 µl), 2.5 mM dNTP mixture (0.5 µl), water (6.45 µl), primer (1 µl), and Taq polymerase (0.25 units). The PCR conditions were: 94°C for 2 min, followed by 27 cycles (for IL-6), or 17 cycles (for β-actin) of 30 s at 94°C, 30 s at 56°C, and 2 min at 72°C, with final extension at 72°C for 10 min. The PCR products were separated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. Real-time PCR is carried out in a 20 µl solution containing SYBR Premix (10 µl), RT template (3 µl), water (6 µl) and primer (1 µl), using the DNA engine Opticon System (MJ Research, Waltham, MA, USA). The Real-time PCR conditions were: 94°C for 10 s, followed by several cycles of 10 s at 94°C, 20 s at 56°C, and 30 s at 72°C. The value of IL-6 mRNA was divided by the corresponding value of β-actin (19).

Measurement of IL-6 by enzyme-linked immunosorbent assay (ELISA)

HaCaT cells were seeded at 1.0 × 10⁵ (cells/well) in a 24-well plate and grown for 2 days. HaCaT cells were stimulated for various periods, and the incubation medium (250 µl) was collected after centrifugation to remove floating cells. Human IL-6 was measured by a sandwich-ELISA method using a capture antibody for human IL-6, biotin-conjugated anti-human IL-6 antibody, and avidin-horseradish peroxidase (all antibodies from eBioscience, Inc., San Diego, CA, USA) according to the manufacturer’s protocol. The absorbance at 450 nm was measured by a plate-reader (SUNRISE; TECAN Group, Ltd., Maennedorf, Switzerland), after incubation with the substrate (o-phenylenediamine dihydrochloride, OPD, from Sigma-Aldrich Co.) for horseradish peroxidase. The amount of human IL-6 in a well was calculated as pg/well (19).

SDS-polyacrylamide gel electrophoresis and immunoblotting

Samples used were prepared as follows: HaCaT cells were seeded onto six-well plates at a density of 2 × 10⁵ (cells/well). The cells were cultured overnight in 0.1% serum DMEM, and then they were incubated with drugs for various periods of time. The incubation medium was aspirated after the reaction, and the cells
were dissolved in Laemmli sample buffer (final concentration: 75 mM Tris-HCl, 2% SDS, 15% glycerol, 3% 2-mercaptoethanol, pH 6.8) and boiled at 95°C for 5 min.

Electrophoresis was performed on 11% acrylamide gels. Proteins were transferred electrically from the gel onto a polyvinylidene difluoride membrane (Millipore Corporation, Billerica, MA, USA) by a semidyblotting method. The blots were blocked for 1 h with 5% skim milk in tris-buffered saline supplemented with 0.1% Tween 20 and they were incubated with anti-phospho-ERK (Thr202/Tyr204) antibody, anti-ERK2 antibody, or anti-PKC-δ antibody (1:1000 dilution) overnight at 4°C. After several washes, the blots were incubated at 25°C for 1 h with a 1:4000 dilution of secondary antibody (horseradish peroxidase-conjugated anti-rabbit IgG antibody or anti-mouse IgG antibody). Blots were developed using enhanced chemiluminescence on Hyperfilm (Amersham Biosciences, Buckinghamshire UK) (21).

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

$[Ca^{2+}]_i$ was measured by monitoring the intensity of Fura 2 fluorescence. HaCaT cells were treated with trypsin, and the suspended cells were loaded with 1 µM 1-[6-amino-2-[(5-carboxy-2-oxazolyl)-5-benzofuranyloxy]-2-(2-amino-5-methylphenoxy)ethane-N,N,N',N'-tetraacetic acid pentaacetoxymethylester (Fura 2-AM) for 15 min at 37°C. Then the cells were washed twice and finally suspended in modified Tyrode solution (137 mM NaCl, 2.7 mM KCl, 1 mM MgCl$_2$, 0.18 mM CaCl$_2$, 5.6 mM glucose, and 10 mM HEPES, pH 7.4). $[Ca^{2+}]_i$ was measured in 1.5 ml of the cell suspension in quartz cells with constant stirring at 37°C, using a fluorescence spectrophotometer (F-2000; Hitachi, Tokyo). Fura 2 fluorescence at 510 nm was monitored every 1 s with excitation at 340 and 380 nm. $[Ca^{2+}]_i$ was calculated by using the K$_d$ value of Fura 2 for Ca$^{2+}$ (224 nM) (19).

**Materials**

DMEM was purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo). Fetal bovine serum, ATP, UTP, and phorbol 12-miristate 13-acetate (PMA) were purchased from Sigma-Aldrich Co. 3-[1-[3-(Dimethylamino)propyl]-1H-indol-3-yl]-4-[(1H-indol-3-yl)-1H-pyrrrole-2,5-dione (GF109203X) was purchased from Wako Pure Chemicals Co., Ltd. (Osaka). Fura 2-AM and O,O'-bis(2-aminophenyl)ethyleneglycol-N,N,N',N'-tetraacetic acid, tetraacetoxymethyl ester (BAPTA-AM) were obtained from Dojindo Laboratories (Kumamoto). (2-Aminoethoxy)diphenylborane (2-APB) was obtained from Alfa Aesar (Lancashire, England). SYBR Premix Ex Taq was purchased from Takara Bio Inc. (Shiga, Japan). Anti-phospho-ERK (Thr202/Tyr204) antibody and anti-ERK2 antibody were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-PKC-δ antibody was obtained from Sigma-Aldrich Co. All other chemicals used were of reagent grade or the highest quality available.

**Data analysis**

Data are expressed as the mean ± S.E.M. Significant differences were determined by Tukey’s-Kramer test.

**Results**

Involvement of ERK in IL-6 production induced by UTP

UTP stimulated IL-6 synthesis and secretion from human HaCaT keratinocytes in a concentration-dependent manner with an EC$_{50}$ value of about 1 µM (Fig. 1A). UTP-induced IL-6 production was inhibited by PD98059, a MAPK kinase (MEK) inhibitor (Fig. 1B), suggesting that the ERK signaling pathway is
involved in UTP-induced IL-6 production. Next, we
analyzed whether UTP caused the phosphorylation of
ERK (Fig. 2). UTP increased ERK phosphorylation in a
time-dependent manner with a peak at 10 min (Fig. 2A).
UTP-induced ERK phosphorylation was concentration-
dependent with an EC$_{50}$ of about 0.5 µM (Fig. 2B).
Furthermore, PD98059 suppressed the phosphorylation
of ERK induced by UTP (Fig. 2C). These results
indicate that the ERK pathway is involved in UTP-
induced IL-6 production.

Involvement of [Ca$^{2+}$]i elevation in ERK phosphory-
lation and IL-6 production induced by UTP

Next, we tried to analyze the relationship between
intracellular Ca$^{2+}$ mobilization and ERK phosphoryla-
tion induced by UTP. The pretreatment of cells with
BAPTA-AM for 30 min resulted in a concentration-
dependent inhibition of UTP-induced ERK phosphoryla-
tion (Fig. 3A). UTP-induced IL-6 mRNA expression
was potently inhibited by BAPTA-AM treatment
(Fig. 3B). These results suggest that an increase in
[Ca$^{2+}$]i would be involved in ERK phosphorylation and
IL-6 mRNA expression by UTP. A23187, a Ca$^{2+}$ iono-
phore, increased IL-6 mRNA expression in a time-
dependent manner with a peak of 1 h (Fig. 4A) and in a
concentration-dependent manner with an EC$_{50}$ of about
0.5 µM (Fig. 4B). A23187 also increased ERK phos-
phorylation potently (Fig. 4C). Furthermore, 2-APB, an
IP$_3$-receptor blocker, suppressed UTP-induced ERK
phosphorylation and IL-6 mRNA expression (Fig. 5: A
and B). In addition, UTP caused [Ca$^{2+}$]i elevation after a
removal of extracellular Ca$^{2+}$ with EGTA (Fig. 5C).
These results suggest that IP$_3$-sensitive [Ca$^{2+}$]i elevation
is involved in ERK phosphorylation and IL-6 mRNA
expression induced by UTP.

Effect of PKC downregulation on ERK phosphorylation
and IL-6 expression by UTP

It has been shown that PKC is involved in ERK phos-
phorylation and IL-6 production in NIH-3T3 cells (22).
Therefore, we examined the involvement of PKC in
UTP-induced ERK phosphorylation and IL-6 mRNA
expression in HaCaT cells using a method of PKC
downregulation. The treatment of the cells with 100 nM
PMA for 24 h resulted in downregulation of PKC-δ
(Fig. 6C). In these cells, UTP still caused ERK phos-
phorylation and IL-6 mRNA expression (Fig. 6: A and B). In addition, GF109203X, a PKC inhibitor, had no effect on UTP-induced ERK phosphorylation and IL-6 mRNA expression (data not shown). These results suggest that PKC may not be involved in ERK phosphorylation and IL-6 expression induced by UTP in HaCaT cells.

**Effect of pertussis toxin (PTX) on UTP-induced ERK phosphorylation and IL-6 mRNA expression in HaCaT cells**

Since P2Y<sub>10</sub> and P2Y<sub>4</sub> receptors are coupled to G<sub>i</sub> in addition to G<sub>q/11</sub> (23 – 25), we examined the effect of PTX on ERK phosphorylation and IL-6 mRNA expression in HaCaT cells. UTP increased ERK phosphorylation in PTX-treated HaCaT cells to an extent similar to that in PTX-untreated cells (Fig. 7A). UTP-induced IL-6 mRNA expression was not reduced by PTX (Fig. 7B). These results suggest that G<sub>i</sub> is not involved in ERK phosphorylation and IL-6 mRNA expression induced by UTP in HaCaT cells.

**Transactivation of epidermal growth factor (EGF) receptor by UTP in HaCaT cells**

It has been shown that ERK phosphorylation can be induced by transactivation of growth factor receptors in several G protein-coupled receptors (26). Therefore, we examined the effect of AG1478, an inhibitor of EGF...
receptor, on UTP-induced ERK phosphorylation and IL-6 mRNA expression (Fig. 8). ERK phosphorylation and IL-6 expression by UTP were partially suppressed by AG1478 (Fig. 8: A and B), showing that UTP-induced ERK phosphorylation is mediated in part by transactivation of the EGF receptor in HaCaT cells.

Discussion

In the present study, we showed that stimulation of P2Y2/P2Y4 receptor increased IL-6 production mediated by IP3-sensitive [Ca2+]i elevation and ERK phosphorylation.

It has been shown that cytokine production is regulated by the MAPK superfamily (27). For instance, the MEK/ERK pathway is involved in histamine-induced IL-8 and IL-6 production in human keratinocytes (14). PD98059 at the concentration of 30 µM suppressed UTP-induced IL-6 production and ERK phosphorylation in HaCaT cells. These results suggest that MEK/ERK signaling is important for IL-6 production induced by UTP in HaCaT cells, but some other pathway would exist in IL-6 production by UTP in HaCaT cells.

It is generally believed that intracellular Ca2+ plays an important role in UTP-induced IL-6 production (28). We demonstrated that BAPTA-AM, an intracellular Ca2+ chelator, suppressed UTP-induced ERK phosphorylation and IL-6 mRNA expression (Fig. 3) and that A23187, a Ca2+ ionophore, induced IL-6 mRNA expression in a similar manner to UTP (Fig. 4). Furthermore, 2-APB, an IP3-receptor blocker, suppressed UTP-induced ERK phosphorylation and IL-6 mRNA expression.
Fig. 8. Effect of AG1478 on UTP-induced ERK phosphorylation and IL-6 mRNA expression. A: Effect of AG1478 on UTP-induced ERK phosphorylation. HaCaT cells were preincubated with AG1478 (5 µM) for 30 min and incubated with UTP (10 µM) for 20 min. ERK phosphorylation was analyzed by Western blotting as described in Materials and Methods. B: Effect of AG1478 on UTP-induced IL-6 mRNA expression. HaCaT cells were preincubated with AG1478 (5 µM) for 30 min and incubated with UTP (10 µM) for 1 h. IL-6 mRNA expressions were analyzed by RT-PCR as described in Materials and Methods. Data are expressed as the mean ± S.E.M. from three determinations. Significant differences from the values without drugs are shown as *P<0.05.

Although PD98059 partially suppressed UTP-induced IL-6 production, BAPTA-AM completely inhibited UTP-induced IL-6 mRNA expression. These results suggest that ERK signaling is one of the major pathways in UTP-induced IL-6 production, but some other pathways, possibly involving [Ca²⁺], elevation, are involved in IL-6 production by UTP.

It is known that ERK is regulated by various signaling pathways (32). There are many reports that PKC is involved in ERK phosphorylation (33 – 35). It is known that calmodulin is involved in opioid-receptors-mediated ERK phosphorylation in astrocytes (36). However, another report suggested that MMP9, matrix metalloprotease-9 (37), is important for PMA-induced ERK phosphorylation in astrogliaoma cells (38). In the present study, however, UTP-induced ERK phosphorylation was not suppressed in PKC-downregulated HaCaT cells (Fig. 6A). Therefore, it is assumed that PKC is not involved in UTP-induced ERK phosphorylation in this cell line. It is known that PKC is involved in cytokine production in various kinds of cells (39). Although it has been shown that histamine-induced IL-6 and IL-8 productions are suppressed by a specific PKC inhibitor, Ro-31-8220 (14), in human keratinocytes, our present study demonstrated that PKC downregulation did not affect UTP-induced IL-6 mRNA expression (Fig. 6B). It remains to be solved why the different results were obtained about PKC involvement in IL-6 production.

It is known that P2Y₂ and P2Y₄ receptors, which are sensitive to UTP, are coupled with G₉/₁₁ and G₁ proteins. Although it is possible that Gᵢ is involved in UTP-induced ERK phosphorylation, the phosphorylation was not affected by PTX treatment (Fig. 7A), suggesting that UTP-induced ERK phosphorylation is independent of Gᵢ signaling in HaCaT keratinocytes.

We have shown that [Ca²⁺] elevation resulted in ERK phosphorylation in HaCaT cells. AG1478, an inhibitor of EGF-receptor phosphorylation, suppressed UTP-induced ERK phosphorylation and IL-6 mRNA expression (Fig. 8). It is known that EGF and PDGF receptors are transactivated by stimulation of P2Y receptor in Muller glial cells (40). It is thought that the G₉/₁₁/PLC pathway mediates an activation of matrix metalloprotease, resulting in release of EGF-receptor ligand for activation of the EGF receptor (41). In addition, Src and Pyk2, non-receptor tyrosine kinases, may mediate EGF-receptor transactivation induced by P2Y-receptor stimulation (42). Since the phosphorylation of Pyk2 is enhanced by [Ca²⁺] elevation (43), one of the mechanisms may be that UTP-induced, G₉/₁₁/IP₃-mediated [Ca²⁺] elevation results in EGF-receptor transactivation and ERK phosphorylation through the phosphorylation...
of Pyk2 in HaCaT cells.

In conclusion, the stimulation of P2Y<sub>2</sub>/P2Y<sub>4</sub> receptor by UTP increased IL-6 production, mediated through IP<sub>3</sub>-sensitive [Ca<sup>2+</sup>]<sub>e</sub> elevation and ERK phosphorylation in HaCaT cells. The transactivation of EGF receptor was partially involved in UTP-induced ERK phosphorylation. Further study is necessary to clarify the detailed molecular mechanism of IL-6 production via P2Y<sub>2</sub>/P2Y<sub>4</sub> receptors.

References


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