THE FEMALE SEX HORMONE ESTROGEN (Estradiol, E2) is a crucial hormone which regulates many physiological processes in mammals including reproduction, mammary gland development, bone integrity, and metabolism [1]. In addition, it plays an important role in neuro- and cardiovascular protection through anti-inflammatory effects [2-5]. These effects are mediated by two classical nuclear estrogen receptors (ERs), ERα and ERβ, which are ligand-activated transcriptional factors that bind directly to the promoter regions of genes. Not only does E2 carry out genomic actions through transcriptional activation by classical nuclear ERs, it can also activate rapid signaling pathways within several minutes after stimulation [6]. These rapid pathways are initiated at the plasma membrane and induce calcium influx, NO production, and also nuclear transcriptional events independent of nuclear ERs [7]. Recent studies have shown that G protein-coupled estrogen receptor1 (GPER), also called GPR30 (rat GPR41), exerts physiological effects on E2 in rapid signaling events [8, 9]. GPER is a seven-transmembrane spanning G protein-coupled receptor (GPR) and has been shown to bind E2 on plasma membrane [10]. We have shown that GPER induced by oxidative stress leads to apoptotic cell death through p53 activation [11]. On the other hand, GPER enhances cell proliferation in breast cancer cells [12, 13]. These observations indicate that GPER has different physiological effects depending on distinct cell types. Several reports have revealed that among the physiological effects of GPER is its ability to mediate anti-inflammatory effects. In inflammatory model animals, GPER negatively regulates TNFα-induced IL-6 production in human breast cancer cells via NF-κB pathway

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Abstract. Estrogen is known to have anti-inflammatory effects, that are thought to be mediated by the classical estrogen receptors (ERs), ERα and ERβ. G protein coupled estrogen receptor1 (GPER) is a novel membrane-type estrogen receptor that can mediate non-genomic estrogenic responses. Although there have been several reports asserting that the participation of GPER in anti-inflammatory effects is induced by estrogen, the role of GPER remains poorly understood. In this study, we investigated the involvement of GPER in the regulation of a representative inflammatory cytokine, IL-6. We first examined the expression of IL-6 mRNA by TNFα stimulation in the transfection of GPER-expression plasmid into HeLa cells. Exogenous GPER significantly inhibited TNFα-induced IL-6 expression, and blocked NF-κB promoter activity inducing the expression of IL-6 in a dose-dependent manner. The promoter activity was restored almost to control level by transfection with the C-terminal deletion mutant of GPER. Similar results have been observed in endogenous GPER using SKBR3 cells which do not express the classical ERs. The data have been validated by treatment of GPER with siRNA. These findings indicate that GPER negatively regulates TNFα-induced IL-6 expression, probably through inhibition of NF-κB promoter activity by a signal(s) derived from the C-terminal region of GPER.

Key words: GPER, IL-6, NF-κB, E2, G-1
activated GPER reduced the pathophysiological conditions induced by inflammatory processes, including the production of cytokines observed in multiple sclerosis, stroke, and ischemia-reperfusion injury [14-18]. However, the molecular mechanisms of anti-inflammatory effects via GPER signaling remain poorly understood. In the present study, we examined the effects of GPER on TNFα-induced expression of interleukin 6 (IL-6), one of the representative inflammatory cytokines. We showed that GPER inhibits TNFα-induced IL-6 expression through down-regulation of nuclear factor-kappa B (NF-κB) promoter activity.

Materials and Methods

Reagents
E2, p3xFLAG-CMV10, siRNA duplexes targeting human GPER and control siRNA were purchased from Sigma-Aldrich (St. Louis, MO, USA). GPER agonist G-1 [19] was obtained from Cayman Chemical (Ann Arbor, MI, USA). TNFα was from R&D Systems (Minneapolis, MN, USA). The pGL4.32 which contains NF-κB response element that drives firefly luciferase reporter gene, and transfection control plasmid pGL4.74 were purchased from Promega (Madison, WI, USA).

Cell culture
HeLa human cervical cancer cells were obtained from the American Tissue Culture Collection (ATCC) and cultured in DMEM medium (Wako, Osaka, Japan) containing 5% heat-inactivated fetal bovine serum (FBS), and 100U/mL penicillin/streptomycin. SKBR3 human breast carcinoma cells were from ATCC and maintained in DMEM/Ham’s F-12 medium (Wako) with 10% heat-inactivated FBS, and 100U/mL penicillin/streptomycin. All cell lines were maintained in 5% CO2 at 37°C. In all experiments, the medium and FBS were replaced by phenol red free medium and charcoal-stripped FBS. In the experiments with SKBR3 cells in which the phenol red free medium and charcoal-stripped FBS were used, the ligand stimulation of endogenous GPER was performed by treating cells with G-1/ethanol (EtOH) or E2/DMSO.

Plasmid construction
The GPER expression plasmid, pFLAG-GPER, was constructed by inserting full-length cDNA of human GPER into p3xFLAG-CMV10 to express GPER protein tagged FLAG peptides at N-terminal as described previously [10]. The GPER (1-153) fragment was generated by PCR from pFLAG-GPER as a template using forward primer, CCCAGCTTATGGATGTGACTTCCCAAGCC, and reverse primer, AAA GGATCCCTAGAAGCTCATCCAGGTGAGGA. The amplified product was digested with Hind III and cloned into p3xFLAG-CMV10 to obtain pFLAG-GPER (1-153). The GFP expression plasmid, pFLAG-GFP was generated as control expression plasmid. Nucleotide sequences of all PCR-amplified products were confirmed by Big Dye Terminator v3.1 Cycle Sequencing Kit® with primers and 3130x Genetic Analyzer (Life Technologies, Foster City, CA, USA).

Transfection and reporter assay
1.5 × 10^4 (HeLa) or 4 × 10^4 (SKBR3) cells were plated into 96-well plates the day before transfection. Culture medium was replaced with phenol red free medium containing charcoal-stripped FBS on the day of transfection. DNA transfection into HeLa cells was performed using PolyFect® (Qiagen, Hilden, Germany) with a DNA mixture containing 250 ng of expression plasmid (pFLAG-GPER, pFLAG-GPER (1-153), or pFLAG-GFP), 125 ng of pGL4.32 and 5 ng of pGL4.74. After 28 h, cells were washed with phenol red free medium and then stimulated with 10 ng/mL TNFα for 3 h. On the other hand, DNA and siRNA transfection into SKBR3 cells was performed using X-tremeGENE siRNA Transfection Reagent® (Roche Diagnostics, Basel, Switzerland) with a mixture containing 250 ng of pGL4.32 and 5 ng of pGL4.74. After 28 h, cells were washed with phenol red free medium and then stimulated with 10 ng/mL TNFα for 3 h. On the other hand, DNA and siRNA transfection into SKBR3 cells was performed using X-tremeGENE siRNA Transfection Reagent® (Roche Diagnostics, Basel, Switzerland) with a mixture containing 100 ng of pGL4.32, 2 ng of transfection control pCMV-hRluc, and 100 ng siRNA. Cells were incubated overnight, and then washed and stimulated with 40 ng/mL TNFα for 16 h in the presence of 10 µM E2/DMSO or 10 µM G-1/EtOH. Luciferase activity was then measured with the Dual Luciferase Reporter Assay System® (Promega) according to the manufacturer’s instruction. Firefly luciferase activity was normalized to the transfection control provided by the Renilla luciferase activity.

Western blot analysis
Western blot analysis was carried out as described previously [20-22]. Cells were extracted in lysis buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.5 mM dithiothreitol) containing Protease inhibitor Cocktail® (Nakarai, Osaka, Japan). Cell lysates were resolved on SDS-PAGE and...
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Statistical analysis
The results were calculated as the mean ± standard deviation. Statistical analysis was done using Student’s t test for paired observations. Results were considered as statistically significant if the P value was < 0.05.

Results
Exogenous expression of GPER reduced TNFα-induced IL-6 expression in HeLa cells
To elucidate the role of GPER in inflammatory cytokine secretion, expression levels of IL-6 mRNA were examined in the presence of TNFα by introducing the GPER expression plasmid into HeLa cells expressing no or very low levels of GPER protein [24]. The expression of exogenous FLAG-tagged GPER or GFP protein in the HeLa cells was confirmed by Western blotting (Fig. 1A). Under this experimental condition, the expression of IL-6 mRNA was observed by TNFα treatment in control GFP-expressed cells, while IL-6 expression was significantly decreased by exogenous expression of GPER in comparison with that of GPER-unexpressed cells (Fig. 1B). These findings indicate that GPER may function as a negative regulator in IL-6 expression.

Fig. 1 GPER negatively regulates TNFα-induced IL-6 expression.
(A) HeLa cells were transfected with FLAG-GPER or control FLAG-GFP expression plasmid. After 28 h, cells were harvested and exogenous protein expression was verified by Western blotting using anti-FLAG antibody.
(B) HeLa cells were transfected with each plasmid as mentioned in (A), and 28 h later, they were either unstimulated or stimulated with TNFα (10 ng/mL) for 3 h. IL-6 mRNA expression levels were quantified by RT-qPCR analysis. Data are expressed as means ± SD. * P<0.05.
Exogenous GPER down-regulates NF-κB promoter activation induced by TNFα stimulation

Since it is well-known that IL-6 mRNA expression upon TNFα stimulation is largely mediated by the NF-κB pathway, we next examined the effect of exogenous GPER on the transcriptional activation of transcriptional factor NF-κB by luciferase reporter assays with NF-κB reporter plasmid. As shown in Fig. 2A, TNFα stimulation resulted in a NF-κB promoter activity more than 100-fold higher than that in unstimulated control GFP-expressed HeLa cells. Consistent with the inhibitory effect on IL-6 expression (Fig. 1B), exogenous GPER significantly inhibited the promoter activation (Fig. 2A), in a dose-dependent manner (Fig. 2B).

Fig. 2 GPER negatively regulates TNFα-induced transcriptional activity of NF-κB.
(A) HeLa cells were co-transfected with pGL4.32 (NF-κB-luciferase reporter), pGL4.74 (transfection control), and pFLAG-GPER or pFLAG-GFP and 28 h later, they were either unstimulated or stimulated with TNFα (10 ng/mL) for 3h. The luciferase activities were then measured with the Dual Luciferase Reporter Assay System. Data are expressed as means ± SD. ** P<0.01.
(B) HeLa cells were transfected with each plasmid as mentioned in (A), except for the amount of GPER expression plasmid. We used total 250 ng of expression plasmid that consisted of various proportions of pFLAG-GPER and pFLAG-GFP. TNFα stimulation and measurement of reporter activity were performed as mentioned in (A). Data are expressed as means ± SD. ** P<0.01.
(C) HeLa cells were transfected with pGL4.32, pGL4.74, and pFLAG-GPER, pFLAG-GFP, or pFLAG-GPER (1-153). TNFα stimulation and measurement of reporter activity were performed as mentioned in (A). Data are expressed as means ± SD. ** P<0.01.
We validated by dot plot analysis that the expression level of exogenous GPER was dose-dependent (Supplementary Fig. 1A). Furthermore, we observed that the NF-κB promoter activity also was inhibited by exogenous expression of GPER in another dose response experiment that was varied in the amount of GPER expression plasmid used (Supplementary Fig. 1C). To confirm whether trimeric G protein is involved in the regulation of NF-κB promoter activity, the promoter activity was examined using the plasmid pFLAG-GPER (1-153), which expresses N-terminal 153 residues in GPER and lacks a binding region to trimeric G proteins that mediate non-genomic response [25]. The deletion mutant of GPER restored NF-κB promoter activity, which was blocked by the transfection of full-length GPER up to the level of the control GFP plasmid (Fig. 2C). Dot plot and Western blot analysis showed the expression levels of wild and the deletion mutant GPER are comparable (Supplementary Fig. 1A, B). These results suggest that exogenous GPER inhibits NF-κB promoter activation induced by TNFα stimulation, probably through a signal(s) derived from the C-terminal region of GPER.

**Endogenous GPER in SKBR3 cells reduced TNFα-induced IL-6 expression**

Based on the above results, we used a human breast cancer cell line, SKBR3, that expresses GPER but lacks ERα and ERβ, to examine whether endogenous GPER could inhibit inflammatory cytokine expression [26]. The expression levels of IL-6 mRNA were up-regulated in SKBR3 cells after TNFα stimulation (Fig. 3A). To examine the effect of GPER on TNFα-induced IL-6 expression, cells were treated with E2 in addition to TNFα stimulation. Treatment with E2 significantly inhibited TNFα-induced IL-6 expression in SKBR3 cells (Fig. 3A). To confirm the inhibitory effect, the regulation of IL-6 expression by GPER was examined using G-1, a specific agonist of GPER. G-1 significantly inhibited the expression of IL-6 mRNA in SKBR cells, and the obtained data were very similar to that observed in E2 treatment (Fig. 3A). To show that GPER actually plays a role in the inhibitory effects, we transfected a specific siRNA for GPER into SKBR3 cells. Treatment with GPER siRNA led to the reduction of GPER expression by approximately 50% (Fig. 3B). In control siRNA, NF-κB promoter activity was increased by TNFα stimulation, and the activity was decreased by treatment with G-1, although the decrease was not statistically significant. The inhibitory effect of G-1 ceased to be observed in GPER siRNA transfected cells (Fig. 3B). These data revealed that endogenous GPER negatively regulates TNFα-induced IL-6 mRNA expression through inhibition of NF-κB promoter activation.

**Discussion**

In this study, we examined the participation of GPER in the regulation of IL-6 mRNA expression induced by TNFα. GPER negatively regulated TNFα-induced IL-6 expression through inhibition of NF-κB transcriptional activity. The inhibitory effect of GPER might be mediated by an intracellular signal that occurs from the C-terminal region of GPER, since IL-6 expression was restored by transfection with the C-terminal deletion mutant of GPER.

The exogenous GPER constitutively reduced NF-κB promoter activity, independently of E2 ligands (data not shown). The overexpressed GPER may be activated by dimer formation similar to other GPR [27]. The results obtained from exogenous expression of GPER were confirmed using SKBR3 cells that express endogenous GPER, and the expression of IL-6 mRNA induced by TNFα was significantly reduced in SKBR3 cells in response to E2 or G-1, as shown in Fig. 3A. These results were consistent with a previous report by Blasko et al. indicating that LPS-induced IL-6 or TNFα production in macrophage cells is inhibited by 10 µM of G-1 [15]. They also showed that GPER is capable of mediating second messenger production in the promyelocytic immune cells in response to 10 µM of E2 or G-1 [15]. We observed that the treatment of SKBR3 with 1 µM of G-1 showed only a slight decrease in TNF-α induced NF-κB promoter activity (data not shown). We could not observe any suppressive effects of GPER on IL-6 expression and promoter activity in the NF-κB reporter by addition of low concentration (e.g. 1 nM) of E2 (data not shown). GPER siRNA transfection assay showed that endogenous GPER in SKBR3 cells down-regulated TNFα-induced IL-6 mRNA expression via inhibition of NF-κB promoter activation (Fig. 3B). Previous studies indicated that TNFα and L-6 induce mRNA expression of aromatase that catalyzes estrogen biosynthesis through AP-1 and GAS sites on the promoter region of aromatase gene [28, 29]. Irahara et al. reported that mRNA levels of aromatase are significantly associ-
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Endogenous GPER in SKBR3 cells negatively regulates TNFα-induced IL-6 expression through down-regulation of NF-κB transcriptional activity.  

(A) SKBR3 cells were unstimulated, or stimulated with TNFα (40 ng/mL) for 16 h with 10 µM E2/DMSO or 10 µM G-1/EtOH. IL-6 mRNA expression levels were quantified by RT-qPCR analysis. Data are expressed as means ± SD. * P<0.05, ** P<0.01.  

(B) SKBR3 cells were transfected with GPER or control siRNA, and GPER mRNA expression levels were quantified by RT-qPCR analysis (top). SKBR3 cells were transfected with pGL4.32, pCMV-hRluc (transfection control), and GPER or control siRNA. After 28 h, cells were either unstimulated or stimulated with TNFα (40 ng/mL) for 16 h with 10 µM G-1/EtOH. The luciferase activities were then measured with the Dual Luciferase Reporter Assay System (bottom). Data are expressed as means ± SD. * P<0.05, ** P<0.01.

- ated with TNFα and L-6 mRNA levels in breast cancer tissues, mammary and axillary adipose tissues [30]. From these reports, it is suggested that TNFα itself or TNFα-induced IL-6 promotes aromatase expression in SKBR3 cells. Furthermore, it is conjectured that the excess E2 synthesized by catalytic activity of aromatase might bind to GPER. Thus, GPER signals might act as an autonomous E2 synthesis regulator through inhibiting IL-6 expression, in addition to exerting anti-inflammatory effects.

The C-terminal region in GPER contains domains that can activate intracellular signal pathways such as trimeric G protein and PDZ motif. It was previously reported that the exogenous expression of GPER ele-
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Activated cAMP production, which required a motif for coupling to trimeric G protein [25, 31]. The production of cAMP induces cAMP-dependent kinase activation that can phosphorylate cAMP responsive element binding protein (CREB), with CREB phosphorylation leading to the elevation of the protein levels of intracellular IκB, an inhibitory unit of NF-κB [32]. The signal that occurred from the C-terminal region of GPER may negatively regulate NF-κB through cAMP production. On the other hand, it was also reported that exogenous GPER inhibited c-AMP production in HEK293 cells through PDZ motif in the C-terminal region [33]. Our preliminary results from assays using a macrophage cell line showed that the phosphorylation of some kinases involved in NF-κB pathway was decreased by treatment with G-1, although we could not identify the target molecule(s) of GPER in the NF-κB pathway (data not shown). Further studies are needed regarding the molecular mechanism of NF-κB pathway regulation by a GPER signal that exerts the anti-inflammatory effects of estrogen.

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Disclosure

None of the authors have any competing interests to disclose.

Supplementary Fig. 1

Exogenous GPER negatively regulates TNFα-induced NF-κB promoter activity

(A) Dot plot analysis of exogenous GPER in cells that were transfected with various amounts of FLAG-GPER expression plasmid shown in Fig. 2B. The expression levels of FLAG-GPER and FLAG-GPER(1-153) were also verified by dot plot analysis using anti-FLAG antibody.

(B) Exogenous protein expression in cells that were transfected with pFLAG-GPER or pFLAG-GPER(1-153) was verified by Western blotting using anti-FLAG antibody.

(C) HeLa cells were transfected with each plasmid as mentioned in Fig. 2A. We used a total of 250 ng of expression plasmid that consisted of various proportions of pFLAG-GPER and pFLAG-GFP. TNFα stimulation and measurement of reporter activity were performed as mentioned in Fig. 2A. Data are expressed as means ± SD. ** P<0.01.
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