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Hydrogen peroxide stimulates cell motile activity through LPA receptor-3 in liver epithelial WB-F344 cells

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ABSTRACT

Hydrogen peroxide which is one of reactive oxygen species (ROS) mediates a variety of biological responses, including cell proliferation and migration. In the present study, we investigated whether lysophosphatidic acid (LPA) signaling is involved in cell motile activity stimulated by hydrogen peroxide. The rat liver epithelial WB-F344 cells were treated with hydrogen peroxide at 0.1 or 1 μM for 48 h. In cell motility assays, hydrogen peroxide treated cells showed significantly high cell motile activity, compared with untreated cells. To measure the expression levels of LPA receptor genes, quantitative real time RT-PCR analysis was performed. The expressions of LPA receptor-3 (*Lpar3*) in hydrogen peroxide treated cells were significantly higher than those in control cells, but not *Lpar1* and *Lpar2* genes. Next, to assess the effect of LPA_3 on cell motile activity, the *Lpar3* knockdown cells from WB-F344 cells were also treated with hydrogen peroxide. The cell motile activity of the knockdown cells was not stimulated by hydrogen peroxide. Moreover, in liver cancer cells, hydrogen peroxide significantly activated cell motility of *Lpar3*-expressing cells, but not *Lpar3*-unexpressing cells. These results suggest that LPA signaling via LPA_3 may be mainly involved in cell motile activity of WB-F344 cells stimulated by hydrogen peroxide.

1. Introduction

It is well known that oxidative stress plays an important role in the initiation and progression of many diseases [1,2]. Reactive oxygen species (ROS) are the most important mediators of oxidative stress and produced in aerobic organisms during normal metabolism [1–3]. Hydrogen peroxide is one of the most important ROS and induces oxygen radicals that cause oxidative stress to cells [2]. Hydrogen peroxide can activate the intracellular signaling pathways and regulates a variety of biological functions, such as cell proliferation, migration, differentiation and apoptosis [1–3]. In cancer cells, hydrogen peroxide stimulated malignant properties, including tumor growth and metastatic potency [4,5].

Lysophosphatidic acid (LPA) is an extracellular signaling lipid. It interacts with G protein-coupled LPA receptors (LPA receptor-1 (LPA_1) to LPA_6) and mediates several biological responses [6–8]. In tumor cells, LPA receptors also contribute to the gain of cell proliferation, motility, invasion, angiogenesis and tumorigenicity [8–10]. However, each LPA receptor indicates the different cellular effects, depending on the types of cells. In fact, LPA_2 and LPA_3 increased cell migration and invasion in rat neuroblastoma cells, but not LPA_1. Especially, LPA_3 markedly stimulated tumorigenicity as well as migration ability [9]. In rat liver tumor cells, LPA_3 enhanced cell migration, invasion, tumorigenicity and drug resistance [11]. Moreover, LPA_1 inhibited and LPA_3 enhanced cell motility and invasion of hamster pancreatic cancer cells [12]. By contrast, LPA_3 suppressed cell migration of rodent lung cancer cells and inhibited angiogenesis in mouse mammary tumor cells [13,14].

In our recent studies, we showed that 12-O-tetradecanoylphorbol-13-acetate (TPA) which is a tumor promoting agent stimulated cell migration activity of rat liver epithelial WB-F344 cells, correlating with the elevated expression level of *Lpar3* [15]. Moreover, estrogens regulated cell motile activity of WB-F344 cells through the different induction of LPA receptors [16]. The cell motile activities stimulated by TPA or estrogens were inhibited by LPA receptor knockdown cells [15,16]. In the present study, to investigate an involvement of LPA signaling on cell motile activity mediated by hydrogen peroxide, WB-F344 cells were treated with hydrogen peroxide. We demonstrate that hydrogen peroxide stimulates cell motile activity of WB-F344 cells through the induction of LPA_3.

Abbreviations: LPA, lysophosphatidic acid; LPA_3, LPA receptor-3; ROS, reactive oxygen species; RT, reverse transcription; PCR, polymerase chain reaction.

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2. Materials and methods

2.1. Cell culture

WB-F344 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Nacalai Tesque Inc., Kyoto, Japan) containing 10% fetal bovine serum (FBS) in 5% CO_2 atmosphere at 37 °C. To generate the *Lpar3* knockdown cells (WB-shRNA3–2 cells) from WB-F344 cells, we used HuSHTM short hairpin RNA (shRNA) plasmid panel (29-mer) for the *Lpar3* (Origene, Rockville, MD) with GeneJuice Transfection Reagent (Merck KGaA, Darmstadt, Germany) according to the manufacturer's instructions. After transfection, cells were selected with puromycin for 2 weeks to obtain a stable clone [15,16].

2.2. Toxic effect of hydrogen peroxide on cell proliferation of WB-F344 cells

Cells were plated at 5000 cells/well in a 96-well plate and cultured with 100 μl of DMEM containing 10% FBS. Cells were treated with hydrogen peroxide (Nacalai Tesque Inc.) at a concentration of 0.1, 1, 10 and 100 μM every 24 h for 3 days. To measure the cell proliferation rate, a Cell Counting Kit-8 (CCK-8) (Dojin Chemistry, Kumamoto, Japan) was used. The assay was always done in triplicate [9,11,12].

2.3. Effect of hydrogen peroxide on cell motile activity of WB-F344 cells

Based on cell proliferation assay, we investigated the effect of hydrogen peroxide on cell motile activity of WB-F344 cells, using a Cell Culture Insert (BD Falcon, NJ, USA) with 8 μm pore size. Cells were pretreated with hydrogen peroxide at 0.1 and 1 μM concentrations for 48 h which were added every 24 h, and were then seeded into the filter at 1×10^5 cells in 200 μl serum-free DMEM (upper chamber) and placed in 24-well plates (lower chamber) containing 800 μl of DMEM containing 10% FBS. Cells were further incubated for 24 h in 5% CO_2 atmosphere at 37 °C. Cells remaining in the upper side of the filter were removed with cotton swabs. After Giemsa staining, the number of cells moved to the lower side of the filter was counted [9–16]. To assess the effect of LPA treatment on cell motility, cells pretreated with hydrogen peroxide were also seeded into the filter at 1×10^5 cells in 200 μl serum-free DMEM and placed in 24-well plates containing 800 μl of 5% charcoal stripped FBS (Sigma Biochemicals, St. Louis, MO, USA) – DMEM with or without LPA (10 μM) (Avanti Polar Lipids, Inc., AL, USA) [9,11].

2.4. Scrape assay

Cells were plated onto a 6-well plate and cultured until confluence in DMEM containing 10% FBS. A 1000 μl tip was used to make a scrape line. The wells were washed twice with PBS and then incubated in 10% FBS DMEM with or without hydrogen peroxide (0.1 and 1 μM). The photographs were taken at 0 and 14 h after scrape [13,14].

2.5. Effects of LPA signaling inhibitors on cell motile activity

After the pretreatment of hydrogen peroxide, cells were also treated with 100 ng/ml pertussis toxin (PTX) (Wako Pure Chemical Industries) for 24 h, 5 μM U-73122 (Cayman Chemical Co., Ann Arbor, MI, USA) for 30 min, 1 μM Y-27632 (Mitsubishi Pharma Co., Osaka, Japan) for 30 min or 10 μM dioctylglycerol pyrophosphate (DGPP) (Avanti Polar Lipid) for 30 min. Cells were then seeded into the filter at 1×10^5 and incubated for 16 h with LPA (10 μM) [9,10,17,18].

2.6. Quantitative real time reverse transcription (RT) - polymerase chain reaction (PCR) analysis

Using RNA extracted from each cell, the first-strand cDNA was synthesized with Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics Co. Ltd., Mannheim, Germany). To measure the expression levels of LPA receptor genes, quantitative real time RT-PCR analysis using a SYBR *Premix Ex Taq* (Tli RNaseH Plus) (TaKaRa Bio, Inc., Shiga, Japan) and a Smart Cycler II System (TaKaRa) was performed according to the manufacturer's protocol. The data for LPA receptor genes were normalized to rat *Gapdh* [15,16].

3. Results and discussion

WB-F344 cells expressed *Lpar1* and *Lpar2* genes, but the expression of *Lpar3* gene was relatively low [15,16]. Recently, we investigated cell motile activity of WB-F344 cells treated with TPA or estrogens. TPA, 17β-estradiol and ethinyl estradiol stimulated cell motile activity of WB-F344 cells, correlating with the elevated *Lpar3* gene expression. Those activities were suppressed by *Lpar3* knockdown cells [15,16]. Therefore, in this study we also used WB-F344 cells to assess whether LPA signaling is involved in cell motility mediated by hydrogen peroxide.

To determine the concentration of hydrogen peroxide used in the cell motility assay, cells were treated with hydrogen peroxide at 0.1, 1, 10 and 100 μM for 3 days, which were added every 24 h. The each cell growth rate was measured by CCK-8 (Dojin Chemistry). Hydrogen peroxide at concentrations of 10 and 100 μM significantly suppressed cell growth of WB-F344 cells, but not 0.1 and 1 μM (data not shown). Base on these results, we measured cell motile activity of WB-F344 cells treated with hydrogen peroxide at 0.1 and 1 μM for 48 h which were added every 24 h. In the cell motility assay with Cell Culture Insert, hydrogen peroxide treated cells showed significantly high cell motility, compared with untreated cells (Fig. 1A). In scrape assay, cells were incubated with or without hydrogen peroxide for 14 h after scrape. The cell motile activity of hydrogen peroxide treated cells was markedly higher than that of untreated cells (Fig. 1B).

LPA treatment significantly increased cell motile activity stimulated by hydrogen peroxide (Fig. 2A). Therefore, to assess whether LPA signaling may contribute to cell motility of WB-F344 cells stimulated by hydrogen peroxide, cells were treated with G protein inhibitors. After the pretreatment of hydrogen peroxide, cells were treated with G protein inhibitors, PTX, U-73122 or Y-27632. PTX is an inhibitor of Gi protein. U-73122 inhibits PLC which is a downstream effector of Gq and Y-27632 inactivates ROCK which is a downstream effector of G12/13 [9–11,17,18]. The cell motile activity stimulated by LPA treatment was significantly inhibited by PTX and U-73122, but not Y-27632 (Fig. 2B). LPA_1 and LPA_2 couple to Gi, Gq and G12/13, while LPA_3 links to Gi and Gq, but not G12/13 [8,19]. Moreover, cells were also pretreated with DGPP which is used for the antagonist of LPA_1/LPA_3 [20]. The cell motility of WB-F344 cells treated with LPA in the presence of DGPP was markedly suppressed than that of untreated cells (Fig. 2B).

Next, we measured the expression levels of LPA receptor genes in WB-F344 cells treated with hydrogen peroxide at 0.1 and 1 μM for 48 h, using real time RT-PCR analysis. The *Lpar3* expression in hydrogen peroxide treated cells was significantly higher than that in untreated cells, but not *Lpar1* and *Lpar2* (Fig. 3A). Since hydrogen peroxide induced the *Lpar3* expression in WB-F344 cells, *Lpar3* knockdown (WB-shRNA3-2) cells were treated with hydrogen

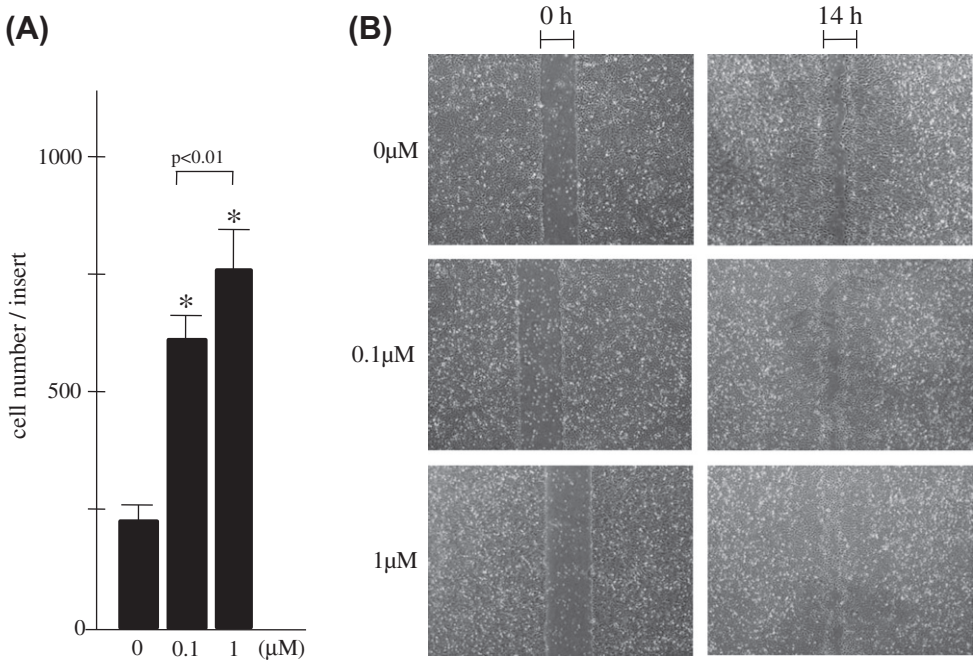

Fig. 1. (A) The cell motility assay with a Cell Culture Insert. Cells were pretreated with hydrogen peroxide at a concentration of 0.1 and 1 μM for 48 h, and were seeded in the filter at 1×10^5 cells in 200 μl serum-free DMEM (upper chamber) and placed in 24-well plates (lower chamber) containing 800 μl of DMEM containing 10% FBS. Columns indicate the mean of three studies. Bars indicate SD. * $p < 0.01$ vs. untreated (control) cells. (B) Representative results of scrape assay. After scrape, cells were incubated for 14 h with or without hydrogen peroxide at a concentration of 0.1 and 1 μM.

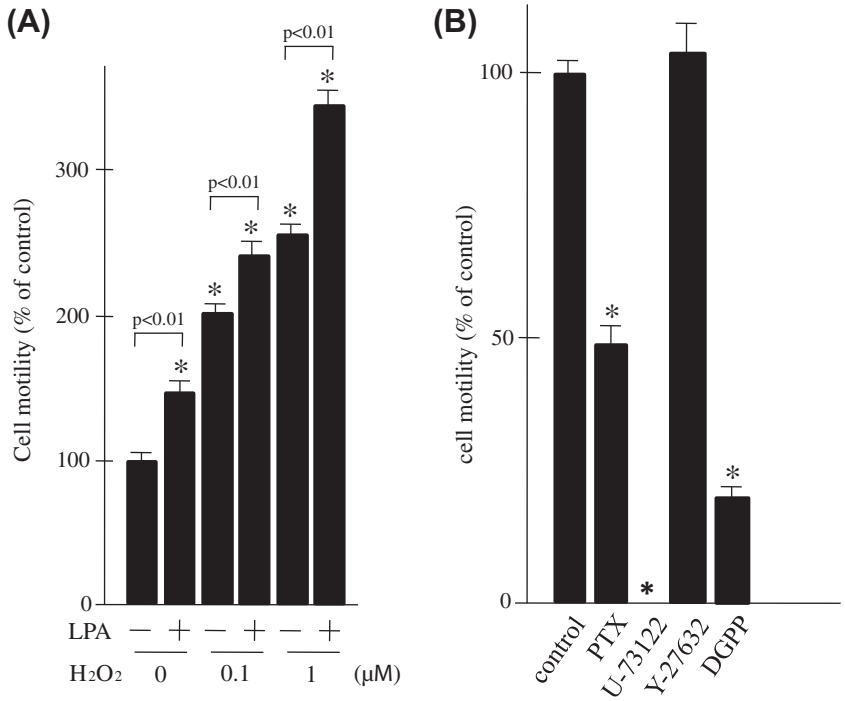

Fig. 2. (A) Effects of LPA on cell motility of WB-F344 cells stimulated by hydrogen peroxide. Cells pretreated with hydrogen peroxide (0.1 and 1 μM) were seeded into the filter at 1×10^5 cells in 200 μl serum-free DMEM and placed in 24-well plates containing 800 μl of 5% charcoal stripped FBS – DMEM with or without LPA (10 μM). (B) Effects of LPA signaling inhibitors on cell motile activity of WB-F344 cells. After the pretreatment of hydrogen peroxide, cells were also treated with PTX (100 ng/ml) for 24 h, U-73122 (5 μM) for 30 min, Y-27632 (1 μM) for 30 min or DGPP (10 μM) for 30 min. Cells were seeded into the filter at 1×10^5 and incubated for for 16 h with LPA (10 μM). Columns indicate the mean of three studies. Bars indicate SD. * $p < 0.01$ vs. untreated (control) cells.

peroxide. The *Lpar3* expression and cell motile activity stimulated by hydrogen peroxide were completely suppressed in WB-shRNA3-2 cells (Fig. 3B and C). Therefore, these results suggest that LPA_3 may be mainly involved in cell motile activity stimulated by hydrogen peroxide. However, it remains to be clarified why LPA_3 was induced by hydrogen peroxide treatment.

Finally, we examined the effect of hydrogen peroxide on cell motility of *Lpar3*- expressing and unexpressing cells. RH7777 rat

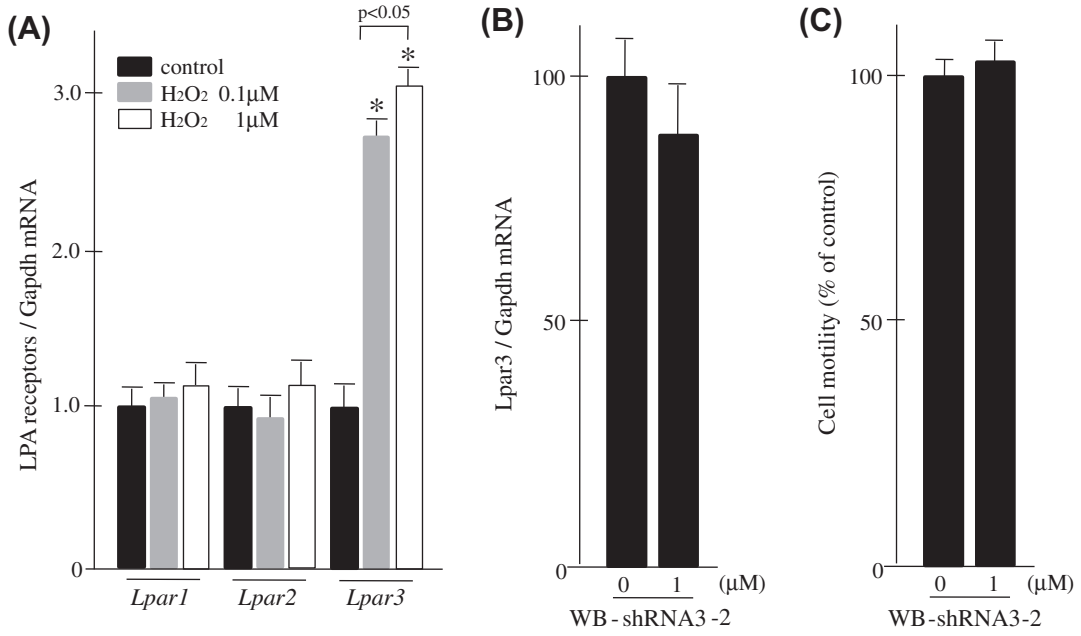

Fig. 3. (A) Quantitative real time RT-PCR analysis for *Lpar1 Lpar2* and *Lpar3* gene expressions in WB-F344 cells treated with hydrogen peroxide for 48 h. Columns indicate the mean of three studies. Bars indicate SD. * $p < 0.01$ vs. untreated (control) cells. (B), (C) The *Lpar3* gene expression level and cell motile activity in WB-shRNA3-2 cells treated with hydrogen peroxide for 48 h. Columns indicate the mean of three studies. Bars indicate SD. * $p < 0.01$ vs. untreated (control) cells.

hepatoma cells did not express *Lpar1*, *Lpar2* and *Lpar3* genes [11,21,22]. The *Lpar3*-expressing RHa3G8 cells were generated from RH7777 cells. As control cells, the *Lpar3*-unexpressing RH7777AB cells were also used [11]. While hydrogen peroxide did not stimulate cell motile activity of RH7777AB cells, the cell motility of RHa3G8 cells was significantly enhanced by hydrogen peroxide treatment (Fig. 4). Therefore, it suggests that LPA_3 may act as a positive regulator of cell motility induced by hydrogen peroxide in RH7777 cells as well as WB-F344 cells.

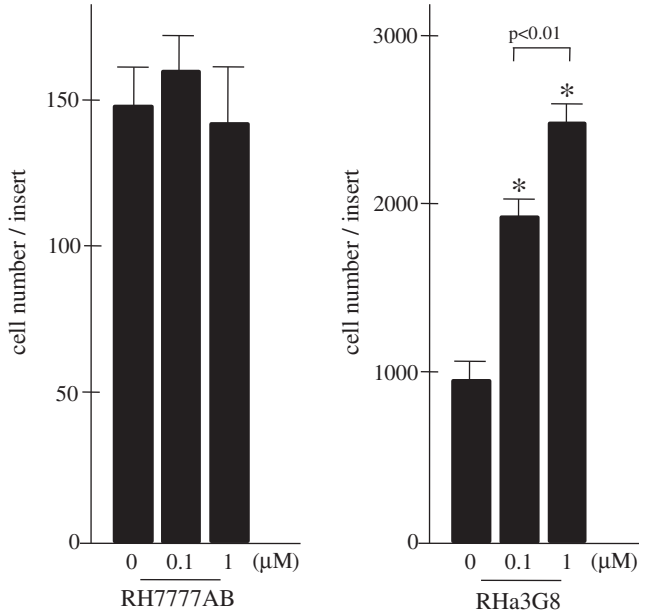

Fig. 4. The cell motility assay with a Cell Culture Insert. *Lpar3*-unexpressing RH7777AB (control) cells and *Lpar3*-expressing RHa3G8 cells were pretreated with hydrogen peroxide at a concentration of 0.1 and 1 μM for 48 h, and were seeded in the filter at 1×10^5 cells in 200 μl serum-free DMEM (upper chamber) and placed in 24-well plates (lower chamber) containing 800 μl of DMEM containing 10% FBS. Columns indicate the mean of three studies. Bars indicate SD. * $p < 0.01$ vs. untreated (control) cells.

Hydrogen peroxide can activate the intracellular signaling pathways, such as protein kinase C, epidermal growth factor, transcription factor activator protein-1 and mitogen-activated protein kinase [1,2]. The several biological responses are stimulated through the activation of those signaling pathway [1,2]. Previously, it has been reported that hydrogen peroxide stimulated cell proliferation and motile activity of normal cells [2,3]. In cancer cells, hydrogen peroxide also enhanced tumor growth and metastatic potential as well as cell migration [2–5]. In this study, we demonstrated that hydrogen peroxide stimulated cell motile activity of WB-F344 cells through the induction of *Lpar3* expression. Moreover, the stimulated cell motile activity by hydrogen peroxide was significantly enhanced by LPA treatment and inhibited by *Lpar3* knockdown. Therefore, the investigation whether LPA signaling via LPA_3 on cell motility stimulated by hydrogen peroxide may cooperate with other intracellular signaling should be further required.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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