Biochemical and Biophysical Research Communications 441 (2013) 47–52

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Inhibitory effects of LPA_1 on cell motile activities stimulated by hydrogen peroxide and 2,3-dimethoxy-1,4-naphthoquinone in fibroblast 3T3 cells

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ARTICLE INFO

Article history:
Received 3 October 2013
Available online 14 October 2013

Keywords:
LPA
LPA_1
Cell motility
Hydrogen peroxide
2,3-dimethoxy-1,4-naphthoquinone

ABSTRACT

Reactive oxygen species (ROS) are known to mediate a variety of biological responses, including cell motility. Recently, we indicated that lysophosphatidic acid (LPA) receptor-3 (LPA_3) increased cell motile activity stimulated by hydrogen peroxide. In the present study, we assessed the role of LPA_1 in the cell motile activity mediated by ROS in mouse fibroblast 3T3 cells. 3T3 cells were treated with hydrogen peroxide and 2,3-dimethoxy-1,4-naphthoquinone (DMNQ) at concentrations of 0.1 and 1 μM for 48 h. In cell motility assays with Cell Culture Inserts, the cell motile activities of 3T3 cells treated with hydrogen peroxide and DMNQ were significantly higher than those of untreated cells. 3T3 cells treated with hydrogen peroxide and DMNQ showed elevated expression levels of the *Lpar3* gene, but not the *Lpar1* and *Lpar2* genes. To investigate the effects of LPA_1 on the cell motile activity induced by hydrogen peroxide and DMNQ, *Lpar1*-overexpressing (3T3-a1) cells were generated from 3T3 cells and treated with hydrogen peroxide and DMNQ. The cell motile activities stimulated by hydrogen peroxide and DMNQ were markedly suppressed in 3T3-a1 cells. These results suggest that LPA signaling via LPA_1 inhibits the cell motile activities stimulated by hydrogen peroxide and DMNQ in 3T3 cells.

1. Introduction

Lysophosphatidic acid (LPA) is a biological signaling lipid that interacts with G protein-coupled LPA receptors (LPA receptor-1 (LPA_1) to LPA_6). LPA signaling via LPA receptors mediates a variety of cellular responses, such as cell proliferation, differentiation, motility and morphogenesis [1–4]. LPA receptors also contribute to the acquisition of malignant potency by tumor cells, including ovary, colon and liver cancer cells [5–8]. In our recent study, we demonstrated that LPA_2 and LPA_3 increased the cell motility and invasion of neuroblastoma cells, while LPA_1 inhibited these activities [9]. In particular, LPA_3 markedly stimulated the tumorigenicity of neuroblastoma and liver tumor cells [9,10]. In contrast, LPA_3 suppressed cell motile activity and angiogenesis of lung and mammary tumor cells [11,12]. Therefore, it has been considered that each LPA receptor has diverse cellular effects, depending on the cell types involved.

It is widely accepted that oxidative stress is involved in the initiation and progression of several diseases [13,14]. Reactive oxygen species (ROS) are produced in aerobic organisms during normal metabolic processes and mediate oxidative stress [13–15]. Hydrogen peroxide is one of the most important ROS and produces oxygen radicals that cause oxidative stress to cells [13]. Hydrogen peroxide can activate the intracellular signaling pathways to regulate a variety of biological functions, such as cell proliferation, motility, differentiation and apoptosis [13–15]. 2,3-Dimethoxy-1,4-naphthoquinone (DMNQ) is a redox cycling quinone that promotes the generation of ROS [16,17]. DMNQ induces apoptosis and cell necrosis, and stimulates cell proliferation [18].

Recently, we reported that LPA_3 enhanced the cell motile activity stimulated by hydrogen peroxide in liver epithelial cells [19]. In the present study, to assess the role of LPA_1 in cell motile activity regulated by ROS, *Lpar1*-overexpressing cells were generated from mouse fibroblast 3T3 cells that express endogenous LPA_1 and treated with hydrogen peroxide and DMNQ. We demonstrate that LPA_1

Abbreviations: LPA, lysophosphatidic acid; LPA_1, LPA receptor-1; ROS, reactive oxygen species; DMNQ, 2,3-dimethoxy-1,4-naphthoquinone; RT, reverse transcription; PCR, polymerase chain reaction; DGPP, diactylglycerol pyrophosphate.

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http://dx.doi.org/10.1016/j.bbrc.2013.10.009

acts as a negative regulator of the cell motile activities stimulated by hydrogen peroxide and DMNQ in 3T3 cells.

2. Materials and methods

2.1. Cell culture

Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Wako Pure Chemical Industries Ltd., Osaka, Japan) containing 10% fetal bovine serum (FBS) in a 5% CO_2 atmosphere at 37 °C. *Lpar1*-overexpressing (3T3-a1) cells were generated from 3T3 cells as described previously [20].

2.2. Effects of hydrogen peroxide and DMNQ on cell growth

Cells were plated at 4000 cells/well in 96-well plates and cultured in 100 μl of DMEM containing 10% FBS. The cells were treated with hydrogen peroxide (Wako Pure Chemical Industries, Ltd.) or DMNQ (Sigma Biochemicals, St. Louis, MO) at concentrations of 0.01, 0.1, 1 and 10 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 added to the plates. These assays were always performed in triplicate [19,20].

2.3. Effects of hydrogen peroxide and DMNQ on cell motile activity

For motility assays, Cell Culture Inserts (BD Falcon, Franklin Lakes, NJ) with 8-μm pore size were used. The cells were pretreated with hydrogen peroxide or DMNQ at concentrations of 0.1 and 1 μM every 24 h for 48 h. The cells were then seeded on the filters at 5×10^4 cells in 200 μl of serum-free DMEM (upper chamber). The filters were placed in 24-well plates (lower chamber) containing 800 μl of DMEM supplemented with 10% FBS, and incubated for 16 h in a 5% CO_2 atmosphere at 37 °C. The cells remaining on the upper side of the filters were removed with cotton swabs. After Giemsa staining, the numbers of cells that had

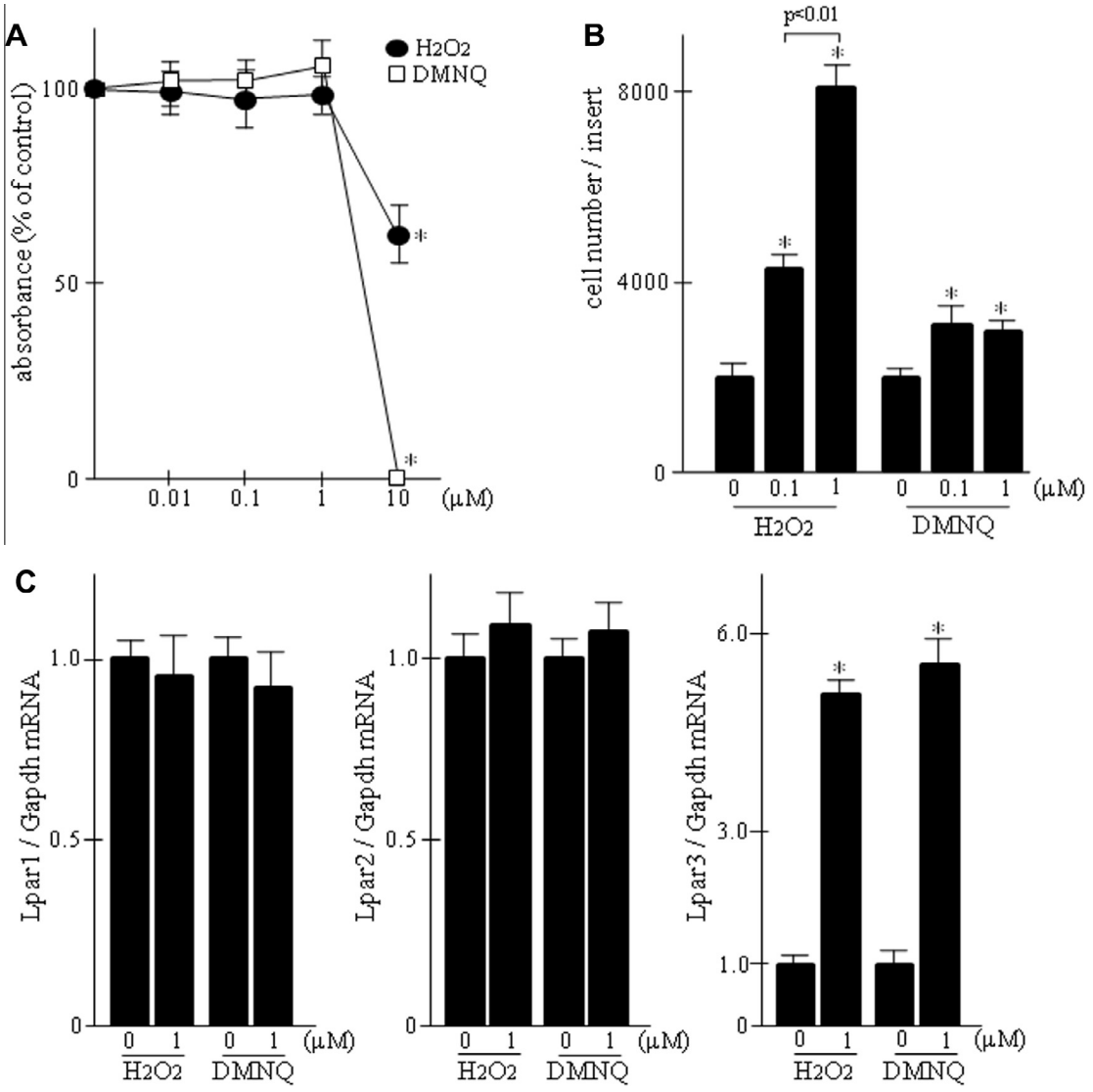

Fig. 1. Effects of hydrogen peroxide and DMNQ on the cell motile activity of mouse fibroblast 3T3 cells. (A) Effects of hydrogen peroxide and DMNQ on the cell proliferation rate of 3T3 cells. Cells were treated with hydrogen peroxide and DMNQ at 0.01, 0.1, 1, 10 and 100 μM every 24 h for 3 days. Data are indicated as the percentages of untreated cells. (B) Cell motility assays of 3T3 cells with Cell Culture Inserts. Cells were pretreated with hydrogen peroxide and DMNQ at concentrations of 0.1 and 1 μM for 48 h. The cells were then seeded in the filters at 5×10^4 cells in 200 μl of serum-free DMEM (upper chamber) and placed in 24-well plates (lower chamber) containing 800 μl of DMEM supplemented with 10% FBS. The data present the means ± SD of three experiments. (C) Quantitative real-time RT-PCR analyses for *Lpar1*, *Lpar2* and *Lpar3* gene expression levels in 3T3 cells treated with hydrogen peroxide and DMNQ at the concentration of 1 μM for 48 h. The data present the means ± SD of three experiments. $^*p < 0.01$ vs. untreated cells.

moved to the lower side of the filters were counted [9–12,19]. In addition, after pretreatment with hydrogen peroxide and DMNQ, some cells were further treated with 10 μM diactylglycerol pyrophosphate (DGPP) (Avanti Polar Lipids, Inc., Alabaster, AL) for 30 min. The cells were then seeded into the Cell Culture Inserts (BD Falcon) at 5×10^4 cells and incubated with 10 μM LPA (Avanti Polar Lipids Inc.) [21].

2.4. Quantitative real-time reverse transcription (RT)-polymerase chain reaction (PCR) analysis for LPA receptor gene expressions

To measure the expression levels of the LPA receptor genes, quantitative real-time RT-PCR analyses using SYBR Premix Ex Taq (Tli RNaseH Plus) (TaKaRa Bio, Inc., Shiga, Japan) and a Smart Cycler II System (TaKaRa Bio, Inc.) were performed according to the manufacturer's protocol. RNA was extracted from cells treated with hydrogen peroxide or DMNQ and first-strand cDNA was synthesized with a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics Co. Ltd., Mannheim, Germany). The expression levels of each LPA receptor gene were normalized to those of mouse *Gapdh* [9–11,19].

2.5. Statistical analysis

Statistical analyses were carried out using Student's *t*-test. The data were recognized to differ significantly for values of $p < 0.01$. The results are given as means ± SD.

3. Results

3.1. Effects of hydrogen peroxide and DMNQ on the cell growth of 3T3 cells

To determine the concentrations for use in the cell motility assays, cells were treated with hydrogen peroxide and DMNQ at 0.01, 0.1, 1 or 10 μM every 24 h for 3 days. The cell proliferation rates

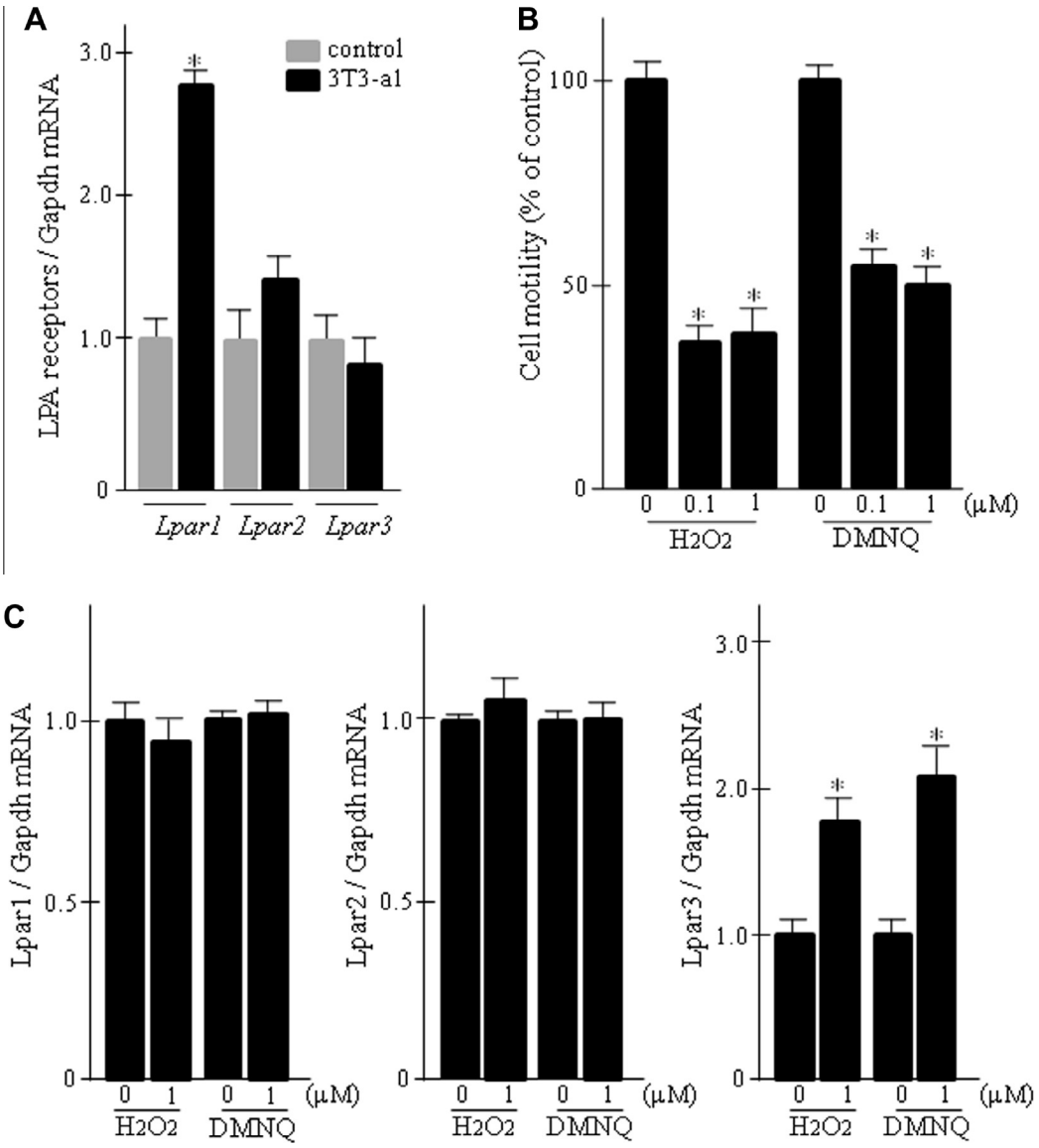

Fig. 2. Cell motile activities of *Lpar1*-overexpressing cells treated with hydrogen peroxide and DMNQ. (A) Quantitative real-time RT-PCR analyses for *Lpar1*, *Lpar2* and *Lpar3* gene expression levels in control (vector) and *Lpar1*-overexpressing (3T3-a1) cells. The data present the means ± SD of three experiments. $^*p < 0.01$ vs. control cells. (B) Cell motile activities of *Lpar1*-overexpressing cells pretreated with hydrogen peroxide and DMNQ at the concentrations of 0.1 and 1 μM for 48 h. The data present the means ± SD of three experiments. $^*p < 0.01$ vs. untreated cells. (C) *Lpar1*, *Lpar2* and *Lpar3* gene expression levels in *Lpar1*-overexpressing cells evaluated by quantitative real-time RT-PCR analyses. Cells were treated with hydrogen peroxide and DMNQ at the concentration of 1 μM for 48 h. The data present the means ± SD of three experiments. $^*p < 0.01$ vs. untreated cells.

were measured using the CCK-8 (Dojin Chemistry). Both hydrogen peroxide and DMNQ significantly inhibited the cell growth of 3T3 cells at 10 μM, but not at 0.01, 0.1 or 1 μM (Fig. 1A).

3.2. Cell motile activities of 3T3 cells treated with hydrogen peroxide and DMNQ

Based on the cell proliferation assays, the cells were treated with hydrogen peroxide and DMNQ at concentrations of 0.1 and 1 μM every 24 h for 48 h. The cells were then seeded into the Cell Culture Inserts and incubated for 16 h. The cell motile activities of 3T3 cells treated with hydrogen peroxide and DMNQ were significantly higher than those of untreated cells (Fig. 1B).

3.3. Expression levels of each LPA receptor gene in 3T3 cells treated with hydrogen peroxide and DMNQ

To evaluate which LPA receptors contributed to the cell motile activity stimulated by hydrogen peroxide and DMNQ, quantitative real-time RT-PCR analyses were performed. The expression levels of the *Lpar3* gene were significantly elevated in 3T3 cells treated with hydrogen peroxide and DMNQ, while those of the *Lpar1* and *Lpar2* genes were not (Fig. 1C).

3.4. Cell motile activities and LPA receptor gene expressions in Lpar1-overexpressing cells treated with hydrogen peroxide and DMNQ

In our previous report, we generated 3T3-a1 cells from 3T3 cells [20]. The expression levels of the *Lpar1* gene in 3T3-a1 cells were confirmed by quantitative real-time RT-PCR analysis, in comparison with control (vector) cells (Fig. 2A). In cell motility assays, the cell motile activities of 3T3-a1 cells treated with hydrogen peroxide and DMNQ were significantly lower than those of untreated 3T3-a1 cells (Fig. 2B). In control cells, the cell motile activity was significantly stimulated by hydrogen peroxide and DMNQ, similar to the case for 3T3 cells (data not shown). The *Lpar3* gene expression levels were significantly elevated in 3T3 cells treated with hydrogen peroxide and DMNQ, while the *Lpar1* and *Lpar2* gene expression levels remained unchanged (Fig. 2C).

3.5. Effects of DGPP on the cell motile activity of Lpar1-overexpressing cells

DGPP is used as an antagonist of LPA_1/LPA_3 [22]. 3T3-a1 cells were pretreated with hydrogen peroxide and DMNQ for 48 h and then treated with DGPP. The cell motile activity was measured in the presence of LPA. In DGPP-treated 3T3-a1 cells, hydrogen peroxide and DMNQ enhanced the cell motile activities, compared with those in DGPP-untreated cells (Fig. 3).

3.6. Effects of hydrogen peroxide and DMNQ on the cell motile activities of neuroblastoma B103 cells

Finally, to confirm whether LPA_1 can suppress the cell motile activity stimulated by hydrogen peroxide and DMNQ in other cell types, we examined *Lpar1*-expressing (lpa1-1) cells, which were generated from rat neuroblastoma B103 cells. AB2-1bf cells were also evaluated as control (vector) cells [9,20]. The cell motile activities of AB2-1bf cells were markedly enhanced by hydrogen peroxide and DMNQ (Fig. 4A). In AB2-1bf cells treated with hydrogen peroxide and DMNQ, the *Lpar2* and *Lpar3* gene expression levels were elevated, while those of the *Lpar1* gene were not (Fig. 4B). In contrast, the cell motile activities stimulated by hydrogen peroxide and DMNQ were significantly suppressed in lpa1-1 cells (Fig. 4C).

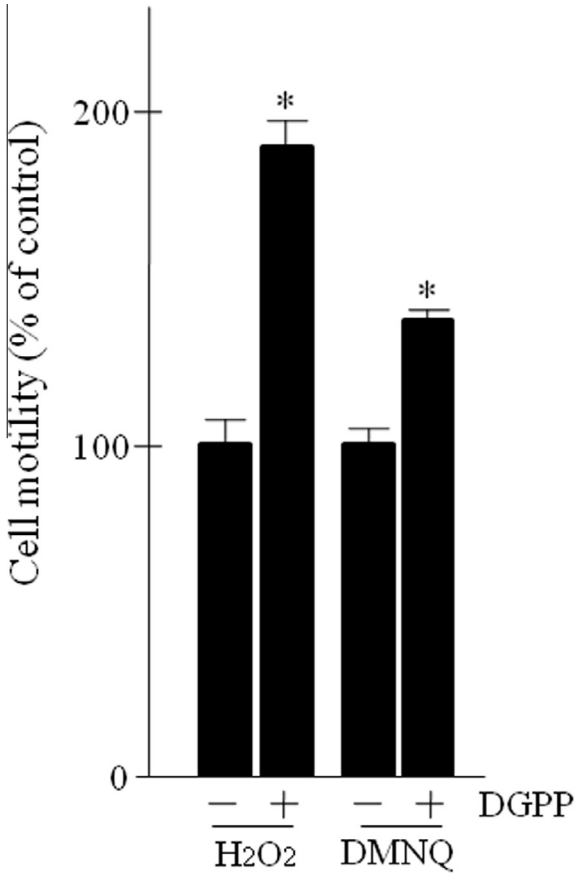

Fig. 3. Effects of DGPP on the cell motile activity of *Lpar1*-overexperssing cells. After pretreatment with hydrogen peroxide and DMNQ, the cells were treated with DGPP (10 μM) for 30 min. The cells were then seeded into filters at 5×10^4 cells and incubated for 16 h with LPA (10 μM). The data present the means ± SD of three experiments. $^*p < 0.01$ vs. untreated cells.

4. Discussion

Hydrogen peroxide stimulates intracellular signaling, such as that by epidermal growth factor, protein kinase C, transcription factor activator protein-1 and mitogen-activated protein kinase [13,14]. The activation of these signaling pathways mediates a variety of biological responses, such as cell proliferation and motile activity [13,14]. In cancer cells, it has been reported that hydrogen peroxide enhances cell growth and metastasis and cell migration [13,23,24]. The exclusive redox cycling quinone DMNQ promotes the generation of ROS, including hydrogen peroxide [16,17]. Several studies involving hepatocytes have shown that the toxicity of DMNQ may be mediated by ROS via one-electron-based redox cycling [16].

Recently, we demonstrated that LPA_3 enhanced the cell motile activity of liver epithelial cells treated with hydrogen peroxide [19]. Since LPA_1 showed inhibitory effects on the cell motility and invasion of neuroblastoma cells [9], we investigated the role of LPA_1 in the cell motile activity induced by hydrogen peroxide and DMNQ. 3T3 cells treated with hydrogen peroxide and DMNQ exhibited high cell motile activities, which were correlated with the induction of *Lpar3* expression. In contrast, the cell motile activities stimulated by hydrogen peroxide and DMNQ were markedly suppressed in *Lpar1*-overexpressing cells, while the *Lpar3* gene expression levels were increased. To confirm whether these inhibitory effects in *Lpar1*-overexpressing cells were mediated by LPA signaling via LPA_1, *Lpar1*-overexpressing cells were treated with DGPP. The results revealed that DGPP prevented the inhibitory effects on the cell motile activities induced by hydrogen peroxide

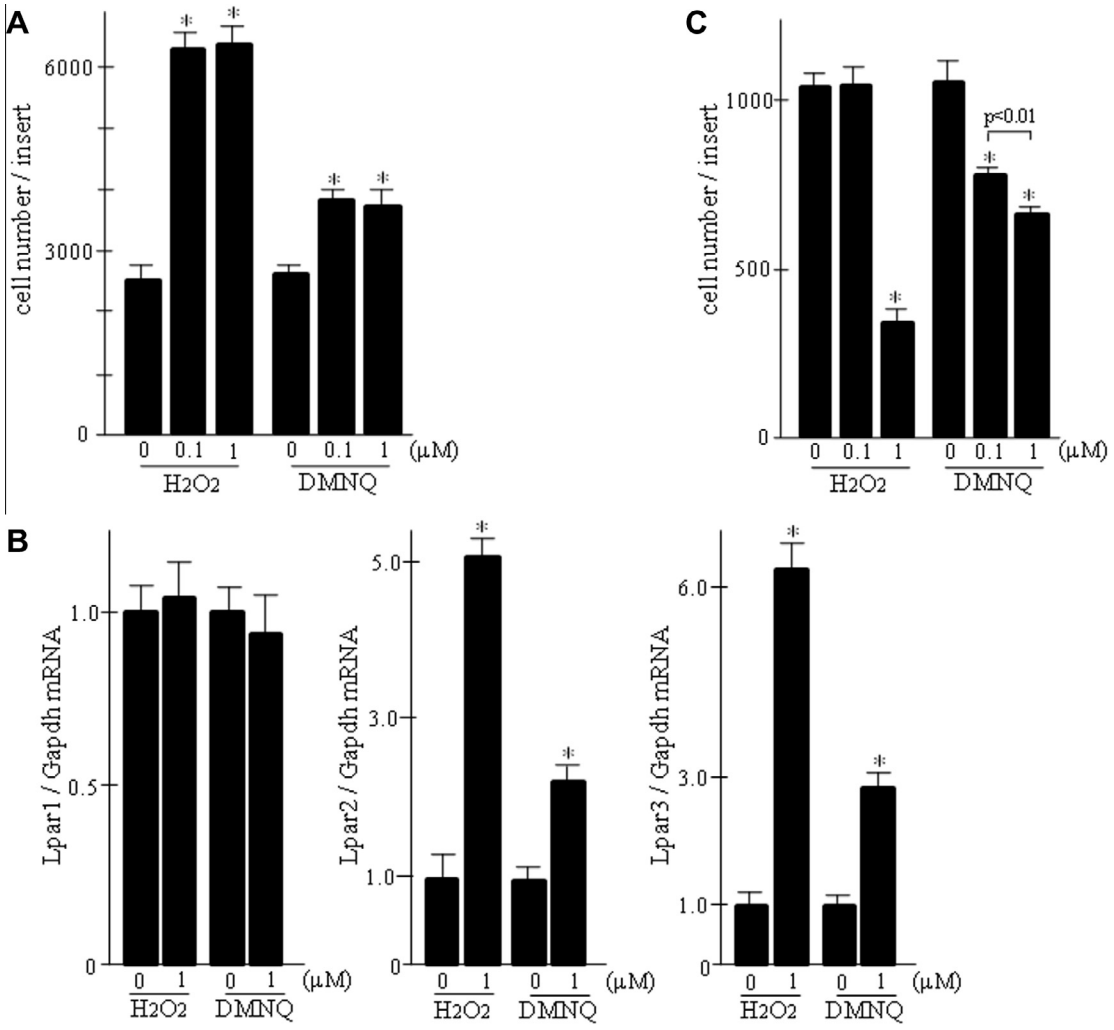

Fig. 4. Effects of hydrogen peroxide and DMNQ on the cell motile activity of neuroblastoma B103 cells. (A) Cell motile activities of AB2-1bf (vector) cells pretreated with hydrogen peroxide and DMNQ at the concentrations of 0.1 and 1 μM for 48 h. The data present the means ± SD of three experiments. (B) *Lpar1*, *Lpar2* and *Lpar3* gene expression levels in AB2-1bf (vector) cells evaluated by quantitative real-time RT-PCR analyses. Cells were treated with hydrogen peroxide and DMNQ at the concentration of 1 μM for 48 h. The data present the means ± SD of three experiments. (C) Cell motile activities of *Lpar1*-expressing (lpa1-1) cells pretreated with hydrogen peroxide and DMNQ at the concentrations of 0.1 and 1 μM for 48 h. The data present the means ± SD of three experiments. $^{*}p < 0.01$ vs. untreated cells.

and DMNQ in the *Lpar1*-overexpressing cells. Taken together, these results suggest that LPA signaling via LPA_1 may suppress the cell motile activity stimulated by hydrogen peroxide and DMNQ in 3T3 cells.

Since the individual endogenous LPA receptors show very low expression levels and no response to LPA treatment is observed in naive B103 cells, we generated clonal B103 cells expressing each LPA receptor and investigated the cellular functions of LPA signaling [20,25,26]. To evaluate the role of LPA_1 in the cell motile activities mediated by hydrogen peroxide and DMNQ in B103 cells, exogenous *Lpar1*-expressing B103 cells were treated with these agents. Although the cell motile activities were increased by hydrogen peroxide and DMNQ in control (vector) cells, exogenous LPA_1 markedly suppressed the cell motile activities in B103 cells, similar to the case for 3T3 cells.

In our recent studies, we reported that 12-*O*-tetradecanoylphorbol-13-acetate, a tumor-promoting agent, enhanced the cell motile activity of liver epithelial cells, which was correlated with elevated *Lpar3* gene expression [27]. Moreover, differential effects of estrogens on the cell motile activities were detected in liver epithelial cells. The cell motile activities and *Lpar3* gene expression levels in cells treated with 17β-estradiol and ethinyl estradiol were significantly higher than those in untreated cells. In contrast, diethylstilbestrol inhibited the cell motile activity with induction of *Lpar1* gene expression [28]. Therefore, it is suggested that LPA_1 and LPA_3 have opposite roles in the cell motile activities of rat liver epithelial cells, similar to the case for cells treated with hydrogen peroxide and DMNQ.

In conclusion, we have demonstrated that LPA_1 acts as a negative regulator of the cell motile activities stimulated by hydrogen peroxide and DMNQ in 3T3 cells. Since ROS mediate oxidative stress and induce several biological functions, LPA signaling via LPA_1 may be a new chemotherapeutic target molecule for the establishment of anticancer agents and LPA_3.

Acknowledgments

This study was supported in part by a Grant-in-Aid (24590493) for Scientific Research (C) from Japan Society for the Promotion of Science, and by Grants from the Ministry of Health, Labor and Welfare of Japan. The authors wish to express their gratitude to Dr. Alison Sherwin for correction of the English and careful reading of the manuscript.

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