Lysophosphatidylate signaling stabilizes Nrf2 and increases the expression of genes involved in drug resistance and oxidative stress responses: implications for cancer treatment

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ABSTRACT The present work elucidates novel mechanisms for lysophosphatidate (LPA)-induced chemoresistance using human breast, lung, liver, and thyroid cancer cells. LPA (0.5–10 μM) increased Nrf2 transcription factor stability and nuclear localization by ≤5-fold. This involved lysophosphatidate type 1 (LPA1) receptors as identified with 1 μM vls-31 (LPA1/2 receptor agonist) and blocking this effect with 20 μM Ki16425 (LPA1/3 antagonist, Ki = 0.34 μM). Knockdown of LPA1 by 50% to 60% with siRNA decreased Nrf2 stability and expressing LPA1, but not LPA2/3, in human HepG2 cells increased Nrf2 stabilization. LPA-induced Nrf2 expression increased transcription of multidrug-resistant transporters and antioxidant genes by 2- to 4-fold through the antioxidant response element. This protected cells from doxorubicin-induced death. This pathway was verified in vivo by orthotopic injection of 20,000 mouse 4T1 breast cancer cells into syngeneic mice. Blocking LPA production with 10 mg/kg per day ONO-8430506 (competitive autotaxin inhibitor, IC50 = 100 nM) decreased expression of Nrf2, multidrug-resistant transporters, and antioxidant genes in breast tumors by ≤90%. Combining 4 mg/kg doxorubicin every third day with ONO-8430506 synergistically decreased tumor growth and metastasis to lungs and liver by >70%, whereas doxorubicin alone had no significant effect. This study provides the first evidence that LPA increases antioxidant gene and multidrug-resistant transporter expression. Blocking this aspect of LPA signaling provides a novel strategy for improving chemotherapy.—Venkatraman G., Benesch, M. G. K., Tang X., Dewald J., McMullen, T. P. W., and Brindley, D. N. Lysophosphatidate signaling stabilizes Nrf2 and increases the expression of genes involved in drug resistance and oxidative stress responses: implications for cancer treatment. *FASEB J.* 29, 000–000 (2015). www.fasebj.org

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A major challenge in cancer treatment is to prevent the development of resistance to existing therapies (1). Chemoresistance is often explained by alterations in drug accumulation or regulation of damage repair mechanisms in residual cancer cells (2). This is particularly important in metastatic cancers, which are frequently associated with treatment resistance and poor outcomes (3, 4). The tumor microenvironment, which includes cytokines, chemokines, and growth factors in the surrounding tissue, could be an important component of therapy resistance (5, 6). The mechanism of protection that is provided by such extrinsic factors against therapeutics may be different from that of acquired resistance, with which it may work in tandem (5, 7). Hence, targeting these derived factors presents an attractive strategy in overcoming resistance by providing additional specificity, decreased toxicity, and protection from metastasis.

The present study concentrated on lysophosphatidate (LPA), which is produced by the secreted enzyme autotaxin (ATX). LPA activates at least 6 G protein-coupled receptors, LPA1,6 (8). The major function of ATX in adults is in wound repair, where ATX is secreted in response to inflammation to stimulate the migration of inflammatory cells, such as neutrophils and macrophages, to the site of injury (9). LPA is also produced in response to local inflammation to stimulate the migration of fibroblasts, leukocytes, and keratinocytes into the injured area (10–12). LPA is also produced in response to local inflammation to stimulate the migration of fibroblasts, leukocytes, and keratinocytes into the injured area (13). LPA is a key regulator of cell proliferation, migration, survival, and angiogenesis. These processes involve the activation of multidrug-resistant transporters, which are involved in the transport of drugs that are used in cancer treatment (14). The activity of ATX is regulated by various factors, including extracellular matrix proteins, growth factors, cytokines, and chemokines (15–17).

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(14). Significantly, dysfunctional LPA signaling in chronic inflammation can progress to cancer in colitis-induced colon cancer (15) and hepatitis (16).

The importance of LPA in breast cancers was demonstrated in mice where expression of ATX, LPA1, LPA4, or LPA3 receptors in mammary epithelial cells promotes the spontaneous development of metastatic tumors (17). Women with breast carcinomas expressing high levels of LPA3 receptors in epithelial cells or ATX in stromal cells have larger tumors, nodal involvement, and higher-stage disease (18). ATX and LPA promote the migration of breast cancer cells (19), an important component of metastasis, and LPA-LPA1 signaling promotes bone metastasis in breast cancer (20, 21). We recently showed that an ATX inhibitor, ONO-8430506, delays breast tumor growth for the first 11 d and lung metastasis at 21 d in an orthotopic syngeneic mouse model (22). ATX is produced in the breast tumor stroma and surrounding adipose tissue rather than by breast cancer cells. The resulting increase in LPA production is part of a vicious cycle of inflammation caused by tumor growth and therapeutic intervention, which fuels further inflammation, tumor growth, metastasis, and treatment resistance (9, 22). LPA also decreases the efficacy of chemotherapeutic drugs. These responses protect against cell death during chemotherapy.

We verified the significance of this LPA signaling in vivo by using a syngeneic orthotopic mouse model of breast cancer. The production of unsaturated LPA species in plasma and breast tumors was blocked with an ATX inhibitor, ONO-8430506 (22, 27). This decreased Nrf2 levels and the expression of antioxidant genes and MDRTs in the breast tumors. Administering a relatively low dose of doxorubicin alone to the mice had little effect on tumor growth. However, a combination of doxorubicin with the ATX inhibitor was effective in blocking tumor growth and metastasis. Also, the effectiveness of ONO-8430506 in decreasing tumor growth was extended from 11 d to about 18 d by combination with doxorubicin. We also showed that Nrf2 expression was higher among treated patients with recurrent breast tumors compared to nonrecurring cancer. This result is compatible with several publications that found that elevated Nrf2 is associated with tumor progression and poor outcomes in cancer patients (28).

The importance of the present work is that it provides the first evidence that LPA increases the expression of antioxidant genes and MDRTs in breast tumors through stabilization of Nrf2. We also established that attenuating LPA signaling in mice by inhibiting ATX provides a novel and well-tolerated strategy for improving the efficacy of doxorubicin in slowing breast tumor growth and metastasis. At present, no cancer treatment involves blocking LPA signaling as an adjuvant therapy; this paradigm could provide a new approach for improving cancer treatment.

**MATERIALS AND METHODS**

**Reagents**

Reagents and chemicals were obtained as follows: LPA (C18:1) (Avanti Polar Lipids, Alabaster, AL, USA); Caspase 3/7 Glo assay kit, GSH-Glo, and Luciferase assay kit (Promega, Mississauga, ON, Canada); penicillin/streptomycin (Life Technologies, Gaithersburg, MD, USA); Ki67425 and doxorubicin HCl (Cayman Chemicals, Ann Arbor, MI, USA); wls-31 (kindly provided by W. Santos and N. Patwardhan, Department of Chemistry, Virginia Tech, Blacksburg, VA, USA) (29, 30). ONO-8430506 (Ono Pharmaceuticals Ltd., Osaka, Japan) was used as an ATX inhibitor in vivo as described previously (22, 27). All other chemicals and reagents were from Sigma-Aldrich (Oakville, ON, Canada).

**Cell culture**

MCF-7 (HTB-22), MDA-MB-231 (HTB-26), MDA-MB-468 (HTB-132), MDA-MB-435 (HTB-131), MDA-MB-435S (HTB-129), 4T1 (CRL-2539), and HeLa (HTB-126), HEK 293T (CRL-11268), A549 (CCCL-185), 4T1 (CRL-2539), and HepG2 (HB-8065). Cells were all purchased from ATCC (Rockville, MD, USA), and 8305C (ACG-135) was from DSMZ (Braunschweig, Germany). All cells were used at low passage number. MCF-7 cells stably overexpressing an inducible, minimal antioxidant response element (ARE) construct (ARE3520) (31) were the kind gift of R. Wolf, Cancer Research UK. 4T1-12B cells were purchased from G. Sahagian (Department of Physiology, Tufts University School of Medicine, Boston, MA, USA). All cell lines were maintained in DMEM + 10% FBS + P/S at 37°C humidified atmosphere containing 5% CO2.

**Plasmids, siRNA, and transient transfections**

cDNA for HA-tagged LPA1, 2 and 3 receptors were purchased from the University of Missouri cDNA Resource Center (Rolla, MO, USA). Enhanced green fluorescent protein (EGFP)-Nrf2 plasmid (215-d9) was from Addgene (Cambridge, MA, USA) (32). Plasmid transfections were achieved with PolyJet (Signagen, Gaithersburg, MD, USA); wls-31 (kindly provided by W. Santos and N. Patwardhan, Department of Chemistry, Virginia Tech, Blacksburg, VA, USA) (29, 30). ONO-8430506 (Ono Pharmaceuticals Ltd., Osaka, Japan) was used as an ATX inhibitor in vivo as described previously (22, 27). All other chemicals and reagents were from Sigma-Aldrich (Oakville, ON, Canada).

**SDS-PAGE gel electrophoresis**

Total cell extracts were sonicated in RIPA buffer containing 100 nM microcystin-LR and protease inhibitor cocktail. Tumor samples were homogenized using the TissueLyser II system (Qiagen), centrifuged, and transferred to fresh tubes. Equal amounts of protein (BCA assay) were loaded in the gel with sample loading dye after boiling. Nitrocellulose membranes were blocked with 5% nonfat dry
milk in TBST buffer (10 mM Tris HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20) and incubated with primary antibodies overnight at 4°C with gentle shaking. Blots were washed 3 times with TBST and incubated with horseradish peroxidase–conjugated secondary antibodies (1:10,000). Immunocomplexes were detected using Immunostar Western C kit (Bio-Rad, Hercules, CA, USA). PVDF membranes were treated with blocking buffer (Li-Cor Biosciences, Lincoln, NE, USA) in PBS containing 0.1% Tween-20 and incubated with primary antibodies overnight at 4°C with gentle shaking. Alexa Fluor 680– and IRDye 800CW– (Li-Cor) conjugated secondary antibodies (1:10,000) were used for staining and the fluorescent signals were visualized in the Li-Cor Imaging System. The antibodies were: Nrf2 (H-300), Nrf2 (C-20), MDR1 (D11), NQO1 (A180) from Santa Cruz (Santa Cruz, CA, USA); PARP [poly(ADP-ribose) polymerase], HMOX1 (H105), K67 (D3B5), and cleaved caspase 3 (D175) from Cell Signaling (Danvers, MA, USA); GAPDH and α-tubulin from Sigma-Aldrich; MRPI (m6) and calnexin from Enzo LifeSciences (Farmingdale, NY, USA); ABCG2 (BXP-21) from Novus Biologicals (Oakville, ON, Canada); HA-tag antibody from Covance (Princeton, NJ, USA); and β-actin from Abcam (Toronto, ON, Canada).

RNA isolation and quantitative RT-PCR

Cells grown in 6 cm dishes were starved for 12 h before treatments for another 12 h. RNAqueous kit (Life Technologies) was used for isolation of mRNA. Genomic DNA was removed using the DNA free kit. qScript cDNA Supermix (Quanta Biosciences, Gaithersburg, MD) or RT2 DNA free kit. qScript cDNA Supermix (Quanta Biosciences, Gaithersburg, MD) or RT-PCR system (Life Technologies). Dissociation curves were established for detection of nonspecific products. Amplified products were verified on a 2% agarose gel. Primers were designed with Primer-BLAST software (NCBI; http://blast.ncbi.nlm.nih.gov/Blast.cgi) with default specificity and prepared by Integrated DNA Technologies Inc. Results were expressed relative to cyclophilin A. Similar results were obtained using hypoxanthine-guanine phosphoribosyltransferase. The primers used were as follows: ABCG2: sense 5′-GGAGGCTCGTGACCAGCGG-3′, ABCG2: sense 5′-AGATGGGTTTCAGACCGTTCAT-3′ antisense 5′-CCAGGCTGATGAGGAGCTT-3′; ABCB1: sense 5′-GCTGATGGTCGGTCCGAA-3′ antisense 5′-TGAAACGCCCACAAGCCTG-3′; ABCB2: sense 5′-TGCAAGGGTCTCCTGTCTATCC-3′ antisense 5′-CCATGCCGATGACCTGCTT-3′; MRP1 (m6): sense 5′-ATCCCACTCTCGGACGAC-3′, ABCC3: sense 5′-GGTCTCTGTCATATGTCCT-3′ antisense 5′-CTGGTCCAGGATCATCTCAGC-3′; ABCC1: sense 5′-GCTGATGTTGAGCCGAGG-3′ antisense 5′-TGAAACGCCCACAAGCCTG-3′; ABCD1: sense 5′-ATCCCACTCTCGGACGAC-3′, ABCD2: sense 5′-GCTGATGTTGAGCCGAGG-3′, ABCD3: sense 5′-ATCCCACTCTCGGACGAC-3′; MRP1 (m6): sense 5′-ATCCCACTCTCGGACGAC-3′, ABCC3: sense 5′-GGTCTCTGTCATATGTCCT-3′ antisense 5′-CTGGTCCAGGATCATCTCAGC-3′; ABCC1: sense 5′-GCTGATGTTGAGCCGAGG-3′ antisense 5′-TGAAACGCCCACAAGCCTG-3′.

Nuclear fractionation

Cells were grown in 15 cm dishes, starved for 12 h, and then treated with LPA or ws-31 for 6 h. Cells were then trypsinized, pelleted, and resuspended in swelling buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT and protease/phosphatase inhibitors) maintained in an ice bath. Cells were then homogenized with a prechilled Dounce homogenizer, and cytoplasm and nuclear fractions were collected by centrifugation (33). Nuclear pellets were resuspended in sucrose buffer I (0.25 M sucrose/10 mM MgCl2) layered over sucrose buffer II (0.88 M sucrose/0.5 mM MgCl2) and centrifuged to obtain a cleaner nuclear pellet. Both cytoplasmic and nuclear fractions were dissolved in RIPA buffer containing protease and phosphatase inhibitors.

Confocal microscopy

Cells were seeded on glass coverslips precoated with fibronectin and grown for 24 h. Transfections were performed after starving cells for 24 h. After treatments, cells were immediately fixed with 4% paraformaldehyde for 30 min, permeabilized with 10% Triton X-100, blocked with 1% normal donkey serum in PBS for 1 h, and washed. Samples were then treated with anti-HA in blocking buffer for 1 h and washed. Coverslips were then treated with the DNA stains Hoechst dye 33342 (Life Technologies), washed, and then mounted with Prolong Gold Antifade (Invitrogen, Carlsbad, CA, USA). Coverslips were then viewed in Leica SP5 confocal microscope (Concord, ON, Canada), and 20 random cells from 3 different experiments were used for quantification.

Doxorubicin accumulation and Flou3-AM efflux

Cancer cells (250,000) were grown on 6-well plates for 24 h, incubated with medium containing 0.1% bovine serum albumin (BSA) or vehicle for 12 h, and treated with 0.5 μM doxorubicin in the presence or absence of 5 μM cyclosporin A or 0.5 mM probenecid. Cells were washed, trypsinized, and subjected to FACs Canto II (BD Biosciences, ON, Canada) for fluorescence measurements. For localization experiments, cancer cells were seeded on glass coverslips, loaded with 5 μM doxorubicin for 4 h, washed, and immediately fixed, permeabilized, and counterstained with Hoescht 33342, and mounted on glass slides. For Flou3-AM efflux, MDA-MB-231 cells were grown in 6-well dishes. After 12 h starvation, the media were changed to medium containing sucrose that had been delipidated with charcoal (34) with or without 10 μM LPA for 24 h. Cells were trypsinized, labeled with 0.4 μM Flou3-AM (Life Technologies) for 1 h, centrifuged, and washed twice; then fresh medium was added with or without 5 mM probenecid. Cells were collected and washed, and at least 100,000 cells were analyzed for mean fluorescence in FACs Canto II.

Mouse model of breast cancer

The syngeneic orthotopic mouse model of breast tumor growth and metastasis has been described elsewhere (22). For experiments with ONO-8430506 and doxorubicin, mice were randomly assigned to 96 well plates in full growth medium. Media was changed after 24 h to starvation medium overnight followed by LPA treatment for 12 h and another 48 h of drug/vehicle treatment. MTT dye (500 μg/ml) was added to cells for 1 to 2 h before measurement of absorbance at 570 nm. Values were expressed relative to no treatment.

Cell viability assays

MTT was used to assess relative cell viability after treatment with doxorubicin or etoposide. Cells were seeded at 5 × 10^4 cells in a 96 well plate in full growth medium. Media was changed after 24 h to starvation medium overnight followed by LPA treatment for 12 h and another 48 h of drug/vehicle treatment. MTT dye (500 μg/ml) was added to cells for 1 to 2 h before measurement of absorbance at 570 nm. Values were expressed relative to no treatment.
divided into 4 groups. Groups 1 and 2 were gavaged daily with water, and groups 3 and 4 were gavaged daily with 10 mg/kg ONO-8430506 in water (22). Groups 1 and 3 received PBS by i.p. injections, while 2 and 4 received 4 mg/kg doxorubicin i.p. every third day starting from 3 d after tumor injection. Doxorubicin was dissolved in PBS by warming and filtering through a 0.22 µm filter.

Glutathione measurements

Reduced glutathione (GSH) was measured from freshly collected tumors using a GSH-Glo assay (Promega) and a GSH standard curve according to the manufacturer’s instructions. Total GSH levels were also measured by performing the assay in the presence of Tris-(2-carboxyethyl) phosphine. Results were normalized to protein concentrations (BCA assay).

Immunohistochemistry

Dako LSAB + Universal Kit (K0679) was used on paraffin-embedded tumor sections (22). Staining was visualized using Dako Envision + Rabbit HRP (K0002) (Burlington, ON, Canada). Images were acquired at ×5 and ×63 using a Zeiss Axioskop 2 imaging system (Carl Zeiss Canada, Toronto, ON, Canada). A magnification of ×5 was used for counting and image analysis.

Pathway gene expression array

Tumors were collected in TRIZol reagent (Invitrogen). mRNA was isolated by chloroform extraction, purified by alcohol precipitation, and treated with a DNA-free kit. RNA (1 µg) was reverse transcribed into cDNA and analyzed using the mouse Oxidative Stress and Antioxidant Defense PCR array and cancer drug resistance PCR arrays (Qiagen). Results were calculated (http://perdataanalysis.sabiosciences.com/pcr/arrayanalysis.php) for statistical significance (P < 0.05) using the ΔΔCt method (35). Actb, GAPDH, and hsp90ab1 were used for normalization.

Ethical approval

Animal procedures complied with the Canadian Council of Animal Care as approved by the University of Alberta Animal Welfare Committee. Human samples were obtained with approval of University of Alberta Heath Research Ethics Board (ID Pro00018758).

Statistical analysis

Results are means ± SEM. Unpaired 2-tailed t test was used to determine P values. One-way or 2-way ANOVA with a Bonferroni post hoc test was used for multiple comparisons. All analyses were performed by GraphPad Prism 6 software (GraphPad Software, San Diego, CA, USA). P < 0.05 was considered statistically significant.

RESULTS

LPA and the effectiveness of chemotherapeutic agents

Anthracyclines, such as doxorubicin, and taxanes are often used as first-line therapy for several cancers, including metastatic breast cancers. We extended our studies on paclitaxel (34) and found that LPA also decreases the effectiveness of doxorubicin in decreasing the viability of triple-negative MDA-MB-231 cells (Fig. 1A). The topoisomerase inhibitor etoposide was similarly less effective when cells were treated with LPA (Fig. 1B). We used 10 µM LPA in these experiments to compensate for LPA breakdown over the 48 h incubation (34). LPA treatment significantly decreased doxorubicin-induced cleavage of PARP and caspase 3/7 activity (Fig. 1C, D). Cells grown in 10% serum medium in the presence of the LPA receptor antagonist (Ki16425) were sensitized to doxorubicin-induced PARP cleavage (Fig. 1E). These results, together with previous work (8), demonstrate that extracellular LPA protects against apoptosis caused by several chemotherapeutic drugs.

Multidrug resistance in cancer cells is often caused by increased export of the drugs through MDRTs. Treatment of MDA-MB-231 cells with LPA did not change the predominant nuclear localization of doxorubicin (Fig. 2A), but it did decrease doxorubicin accumulation (Fig. 2B). This effect was prevented by pretreatment with cyclosporin A or probenecid, which are general MDRT inhibitors. We also used a fluorescent substrate for the MDRTs, Flou3 AM (36). Treating MDA-MB-231 cells with LPA increased its export and this was blocked completely by probenecid (Fig. 2C).

ABCC1 and ABCG2 proteins were detected in a panel of breast cancer cells, but ABCB1 expression was not detected (Supplemental Fig. 1A, B), as expected (37). Treatment with LPA increased ABCC1 and ABCG2 mRNA and protein expression in several breast cancer cell lines (Fig. 2D, E, F). The LPA1/2 receptor agonist wls-31 also increased mRNA for ABCC1 and ABCG2 mRNA in MDA-MB-231 cells (Fig. 2D). Pretreatment with the LPA1/2 receptor antagonist, Ki16425, blocked these LPA-induced increases in mRNA. These results indicate an LPA1-mediated mechanism, and LPA1 is the major LPA receptor in MDA-MB-231 cells (38). We confirmed the involvement of LPA1 receptors because 2 siRNA constructs targeting LPA1 blocked the LPA-induced increases in ABCC1 and ABCG2 mRNA (Fig. 2G). The decrease in mRNA for LPA1 was specific, and there was minimal effect on other LPA receptors (Supplemental Fig. 1C).

Nrf2 is stabilized by LPA-LPA1 receptor–PI3K signaling

Nrf2 is a master regulator of several antioxidant genes and MDRT, including ABCC1 (39, 40) and ABCG2 (41), through their cis-acting ARE. Nrf2 is constantly targeted for ubiquitinylation and proteosomal degradation (32, 42). The chemical activator of Nrf2, tert-butylhydroquinone (tBHQ), increases its stabilization and activation of its downstream targets, including antioxidant genes and drug transporters (43). Nrf2 transcription is elevated in cancers by oncogenic transformation, and mutations that stabilize Nrf2 are often found in various cancers [reviewed in (44)]. We found that Nrf2 expression was much higher in HS578T ductal carcinoma cells compared to the HS578Bst cell line, which was derived from the tissue peripheral to the infiltrating carcinoma of the same patient (Supplemental Fig. 2A). Elevated Nrf2 increases resistance to chemotherapies (45) and suppression of reactive oxygen.
species (46). The role of Nrf2 in cancer cell proliferation and tumorigenesis was recently associated with the Warburg effect (47, 48). MCF-7 cells that were selected for resistance to various drugs show increased Nrf2 expression (49). It has been suggested that blocking Nrf2 signaling could be effective in improving chemotherapy in several cancers. However, we know relatively little about how Nrf2 expression is regulated in cancers.

As expected, treatment of MDA-MB-231 cells with tBHQ increased the transcription of ABCC1, ABCC2, ABCC3, and ABCC6 as well as the antioxidant genes HMOX1 and NQO1 (Fig. 3A). LPA on its own or in combination with tBHQ also increased the expression of mRNA for these genes. LPA alone or with tBHQ also enhanced the protein expression of the antioxidant genes and MDRTs in MDA-MB-231 breast cancer (Fig. 3B). Similarly, LPA increased Nrf2 expression and its regulated genes in 4T1 mouse breast cancer cells (Supplemental Fig. 2B, C), A549 lung cancer cells, and 8305C thyroid cancer cells (Supplemental Fig. 2D).

Nrf2 is mainly regulated through degradation, and therefore, mRNA levels for Nrf2 were unchanged by treatment with tBHQ or LPA (Supplemental Fig. 2E). Nrf2 protein runs at 110 kDa in 8% to 10% separating gels (50), and it is rapidly stabilized by 25 μM tBHQ or the proteosomal inhibitor MG132 (Supplemental Fig. 2F). We verified this with antibodies against the N- and C-terminals in various cancer cells and by using EGFP-Nrf2, which migrates at ~140 kDa (Supplemental Fig. 2F–H). LPA treatment increased Nrf2 protein levels by 2.4-fold within 6 h; this increased up to 5-fold in the presence of tBHQ (Fig. 3B). LPA or the LPA1/2 agonist wls-31 increased the nuclear accumulation of Nrf2 by about 4.5-fold (Fig. 3C).

Figure 1. LPA protects MDA-MB-231 breast cancer cells from doxorubicin-induced apoptosis by increased expulsion of the drug and toxic products. A, B) Cells were serum starved for 12 h and treated with vehicle (0.1% BSA) or LPA (10 μM in 0.1% BSA) for 12 h, followed by various concentrations of doxorubicin or etoposide for 48 h. The relative number of viability cells as defined by MTT oxidisation is expressed relative to no drug treatment for 6 independent assays in (A) and 4 experiments in (B). C) Cells were treated as above with vehicle or 0.5 μM doxorubicin for 0, 24, or 48 h, and PARP cleavage was expressed relative to tubulin expression for 3 independent assays. D) Cells were treated as above with 2.5 μM doxorubicin (Dox) for 12 h in the presence or absence of LPA. ZVAD-FMK was used to inhibit caspase activation. Caspase 3/7 activity was measured by luminescence measurements and expressed relative to no drug treatment for 6 independent assays. E) Cells grown in 10% FBS were pretreated with LPA receptor antagonist Ki16425 (20 μM) for 12 h, followed by 0.5 μM doxorubicin for 48 h. Cell lysates were immunoblotted for PARP cleavage. Results are means ± SEM. *P < 0.05.
Figure 2. LPA through LPA₁ signaling increased the expression of MDRTs and expulsion of doxorubicin and Flou3 in breast cancer cells. A) Cells were fixed after doxorubicin treatment and visualized by fluorescence microscopy for doxorubicin localization. A representative result is shown from 5 experiments. B) Cells were with LPA as before for 12 h followed by pretreatment with inhibitors for 1 h and 0.5 μM doxorubicin treatment for 48 h. Cells were washed, trypsinized, resuspended in PBS + 1% BSA, and analyzed for cellular fluorescence using FACS Canto II. The mean fluorescence was recorded for 100,000 events for 4 independent assays. C) MDA-MB-231 cells were starved as before followed by treatment with delipidated serum with or without 10 μM LPA for 12 h. Trypsinized cells were loaded with Flou3-AM (0.4 μM) for 1 h. Cells were centrifuged, washed, and treated with fresh media with or without 5 mM probenecid. Cellular fluorescence was determined using FACS Canto II. Results for 100,000 cells were expressed as mean fluorescence relative to no treatment for 3 independent assays. D) MDA-MB-231 cells were starved for 12 h and then pretreated with 20 μM Ki16425 for another 6 h before treatment with 10 μM LPA or 1 μM wls-31 for 6 h. mRNA was measured in 6 independent experiments. E) MDA-MB-231 cells were treated with LPA as before for 12 h before immunoblotting for ABCC1 and ABCG2. F) MDA-MB-231, MDA-MB-468, and MCF-7 cells were starved for 12 h and treated with different LPA concentrations for another 12 h. Representative Western blot analyses are shown from 3 independent experiments. G) MDA-MB-231 cells were treated with 50 nM siCTRL or 2 different siRNA constructs targeting LPA₁ for 36 h, and they were then starved for 12 h before treating them with LPA (10 μM) for another 6 h. mRNA was analyzed for ABCC1 or ABCG2 expression. Results are means ± SEM. *P < 0.05.
Figure 3. LPA signaling through LPA₁ increases basal and t-BHQ–induced Nrf2 expression and enhances the expression of antioxidant genes and MDRTs. MDA-MB-231 cells were starved for 12 h and treated with or without 10 μM LPA for 6 h followed by the presence or absence of 10 μM t-BHQ for 6 h. A) mRNA was collected and analyzed from 5 independent experiments by qRT-PCR. B) Nrf2 protein expression was analyzed by immunoblotting after 12 h. Two-tailed t test was used for calculating P values between treatments. C) MDA-MB-231 cells were starved for 12 h and then treated with vehicle (0.1% BSA), 10 μM LPA, or 1 μM wls-31 for 6 h in 3 independent experiments. Nuclear and cytoplasmic fractions were immunoblotted. Nuclear Nrf2 expression was expressed relative to lamin A/C. D) MDA-MB-231 cells were starved for 12 h before pretreatment with 10 μM LY294002 (LY) or 1 μM wortmannin (Wort), 20 μM PD98059 (PD), or 10 μM Gö6983 (Gö) for 6 h. They were then treated with or without 10 μM LPA for 12 h. Nrf2 protein expression was detected by immunoblotting and expressed relative to glyceraldehyde phosphate dehydrogenase (GAPDH) in 3 independent experiments. E) MCF-7 AREc32 cells were transfected with 2 μg of EGFP-Nrf2 or EGFP plasmid, grown for 18 h, and then starved for 12 h before treatments for 24 h. Luciferase assays (continued on next page)
Blocking LPA signaling decreases the expression of Nrf2, antioxidant genes, and MDRTs

We investigated the hypothesis that LPA-induced expression of Nrf2, antioxidant genes, and MDRTs could provide a novel mechanism for producing resistance to several chemotherapeutic agents using a syngeneic orthotopic model of breast cancer. Blocking LPA production and signaling with a potent ATX inhibitor, ONO-8430506, in this model delays tumor growth up to day 11 and the subsequent development of lung metastases, each by about 60%. The efficacy of ONO-8430506 in inhibiting ATX activity in the experiments reported in Fig. 4 was demonstrated by analyzing LPA concentrations in the plasma and breast tumors of the mice. ONO-8430506 (10 mg/kg) markedly decreased LPA concentrations, especially unsaturated LPA species, at 6 h after dosing; this effect was largely maintained at 24 h (22). This inhibition of LPA signaling with ONO-8430506 decreased the protein expression of Nrf2 and NQO1 in the primary tumors (Fig. 4A). This decreased the expression of several Nrf2-regulated antioxidant genes (Fig. 4B), including GPX1, glutathione peroxidase-1; GCLM, glutamate cysteine ligase modifier subunit; SOD1 and SOD2, superoxide dismutase-1 and -2; PRDX4, peroxiredoxin-4; TXNRD1 and TXNRD3, thioredoxin-1 and -3; SCD1, stearoyl CoA desaturase 1; NQO1; IL19, interleukin-19; and ABCB1, ABCC1, and BCL2L1. These genes play important roles in the defense against oxidative stress.

Therefore, we tested the hypothesis that ONO-8430506 should enhance the effects of doxorubicin in decreasing tumor progression. 4T1 breast cancer cells were used because they produce more sustained tumor growth and metastasis compared to 4T1-12B cells (22). We chose a low dose of doxorubicin that had relatively little effect in blocking breast tumor growth (Fig. 5A, inset). However, the effectiveness of both doxorubicin and ONO-8430506 were enhanced significantly when they were combined. ONO-8430506 on its own decreased the growth of breast tumors, but only for approximately 11 d, as expected (22). However, ONO-8430506 continued to decrease tumor volumes until day 17 when combined with doxorubicin (Fig. 5A). Tumor masses on day 12 reflected the tumor volume (Fig. 5B). Doxorubicin treatments had no significant effect on the body weights of the mice until day 15, and ONO-8430506 was well tolerated during the whole experiment (Fig. 5C).

Reduced GSH concentrations in the tumors were decreased by the combination of doxorubicin with ONO-8430506 (Fig. 6A). This demonstrates that blocking LPA signaling increases doxorubicin-induced oxidative stress, leading to increased apoptosis, as seen by cleaved caspase 3 staining (Fig. 6B). Ki67 levels, which are a marker for cell division, were decreased by both doxorubicin and ONO-8430506, but there was no additive effect.

Spontaneous metastases in the lung and liver were decreased by the combination of ONO-8430506 with doxorubicin (Fig. 7A). We also injected 4T1 cells into the tail vein; this resulted in a large number of macroscopic lung nodules. Combination of doxorubicin and ONO-8430506 significantly decreased lung metastasis (Fig. 7B). This demonstrates that ATX inhibition sensitizes cells in both primary tumor and distal sites to doxorubicin therapy.

Expression of Nrf2 and its targets in breast tumors of human patients

We determined Nrf2 expression in breast tumor samples from patients who had received prior taxane/doxorubicin therapy before surgery. Samples were classified as recurring or non recurring on the basis of their disease-free recurrence at 1 year after the lumpectomy. Protein levels of Nrf2 and NQO1 in tumors were higher among patients with recurrent disease compared to those with non recurring disease (Fig. 8). There was no correlation with ATX expression and tumor reoccurrence. This was expected because breast cancer cells express little ATX activity, and ATX expression in the tumors reflects mainly associated adipose tissue (9, 22).

DISCUSSION

LPA aggravates resistance to a variety of chemotherapeutic agents and radiotherapy by various mechanisms [reviewed in (8)]. The present work demonstrates a novel effect of LPA, which, through activation of LPA1, increases Nrf2 stability. This protects breast cancer cells from doxorubicin-induced apoptosis because Nrf2-induced expression of antioxidant genes diminishes oxidative damage that occurs during chemotherapy. In addition, the MDRTs expel several chemotherapeutic agents and oxidation products from cancer cells (Fig. 9). Increased expressions of the MDRTs ABCB1 (55), ABCC1 (56), and ABCG2 (57), as well as several antioxidant genes, are implicated in resistance to cancer therapies (58). We showed that LPA stabilizes Nrf2 and increases the expression of MDRT and antioxidant genes in several cancer cells by stimulating LPA1 and PI3K signaling in cancer cell lines.
Our results indicate that LPA also increases nuclear Nrf2 expression and activity, as shown by the activation of transcription through the ARE. Although previous work also showed that PI3K is a major regulator of nuclear localization of Nrf2 and its transcriptional activity (47, 51, 59–61), it is less clear how this occurs. Multiple phosphorylation sites have been identified on Nrf2, and these are modulated by casein kinase 1/2 (62), ERK (53), PKC (63), and GSK3 (64). One interesting possibility is that repression of GSK3β downstream of PI3K-Akt signaling is involved in increasing in Nrf2 expression (64–66). High levels of oxidative stress could induce GSK3β activation to repress Nrf2 activity. In fact, inhibition of GSK3β blocks the effects of LY294002 on sensitizing lung cancer cells to chemotherapy (67). This would also explain why effects of t-BHQ and LPA are additive: t-BHQ operates by a Keap1-dependent mechanism, which can rapidly stabilize Nrf2 (68) independently of the effects of GSK3β.

**Figure 4.** ATX inhibitor ONO-8430506 decreased Nrf2 expression and the transcription of antioxidant genes and MDRTs in breast tumors. Mouse 4T1-12B breast cancer cells were injected into the mammary fat pad of female Balb/c mice, which was then gavaged daily with vehicle or 10 mg/kg ONO-8430506 (ONO) starting on the day after the injection until day 11 (22). A) Western blot analyses for Nrf2 and NQO1 relative to tubulin expression. B) mRNA from 6 vehicle-treated and 6 ONO-8430506-treated tumors were analyzed using a limited oxidative stress and cancer drug resistance profiler array. Significant changes were reported as relative means and 95% confidence intervals. NQO1, NAD(P)H dehydrogenase, quinone 1; GPX1, glutathione peroxidase 1; GCLM, glutamate–cysteine ligase, modifier subunit; IL19, interleukin 19; PRDX4, peroxiredoxin 4; SCD1, stearoyl–coenzyme A desaturase 1; SOD1, superoxide dismutase 1, soluble; SOD2, superoxide dismutase 2, mitochondrial; TXNRD1, thioredoxin reductase 1; TXNRD3, thioredoxin reductase 3; ABCB1B, ATP-binding cassette, subfamily B (MDR/TAP), member 1B; ABCC1, ATP-binding cassette, subfamily C (CFTR/MRP), member 1; BCL2L1, Bcl2-like 1.

**Figure 5.** Combination of ATX inhibition with doxorubicin treatment produces a synergistic effect in decreasing breast tumor growth in mice. A) 4T1 cells were injected into the mammary fat pad of female Balb/c mice. Mice were gavaged daily with vehicle or 10 mg/kg ONO-8430506. They were injected i.p. with PBS or doxorubicin (4 mg/kg) every third day starting from day 3 after the injection of 4T1 cells. Tumor volumes are shown until day 21; the inset shows an expanded view up to day 12. B) Weights of excised tumor at day 21. C) Body weights of mice (n = 9 mice per group). Results are means ± SEM where large enough to be shown. *P < 0.05.
There is considerable redundancy in LPA receptor expression in tissues (69). LPA1 is the predominant LPA receptor observed in many tissues (70), including breast cancer cells (38) such as MDA-MB-231 cells. Despite the abundance of LPA1, other LPA receptors could also be detected in many tissues and are often activated the same signaling pathways. The role of LPA in tumor growth, migration, and metastasis has been well described. ATX and LPA1/2/3 receptors can all promote mammary tumorigenesis, where LPA1 tumors are highly invasive (17). LPA-LPA1 signaling increases the metastatic potential of colon carcinoma cells (71). LPA-LPA1 signaling can also promote bone metastasis in breast cancer (20, 21).

**Figure 6.** Combination of ATX inhibition with doxorubicin decreases reduced GSH concentrations and cell division, and increases apoptosis in the breast tumors. Tumors obtained at day 12 from the mice described in Fig. 5 were analyzed for (A) reduced GSH concentration and (B) cells that stained positive for cleaved caspase 3 (antibody dilution 1:75) and Ki67 (1:400). Scale bar, 25 μm. *P < 0.05.

**Figure 7.** ATX inhibition combined with doxorubicin decreased spontaneous and experimental metastasis in mice. A) Quantification of lung and liver spontaneous metastases from hematoxylin and eosin–stained sections at day 21 from the experiments described in Fig. 5. Scale bar, 25 μm. B) 4T1 cells were injected into the tail vein, and mice were treated with ONO-8430506 and doxorubicin as in Fig. 5. Lungs were excised on day 14. Representative images of a lung from each group are shown after staining in Bouin solution. The left lung of 8 mouse lungs from each group was counted for macroscopic nodules. Results are means ± SEM. *P < 0.05.
Our use of the ATX inhibitor ONO-8430506 in vivo decreases LPA production, especially unsaturated LPA concentrations, in plasma and breast tumors of mice over 24 h and can decrease LPA signaling, irrespective of tissue LPA-receptor expression. Blocking LPA signaling by this strategy decreased the expressions of Nrf2, antioxidant genes, and MDRTs in breast tumors. These changes increased the efficacy of doxorubicin in decreasing tumor growth and metastasis. Part of the effect on metastasis can be explained by the decreased growth of the primary tumor, but we also observed a direct decrease in metastasis to lungs and liver after injecting the 4T1 cells into the tail vein. We choose a low and well-tolerated dose of doxorubicin, which on its own produced no significant effect on tumor growth. However, this doxorubicin treatment became effective in inhibiting tumor growth and metastasis by about 70% at day 12 when combined with ATX inhibition. The effects of ATX inhibition alone on tumor growth only last about 11 d in this aggressive model of stage IV breast cancer. However, ATX continued to inhibit tumor growth to day 17 when it was administered with doxorubicin. In fact, the combination therapy was synergistic in increasing the efficacy for both therapeutic agents.

An advantage of using ONO-8430506 over LPA receptor antagonists is that these receptors show considerable redundancy in vivo. ONO-8430506 blocks LPA production and thus signaling by each receptor that can activation PI3K and thereby stabilize Nrf2. ATX secretion and activation can be fueled by several factors among which inflammation is a key player. Inflammation in the tumor environment promotes survival of cancer cells and resistance to therapies. The interplay between oxidative stress and inflammation may play a key role in tumor survival, proliferation, and chemoresistance. We hypothesized that ATX inhibition blocks the vicious cycle of inflammation and cytokine production caused by the growth of breast tumors, which causes more cytokine production, leading to more ATX secretion from the surrounding adipose tissue and the tumor stroma. Chemotherapy, and the tissue damage that it produces, also fuels an inflammatory response, which promotes ATX secretion and increased LPA signaling. This process contributes to the progressive development of chemoresistance, which is mediated by LPA as part of a (patho-)physiologic response designed to repair the injured area. The present studies demonstrate that blocking the protective mechanisms associated with LPA signaling improves the efficacy of doxorubicin by increasing oxidative damage and decreasing the growth of breast tumors. The 4T1 model of breast cancer involves an intact immune system, which is essential for the inflammatory actions mediated ATX/LPA signaling. LPA produces chemoresistance by activating several survival pathways. It is well recognized that increased expressions of Nrf2, antioxidant genes, and MDRT are related to chemoresistance. Our results show that the expression of Nrf2 and NQO1 are higher in breast tumors among patients with recurrent disease compared to those with nonrecurring cancer. Also, recent work shows that

**Figure 8.** Expression Nrf2 and NQO1 is increased in tumors from patients with recurrent breast cancer. Banked primary breast tumors from patients were immunoblotted for Nrf2 and NQO1 expression, which was expressed relative to actin. Patients were classified as having recurring (n = 13) or nonrecurring (n = 5) disease at 1 year on the basis of their disease-free status after lumpectomy and subsequent therapy. Results are means ± SEM. *P < 0.05.

**Figure 9.** Proposed mechanism for LPA signaling and therapy resistance by Nrf2 activation.
Nrf2 and NQO1 are increased in BRCA1 mutations, and this is associated with poor outcome in breast cancer patients (74). In fact, oncogenes can increase basal Nrf2 levels (46), which demonstrates that cancer cells are the likely source of increased Nrf2 seen in mixed population cell types from tumors.

The importance of our present work is that it links for the first time LPA signaling to this increased Nrf2 expression and the transcription of antioxidant genes and MDRTs. Our present study also provides a novel and practical solution for decreasing the ability of cancer cells to protect themselves against the effects of chemotherapeutic agents and the oxidative damage that they cause. The ATX inhibitor used in our work is well tolerated by mice and could potentially be readily translated into a clinical setting. We predict that inhibiting the ATX-LPA-Nrf2 axis is a viable strategy for improving the efficacy of existing chemotherapies.

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A and B) Western blots for the expression of ABCC1 and ABCG2 in a panel of breast cancer cell lines. MCF-7 clonal cell line that was selected for resistance to doxorubicin or docetaxel was used as a positive control\(^1\).

C) MDA-MB-231 cells were treated with 50 nM siCTRL or different siRNA constructs targeting LPA\(_1\). After 48 h, mRNA was collected for RT-PCR analysis of receptor expression for LPA\(_{1,2,3}\).

A) Hs578T breast ductal carcinoma cells express higher Nrf2 compared to Hs578Bst cell line (patient-matched peripheral tissue). n=4. Results were expressed relative to tubulin expression. 4T1 cells (B & C), A549 and 8305C cells (D) and MDA MB 231 cells (E) were treated as in Figure 3. F) HepG2 or MDA-MB-231 cells were treated with t-BHQ or the proteosomal inhibitor, MG132 (25 µM), for 4 h prior to collecting the cell lysates. They were immunoblotted for Nrf2, which detects N-terminus. G) A panel of sub-confluent breast cancer cell lines was grown in full growth media and cell lysates were immunoblotted for Nrf2, which detects the C-terminus. H) AREc32 cells grown in 6-well plates were transfected with 2 µg of EGFP plasmid (lanes 1-3) or EGFP-Nrf2 plasmid (lane 4) and incubated for 18 h. They were starved for 12 h followed by treatments with 10 µM t-BHQ or 10 µM MG132 for 4 h. Cell lysates were immunoblotted for Nrf2 expression. The band-shifted EGFP-Nrf2 is shown.
A) HEK 293T cells were transfected with 0.5 µg of EGFP-Nrf2 and HA-tagged LPA₁ or empty vector plasmids/well. They were incubated for another 16 h before starving them for 12 h. Treatments were performed as described for another 12 h. Samples were fixed and then immunostained as described in Materials and Methods. Nuclear GFP fluorescence was determined by ImageJ analysis.

B) HepG2 cells were transfected with HA-tagged LPA₁/₂/₃. After 24 h the cells were collected and immunoblotted for LPA receptor expression (left panel). They were starved for 12 h before treating with LPA for 4 h. Relative Nrf2 to GAPDH was expressed from 3 different experiments (right panel).