Tetracyclines increase lipid phosphate phosphatase expression on plasma membranes and turnover of plasma lysophosphatidate

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Abstract  Extracellular lysophosphatidate and sphingosine 1-phosphate (S1P) are important bioactive lipids, which signal through G-protein-coupled receptors to stimulate cell growth and survival. The lysophosphatidate and S1P signals are terminated partly by degradation through three broad-specificity lipid phosphate phosphatases (LPPs) on the cell surface. Significantly, the expression of LPP1 and LPP3 is decreased in many cancers, and this increases the impact of lysophosphatidate and S1P signaling. However, relatively little is known about the physiological or pharmacological regulation of the expression of the different LPPs. We now show that treating several malignant and nonmalignant cell lines with 1 μg/ml tetracycline, doxycycline, or minocycline significantly increased the expression of the three LPPs and increased expression on the plasma membrane. We tested the physiological significance of these results and showed that treating rats with doxycycline accelerated the clearance of lysophosphatidate, but not S1P, from the circulation. However, administering 100 mg/kg/day doxycycline to mice decreased plasma concentrations of lysophosphatidate and S1P. This study demonstrates a completely new property of tetracyclines in increasing LPP activity and LPA signaling is increased in response to the plasma membrane. We used the physiological significance of these results and showed that treating rats with doxycycline accelerated the clearance of lysophosphatidate, but not S1P, from the circulation. However, administering 100 mg/kg/day doxycycline to mice decreased plasma concentrations of lysophosphatidate and S1P. This study demonstrates a completely new property of tetracyclines in increasing LPP activity and LPA signaling is increased in response to the plasma membrane.

Supplementary key words  autotaxin • matrix metalloproteinase • doxycycline • sphingosine 1-phosphate

Lysophosphatidate (LPA) has potent growth and migratory effects on cells through its activation of six G-protein-coupled receptors (1–3). Extracellular LPA is formed mainly through the hydrolysis of lysophosphatidylcholine by autotaxin (ATX), a secreted enzyme with lysophosphatidic acid D activity (4). ATX activity and LPA signaling are essential for vasculogenesis and the formation of the neural crest in embryos (5, 6). A major function of increasing LPA availability in adult animals is in wound healing (7). ATX activity and LPA signaling is increased in response to inflammation, which normally results in tissue repair and resolution of the inflammation (4, 8). If the inflammation is not resolved, then LPA signaling remains increased and contributes to adverse pathologies seen in asthma, arthritis, fibrosis, Crohn’s disease and ulcerative colitis, atherosclerosis, hepatitis, and multiple sclerosis (4, 9). LPA signaling is also increased in many cancers (10–13), which have been described as “wounds that do not heal” (14). LPA provides part of the signal that leads to increased tumor growth, angiogenesis, metastasis, and the development of resistance to chemotherapy (1, 15). LPA signaling also produces a vicious inflammatory cycle because it increases the production of multiple inflammatory cytokines by cancer cells and in surrounding adipose tissue in the case of breast cancer (16). These cytokines further increase ATX expression. Consequently, considerable effort has been directed to decreasing LPA signaling by various strategies including blocking LPA formation (4, 9, 17) using antibodies against LPA (18) or using LPA receptor antagonists (19).

Another component in controlling the accumulation of extracellular LPA is its dephosphorylation by a family of three enzymes called lipid phosphate phosphatases (LPPs) (1, 20, 21). The LPPs are integral membrane enzymes whose catalytic domains face the extracellular space or the luminal space when the LPPs are located on the plasma membrane.

Abbreviations: ATX, autotaxin; HA, human influenza hemagglutinin (amino acids 98-106, HA-tag); LPA, lysophosphatidic acid or lysophosphatidate (salt form at physiological pH); LPAR1, LPA receptor type 1; LPP, lipid phosphate phosphatase; MMP, matrix metalloproteinase; S1P, sphingosine 1-phosphate.

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membranes or internal membranes, respectively (22). LPPs
dephosphorylate a wide variety of lipid phosphates and
pyrophosphates (20, 23). One of their major functions is
to dephosphorylate extracellular LPA and possibly sphing-
osine 1-phosphate (S1P). Significantly, the activities of
LPP1 and LPP3 are decreased in many cancers (2, 20),
which together with increased ATX activity (4), exposes
tumors to higher extracellular LPA concentrations.

Therefore, an alternative way to reduce LPA signaling
in various inflammatory conditions including cancers is by
increasing the low expressions of LPP1 and LPP3, which
are often observed in different malignancies (20, 24–26).
So far, it has been established that gonadotropin-releasing
hormone increases LPP activity and LPP3 expression in
ovarian cancer cells (27). Overexpression of LPP1 in ova-
rian cancer cell lines increases LPA hydrolysis, which is
associated with a marked inhibition of cell proliferation and
colony-forming activity and increased apoptosis (28). In
SKOV3 and OVCAR-3 ovarian cancer cells, LPP3 over-
expression promotes extracellular LPA hydrolysis, decreases
LPA-stimulated colony formation, and decreases tumor
growth in mice (29). Similarly, increasing the low expres-
sion of LPP1 expression in breast and thyroid cancer cells
increases the dephosphorylation of extracellular LPA and
also attenuates signaling downstream of LPA and other
G-protein-coupled receptors (23). These effects resulted
in a dramatic suppression in breast and thyroid tumor
growth in mouse models (23).

However, designing therapies that will increase the ac-
tivity of an enzyme represents much more of a challenge
compared with creating an enzyme inhibitor. During our
previous work, we used a doxycycline-inducible promoter
to express LPP1 in the cancer cells (23). We observed that
treating cancer cells with increasing doxycycline concen-
trations enhanced the degradation of exogenous LPA,
even in those cells that did not express recombinant LPP1.
We, therefore, initiated the present work to elucidate this
new effect of doxycycline.

Tetracyclines were discovered in 1940s, and they have
developed into a family of broad-spectrum antibiotics with
activities against bacteria, chlamydiae, mycoplasmas, ricket-
siae, and protozoan parasites (30). The bacteriostatic ac-
itivity is caused by preventing the binding of aminoacyl tRNA
to ribosomes, which is necessary for protein synthesis (31).
It is also well documented that tetracyclines inhibit matrix
metalloproteinase (MMP) activities (32). In the present
study, we demonstrate for the first time that doxycycline in-
creases the degradation of extracellular LPA in multiple cell
lines through enhancing the activities of LPP1, LPP2, and
LPP3 and the expression of these enzymes on the plasma
membrane. The physiological relevance of these findings
was demonstrated because doxycycline also increased the
rate of LPA clearance from the circulation of rats, and it
decreased the steady-state concentrations of LPA and S1P
in the plasma of mice. The present work, therefore, establishes
a novel effect of tetracyclines in decreasing the circulating
levels of LPA through increasing the degradation of extrac-
cellular LPA. This property could decrease excessive signal-
ing by LPA through its receptors.

MATERIALS AND METHODS

Reagents

Oleoyl-LPA (233019) was from Avanti Polar Lipids (Alabaster,
AL). Minocycline hydrochloride (475843), marimastat (444289),
mouse anti myc-tag (05-724) antibody, mouse anti HA tag (human
influenzasaegottinigen amino acids 98-106) antibody (05-904),
rabbit anti-integrin α3 (AB1929), and β1 (04-1109) antibodies were
from EMD Millipore (Etobicoke, Ontario, Canada). Doxycycline
hydrochlorate (0219895525) was from MP Biomedicals (Solon, OH).
Rabbit anti EGFR (sc-03) and rabbit anti E-cadherin (H-108) anti-
obodies were from Santa Cruz (Dallas, TX). Tetracycline hydro-
chloride (T7660), fatty acid-free albumin from bovine serum
(A8806), mouse anti-tubulin (T6074) antibody, and protease
inhibitor cocktail (P8340) were from Sigma (St. Louis, MO). The
transfection reagent PolyJet (SL100688) was from SigmaGen Lab-
oratories (Gaithersburg, MD). Sulfo NHS-SS-biotin (PSG0277),
streptavidin agarose (20349), G418 sulfate (11811-031), pENTR/
D-TOPO Cloning Kit (K2400-20), and LR clonase enzyme mix
(11791-019) were from Life Technologies (Grand Island, NY).
Puultra DNA polymerase (600385) was from Agilent Technol-
gies (Santa Clara, CA). Anti-GFP antibody was kindly provided
by Dr. L. Berthiaume (Department of Cell Biology, University
of Alberta, Edmonton, Alberta, Canada), and [γ-32P]ATP
(Blue002250UC) was from PerkinElmer (Woodbridge, Ontario,
Canada). Human recombinant sphingosine kinase-1 (10348)
was from Cayman Chemical (Burlington, Ontario, Canada).

Cell culture and lentivirus generation

Human mammary carcinoma cell lines MDA-MB-231 and MCF-7,
mouse mammary carcinoma cell line 4T1, human mammary epide-
lial cell line MCF10A, and human embryonic kidney 293 (HEK293)
and HEK293T cell lines were from ATCC (Manassas, VA). MCF10A
cells were cultured in DMEM/F12 medium with 10% FBS,
20 ng/ml epidermal growth factor (EGF), 0.5 μg/ml hydrocorti-
sone, 100 ng/ml cholera toxin, and 10 μg/ml insulin. Other cell
lines were maintained in DMEM supplemented with 10% FBS,
C-terminal myc-tagged human LPP1, LPP2, and LPP3 sequences
were first cloned into pENTR/D-TOPO vector and then recombined
into the pENTR/D-TOPO vector and then the lentiviral destination vector
pLenti-PGK-Neo-DEST (Addgene19067) carrying a neomycin
selection marker through LR recombination using Gateway®
Technology. Lentivirus was generated as described previously
(20) by cotransfecting the lentiviral vector and packaging vectors
into HEK293T cells. C-terminal green fluorescent protein (GFP)-
tagged human LPP1, LPP2, and LPP3 sequences were then expressed
by using pEZ3 plasmid. Human LPP1, LPP2, and LPP3 se-
quences were then cloned into the pEY3 vector (Addgene18672) through LR reaction.

Preparation of 32P-labeled LPA and S1P and measurement of LPP activity

[32P]SIP was prepared by incubating 0.4 μmol sphingosine with
0.01 U recombinant sphingosine kinase-1 and 400 μCi [γ-32P]ATP
for 12 h at 37°C in 200 μl of reaction buffer, which contained
20 mM Tris-HCl, pH 7.4; 20% glycerol; 1 mM 2-mercaptoethanol;
1 mM EDTA; 1 mM sodium orthovanadate; 10 mM MgCl2;
and 1 mM ATP. [32P]SIP was extracted into butan-1-ol. [32P]LPA
and was prepared as described previously (33, 34). [32P]LPA
and [32P]SIP were then purified by thin-layer chromatography
as described previously (33, 34), and they were then diluted
with nonradioactive substrate to a specific radioactivity of ~1 ×
was determined as described previously (20).
SIP and total LPP activity against [\(^{3}H\)]phosphatidate (PA) were measured as described by Jasinska et al. (33).

Real-time PCR and Western blotting

LPP1, LPP2, and LPP3 mRNA levels were determined by quantitative RT-PCR using GAPDH as a reference mRNA. Protein levels were measured by Western blotting as described previously (23). Immunoblots were analyzed by Odyssey infrared imaging system (LI-COR Biosciences).

Expression of LPPs on the plasma membrane

The expression of plasma membrane proteins was determined by tagging the exposed proteins with a noncell permeable biotinylated reagent (35). Briefly, cells were cultured in 10 cm dishes and pretreated with or without doxycycline (0.5–2 \(\mu\)g/ml) for 24 h. Cells were placed on ice for 20 min and washed with ice-cold PBS. Cells were incubated with 0.4 mg/ml Sulfo-NHS-SS-biotin in PBS for 30 min on ice and then washed three times with quenching buffer (50 mM glycine in PBS, pH 7.4) and incubated on ice in quenching buffer for 15 min. Cells were washed with PBS and lysed in RIPA buffer. After adjusting the protein concentrations of cell lysates, biotinylated proteins were precipitated using 30 \(\mu\)l streptavidin agarose. The LPPs were resolved by SDS-PAGE and revealed by immunoblotting.

LPA and SIP clearance in the circulation and whole blood of rats

Sprague Dawley rats (300 ± 20 g) were housed with free access to water and food (7001 Teklad 4% fat diet), and they were then pretreated with 50 mg/kg/day doxycycline through ip injection for 3 days. For measuring LPA clearance, rats were anesthetized with ip injection of 50 mg/kg sodium pentobarbital. A straight incision was made at the middle of the abdomen, and the peritoneal cavity was opened. The portal vein and hepatic artery were ligated using 2.0 silk sutures, and the peritoneal cavity was closed. A straight incision was then made at the middle of neck. The right carotid artery was separated by blunt dissection, and catheterization was performed with a 22 G catheter. \(^{[32P]}\)LPA (around 500,000 dpm) was added in 0.5 ml saline containing 0.5 \(\mu\)M nonradioactive LPA, 500 \(\mu\)M Evans blue, and 0.1% BSA and injected into the circulation through the catheter. About 300 \(\mu\)l of blood was collected into an EDTA-coated insulin syringe through the catheter at 30, 60, 90, 120, 180, 240, and 300 s after the injection. Blood samples were centrifuged at 14,000 rpm for 1 min, and 100 \(\mu\)l plasma was collected. HCl (1 M, 50 \(\mu\)l) was added into the plasma and mixed well. Plasma LPA was extracted twice with 100 \(\mu\)l butan-1-ol before measuring the radioactivity. Concentrations of Evans blue were calculated with a standard curve plotted at A620. The value was used to estimate the volume of circulation and the amount of \(^{[32P]}\)LPA in the circulation. 

Clearance of \(^{[32P]}\)SIP in rat circulation was measured as for \(^{[32P]}\)LPA, with the exception that animals were not subject to portal vein and hepatic artery ligation. Blood was collected at 1, 2, 3, 5, and 10 min after injection of \(^{[32P]}\)SIP.

Measurement of plasma LPA and SIP concentrations

Female BALB/c mice (22 ± 2 g) were housed with free access to water and food (7001 Teklad 4% fat diet) and pretreated with 100 mg/kg/day doxycycline for 3 days through ip injection. Mice were euthanized, and blood was collected through the heart using an EDTA-coated syringe. Plasma LPA and SIP concentrations were measured as described previously (17). Blood samples were centrifuged at 14,000 rpm for 1 min, and plasma was collected. Plasma was treated with internal standards (isotope labeled C17:0-LPA and \(^{[13}C_2\)D\(_2\)SIP), and lipid phosphates were extracted into butan-1-ol. Lysophospholipids were measured by LC/MS using electrospray ionization in the negative mode using an Agilent 1200 series LC system coupled to a 3200 QTRAP mass spectrometer (AB Sciex, Concord, Ontario, Canada). The concentrations of different LPA species (C16:0-LPA, C20:4-LPA, C18:2-LPA, C18:0-LPA, and C18:1-LPA) were summed as total LPA concentration in plasma.

Statistical analysis

Results were analyzed by Student’s t-test or by ANOVA for multiple comparisons followed by Student-Newman-Keuls test. \(P < 0.05\) was considered statistically significant.

RESULTS

Tetracyclines increased LPP activity in multiple cell lines

During our previous work (23), we used a doxycycline-inducible promoter to express LPP1 in lentivirus-transduced cells. We observed that doxycycline treatment for 24 h increased the dephosphorylation of exogenous \(^{[32P]}\)LPA (ecto-activity) in HEK293 cells by ~2-fold (Fig. 1A). Because LPP1 and LPP3 activities are decreased in many types of cancer cells, we next determined if doxycycline increased LPP activity in breast cancer cell lines. Treatment with 1 to 50 \(\mu\)g/ml (2 to 100 \(\mu\)M) doxycycline for 24 h increased dephosphorylation of extracellular LPA in human MDA-MB-231 and MCF-7 and mouse 4T1 breast cancer cell lines (Figs. 1B–D). In nontransformed human mammary epithelial MCF10A cell line, a 48 h treatment with doxycycline was required to increase LPP activity (Fig. 1E). We then treated MDA-MB-231 and 4T1 cells with 1 \(\mu\)g/ml tetracycline (2.1 \(\mu\)M) or 1 \(\mu\)g/ml minocycline (2.0 \(\mu\)M) and found that ecto-LPP activity was also increased by these two tetracyclines (supplementary Fig. 1A, B). These combined results demonstrate that tetracyclines increased ecto-LPP activity in multiple cell types. To determine whether the effect of doxycycline resulted from the direct activation of LPPs, we measured the dephosphorylation of exogenous LPA at different times after incubation with 1 \(\mu\)g/ml (2 \(\mu\)M) doxycycline. LPP activity did not increase until after ~20 h of doxycycline treatment (Fig. 1F). These results establish that doxycycline exerts a long-term effect rather than a direct activation of LPP activity.

Because tetracyclines inhibit MMP activity, we determined whether MMP activity controls LPP activity and thus the degradation of LPA. Treatment with 5 \(\mu\)M marimastat, a pan MMP inhibitor, for 24 h did not change ecto-LPP activity in MDA-MB-231 cells (Fig. 1F). The IC\(_{50}\) of
Tetracyclines at 10 μM MMP-2 compared with marimastat: 160 μM MMP isoforms. Tetracycline had a much higher IC₅₀ for marimastat for MMPs are at nanomolar levels (36), and treated cells are shown as *P < 0.05 and **P < 0.01.

Marimastat for MMPs are at nanomolar levels (36), and 5 μM marimastat is more than sufficient to inhibit different MMP isoforms. Tetracycline had a much higher IC₅₀ for MMP-2 compared with marimastat: 160 μM versus 2.3 nM (supplementary Fig. 2A, B). Tetracyclines at 10 μg/ml (21 μM) did not change the amounts of secretion of MMP-2 and MMP-9 (supplementary Fig. 2C). These results indicated that the tetracycline-induced changes in LPP activity do not result from MMP inhibition.

Doxycycline also increased the total LPP activity in cell lysates. In normal MDA-MB-231 cells, treatment with 5 μg/ml (10 μM) doxycycline for 48 h caused a small (~16%) but consistent increase in total LPP activity (Fig. 2A). The same effect of doxycycline was also observed in MDA-MB-231 cells, which stably express myc-LPP1, myc-LPP2, and myc-LPP3 (Fig. 2B–D).

Doxycycline increased the protein levels of LPP1, LPP2, and LPP3 and expression on the plasma membrane

Because existing antibodies are not very effective in detecting endogenous levels of LPPs in cell lysates, we established three stable MDA-MB-231 cell lines expressing myc-tagged human LPP1, LPP2, and LPP3 by using lentivirus. Treatment with 1 μg/ml doxycycline for 24 h increased the expressions of myc-tagged LPP1, LPP2, and LPP3 (Fig. 3A, B). However, treatment with 1–50 μg/ml doxycycline for 24 h did not significantly change mRNA levels of LPPs in both normal (Fig. 3C) and virus-transduced MDA-MB-231 cells expressing myc-LPP1, LPP2, and LPP3 (supplementary Fig. 3). There was no significant change in mRNA levels of LPPs after 3–12 h of treatment with 1 μg/ml doxycycline (Fig. 3D). Therefore, doxycycline appears to increase the protein levels of LPPs through a post-transcriptional mechanism.

LPPs that degrade extracellular LPA are located on plasma membrane, and so we determined whether the amount of LPPs on the cell surface was changed by doxycycline. Plasma membrane proteins were labeled by using a noncell permeable biotinylation reagent and concentrated with streptavidin beads. Doxycycline treatment for 24 h significantly increased the amounts of myc-LPPs on plasma membrane (Fig. 4A, B). To determine whether these effects of doxycycline were specific to LPPs, we measured the levels of other plasma membrane proteins including integrin-α3, integrin-β1, EGF receptor, and E-cadherin. No significant changes in the expressions of these proteins were found after doxycycline treatment (supplementary Fig. 4A–C).

Doxycycline enhanced LPP1, LPP2, and LPP3 stability

Because doxycycline needs at least 20 h to increase LPP activity in cells and it did not affect mRNA levels for the LPPs, it seemed likely that doxycycline suppressed LPP degradation. To test this, we treated MDA-MB-231 cells that stably expressed myc-tagged LPP1, LPP2, and LPP3 with 50 μg/ml cycloheximide to inhibit protein synthesis. LPP1 and LPP2 levels decreased significantly after 10 h of cycloheximide treatment by ~35% and 45%, respectively. Degradation of LPP3 was faster than LPP1 and LPP2. LPP3 levels were decreased by ~45% after treatment for 6 h with cycloheximide. Adding doxycycline together with cycloheximide significantly slowed the degradation of the three LPPs (Fig. 5A–D). Doxycycline also increased the stability of GFP-LPP2 upon cycloheximide treatment in HEK293 cells, which transiently expressed GFP-LPP2 or HA-LPA receptor type 1 (LPAR1). However, doxycycline did not increase the stability of HA-tagged LPAR1 (supplementary Fig. 5A–D).

Protein levels of LPP1, LPP2, and LPP3 were also elevated by treatment with the lysosome inhibitor NH₄Cl (50 mM), but not the proteasome inhibitor MG132 (10 μM) (supplementary Fig. 6A), indicating that LPPs are degraded...
Doxycycline increases lipid phosphate phosphatase activity in lysosomes. We then determine whether doxycycline decreased the degradation of the LPPs by increasing lysosomal pH using a ratiometric probe, LysoSensor™ Yellow/Blue DND-160 (Life Technologies). Doxycycline at concentrations of 2 µM did not change lysosomal pH, and this contrasted with the effects of 50 mM NH₄Cl or 20 µM chloroquine, which increased lysosomal pH from about 4.2 to 10.8 and 6.8, respectively (supplementary Fig. 6B).

![Fig. 2.](http://www.jlr.org/content/suppl/2016/02/16/jlr.M065086.DC1.html)

**Fig. 2.** Doxycycline increased total LPP activity against [³H]PA. Treatment with doxycycline at 5 µg/ml (10 µM) for 48 h increased total LPP-activity in normal MDA-MB-231 cells (A), MDA-MB-231 cells expressing myc-LPP1 (B), MDA-MB-231 cells expressing myc-LPP2 (C), and MDA-MB-231 cells expressing myc-LPP3 (D). Results are means ± SD from four independent experiments with normal MDA-MB-231 cells or three MDA-MB-231 cells expressing myc-tagged LPPs. Significant differences between drug-treated and nontreated cells are shown as * \( P < 0.05 \).

![Fig. 3.](http://www.jlr.org/content/suppl/2016/02/16/jlr.M065086.DC1.html)

**Fig. 3.** Doxycycline increases the expression of LPPs. A: Protein expressions of myc-tagged human LPP1, LPP2, and LPP3 in MDA-MB-231 cells with or without doxycycline (Dox) treatment at 1, 5, and 10 µg/ml (2, 10, and 20 µM) for 24 h. B: Quantification of expression levels of myc-LPP1, LPP2, and LPP3 in A. C: mRNA levels of LPP1, LPP2, and LPP3 relative to GAPDH mRNA in MDA-MB-231 cells after 24 h treatment with 1 µg/ml (2 µM) doxycycline treatment for different times. Results are means ± SD from three independent experiments. Significant differences between drug-treated and nontreated cells are shown as * \( P < 0.05 \).
clearance from the circulation. The clearance of \([^{32}P]LPA\) frequently, we chose this strategy for measuring the effects of and from 75% to 85% at 60 s after injection of \([^{32}P]LPA\), from the plasma was increased from 61% to 79% at 30 s increased LPA dephosphorylation in the blood relative to because LPPs are expressed on blood cells. Doxycycline (Fig. 6A).

In the present study, we report for the first time doxycycline increases the degradation of exogenous LPA by increasing the expressions of LPP1, LPP2, and LPP3 on the surface of cells. This increases the “ecto-activity” of the LPPs in degrading extracellular LPA. The mechanism for this effect involved the action of doxycycline in increasing the protein levels of LPP1, LPP2, and LPP3 by delaying their degradations. These results do not depend on the use of an myc-tag to identify the LPPs because doxycycline also increased the stability of GFP-tagged LPP2. The effect was specific because there was no effect of doxycycline on other plasma membrane proteins including integrin-\(\alpha3\), integrin-\(\beta1\), EGF receptor, E-cadherin, and also HA-tagged LPAR1.

LPPs are plasma membrane proteins, which are likely to be degraded in lysosomes when they are internalized. This is compatible with the lack of effect of the proteasome
Doxycycline increases lipid phosphate phosphatase activity

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MG132, on LPP levels and their increased expression in the presence of NH₄Cl, which increases lysosomal pH to inhibit lysosomal proteases. However, doxycycline did not increase lysosomal pH. We were also not able to detect an effect of doxycycline on the cycling of the LPPs to and from the plasma membrane. The effects of doxycycline on LPP stability did not depend on the inhibition of MMP activity. The IC₅₀ of tetracycline was ~160 μM for inhibiting MMP2 activity, which is much higher than that of marimastat, ~2.3 nM. For other MMP isoforms, the IC₅₀ of marimastat are within nanomolar range (36). We showed that marimastat as high as 5 μM did not affect LPP activities.

As a consequence of the doxycycline-induced increase in localization of the LPPs on the plasma membrane, there is an increase in the dephosphorylation of extracellular LPA in multiple cell lines including cancer cells and in whole blood. Significantly, all three LPPs were effective in dephosphorylating extracellular LPA. We established the physiological significance of these findings in vivo by showing that doxycycline increased the clearance of LPA from the circulation in rats and decreased the steady-state concentrations of LPA in the plasma of mice.

The kinetics of the turnover of S1P in the circulation differed from that in LPA. S1P was not cleared rapidly by the liver. Doxycycline treatment did not increase S1P dephosphorylation significantly in the circulation or in whole blood. This is probably explained because dephosphorylation of extracellular S1P depends on the expression of LPP3, with LPP1 and LPP2 having little effect. These results indicate that although the LPPs show relatively little specificity for different lipid phosphates when these are in detergent micelles (40), there does appear to be selectivity for the ecto-activities in intact cells. Our conclusion that LPP3 acts preferentially on extracellular S1P is compatible with studies on the dephosphorylation of FTY720-P, which is an analog of S1P. Lysates from cells that overexpressed LPP1, LPP2, and LPP3 showed that only LPP3 dephosphorylated FTY720-P (41). LPP3 also acted as an ecto-phosphatase in intact cells in controlling the equilibrium between FTY720 and FTY720-P that was observed in vivo (41).

Fig. 5. Doxycycline decreases the degradation of LPPs. A: Protein levels of myc-LPP1, myc-LPP2, and myc-LPP3 in stable MDA-MB-231 cells treated with 50 μg/ml cycloheximide (Chx) for 6, 8, 10, and 24 h in the presence or absence of 1 μg/ml doxycycline (Dox). The first two lanes on the left side are cells without treatment (NT) and cells treated with 1 μg/ml doxycycline for 24 h. B–D: Quantification of protein levels of myc-LPP1 (B), myc-LPP2 (C), and myc-LPP3 (D) in A. Results are means ± SD from three independent experiments. Significant differences between cells with or without doxycycline treatment are shown as * P < 0.05.
Doxycycline did decrease the steady-state level of circulating S1P in treated mice, but this reflects a balance of S1P production and removal. The effect could have resulted from increased LPP5 expression on plasma membranes throughout the body. It could also have been caused secondary to the decrease in LPA concentrations because LPA can increase S1P release from cells (42). However, we think that this is unlikely because decreasing plasma LPA concentrations with an ATX inhibitor did not lower the concentration of circulating S1P (13). A further possibility is that tetracyclines exert an anti-inflammatory effect (43, 44), which might indirectly decrease plasma S1P concentrations (45). Consequently, doxycycline can decrease the extracellular concentrations of two potent lysosphospholipid mediators, LPA and S1P, in part by increasing the combined ecto-activities of LPP1, LPP2, and LPP3. This situation contrasts with our previous study in which we increased LPP1 expression only in the breast cancer cells that were injected into the mice. In this case, there were no significant changes in plasma LPA or S1P concentrations in mice with or without 100 mg/kg/day doxycycline treatment for 3 days, n = 6. Results are means ± SD. Significant differences between animals with or without doxycycline treatment are shown as * P < 0.05 and ** P < 0.01.

![Supplemental Material can be found at: http://www.jlr.org/content/suppl/2016/02/16/jlr.M065086.DC1.html](http://www.jlr.org/content/suppl/2016/02/16/jlr.M065086.DC1.html)

Fig. 6. Doxycycline increases the turnover of LPA in blood but not S1P. A: Effect of doxycycline on the turnover of $[^{32}P]$LPA in circulation of rats. Rats with or without pretreatment of 50 mg/kg/day doxycycline (Dox) for 3 days were subjected to portal vein and hepatic artery ligation before experiment. Results from rats without ligation are also shown, n = 6. B: Effect of doxycycline on $[^{32}P]$LPA degradation in whole blood. Blood was collected from rats with or without pretreatment with 50 mg/kg/day doxycycline (Dox) for 3 days, and incubated with $[^{32}P]$LPA for the indicated time, n = 5. C: Removal of $[^{32}P]$S1P from the circulation of rats with or without portal vein and hepatic artery ligation. D: Removal of $[^{32}P]$S1P from circulation of rats with or without 50 mg/kg/day doxycycline treatment. E: Degradation of $[^{32}P]$S1P in whole blood from rats with or without 50 mg/kg/day doxycycline treatment. F: Effect of doxycycline on plasma S1P and LPA concentrations in mice with or without portal vein and hepatic artery ligation for 3 days, n = 6. Results are means ± SD. Significant differences between animals with or without doxycycline treatment are shown as * P < 0.05 and ** P < 0.01.
increased the dephosphorylation of LPA and SIP in intact cells. Therefore, this additional side effect of tetracyclines should be considered when using the Tet-On/Off system. LPA and SIP are very potent signaling molecules, and their signaling effects contribute to the progression of several inflammatory conditions such as asthma, arthritis, fibrosis, Crohn’s disease, ulcerative colitis, atherosclerosis, hepatitis, multiple sclerosis, and cancers. Increasing LPA degradation and decreasing its signaling actions have positive effects as cancer treatments. One strategy for achieving this is to increase the low expression of LPP1 and LPP3 that doxycycline has the capacity to increase the degradation and decreasing its signaling actions have positive effects as cancer treatments. One strategy for achieving this is to increase the low expression of LPP1 and LPP3.

REFERENCES


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