Activity based proteomic profiling of lysophosphatidic acid treated cancer cells

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Ovarian cancer is a debilitating disease lacking effective treatments. A key feature of the disease is elevated levels of the mitogenic lipid lysophosphatidic acid (LPA) found in the ascites fluid surrounding tumors. LPA evokes a wide array of pro-tumorgenic effects in cells and was recently shown to stimulate the expression of a cancer-associated protease, urokinase type plasminogen activator (uPA). To discern whether LPA treatment resulted in active uPA, I applied a novel proteomic technique, activity-based protein profiling (ABPP), that specifically monitors the amount of protein activity rather than abundance. I utilized ABPP to examine the effect of the bioactive lipid LPA on uPA in a human ovarian cancer cell line SKOV-3. To achieve this I first developed a new strategy for analysis of secreted proteins and then determined that treatment of SKOV-3 cells with LPA does indeed result in increases of active uPA. In addition to this finding, I also detected elevated uPA activity upon treatment of structurally distinct forms of LPA that vary in acyl chain length. This finding has not previously been reported and demonstrates the power of ABPP to identify changes in the functional state of low abundance enzyme activities.

Introduction

Ovarian cancer is a debilitating disease for which there are not effective treatments. Unlike other cancers, which can be detected early, ovarian cancer often lies unnoticed until it spreads to the intra-abdominal area at large. In fact, only 25% of ovarian cancers are diagnosed at an early stage while 60% are diagnosed after the cancer has spread [1]. To date, several potential tumor markers including CA-125, growth factors, and lipids have been found at elevated levels in the ascites fluid of ovarian tumors [2-5]. One hallmark of the disease, however, is the mitogenic lipid lysophosphatidic acid (LPA; 1-acyl-glycerol-3-phosphate), which elicits growth factor like responses from cells [6-8] and is also often found at high levels ranging from 2 to 80 uM [3].

LPA has been studied for many years. Once believed only to be a precursor for complex lipids, LPA has emerged as a bioactive lipid capable of producing a wide range of cellular responses. Some of the notable cancer related LPA-induced cellular effects include cell proliferation [8, 9], cell survival and apoptosis suppression [10], cell migration (both chemotaxis and chemokinesis) [11, 12], tumor cell invasion [13, 14], wound healing [15], and cell de-differentiation [16]. This broad bioactivity is mediated, primarily, through activation of a family of G-protein coupled receptors (GPCR) namely the LPA1, LPA2, LPA3, and LPA4 receptors [17-20]. Thus far two distinct enzymatic routes for LPA biosynthesis have been identified. Autotaxin, a secreted enzyme with lysophospholipase D activity, can generate LPA via the hydrolysis of lysophosphatidylcholine [21, 22]. This extracellular LPA has been shown to be produced by activated platelets, fibroblasts, and injured cells. Alternatively LPA can be generated by the removal of a fatty acid chain from phosphatidic acid by the phospholipase A1 or A2 enzymes [21, 22].

In vitro migration and invasion assays have confirmed that LPA promotes cancer pathogenesis in cancer cells through the activation of LPA receptors. Like other bioactive molecules, however, LPA has many downstream effects due to the complex nature of signal transduction pathways. An interesting new hypothesis on LPA signaling in ovarian cancer cells suggests that LPA promotes cancer metastasis by upregulation of urokinase type plasminogen activator (uPA) [23].

uPA is a serine protease known for its ability to convert the zymogen plasminogen to its active form plasmin and was originally administered after heart attacks and strokes to help dissolve clots [24]. Similarly, uPA has been shown to be effective in degrading epithelial basement membranes [25] - a key step for cancer cell metastasis. In addition, uPA stimulates cellular migration and proliferation presumably through activation of a specific cell
surface receptor [26]. uPA is also present in ascites fluid and production can be localized to ovarian cancer cells [27]. Furthermore, with respect to ovarian cancer, elevated uPA levels correlate with poor prognosis [28, 29]. LPA’s ability to upregulate uPA is not universal. In fact, thus far LPA has been shown only to upregulate uPA in ovarian cancer cells [23] via the LPA2 and LPA3 receptors which are overexpressed in most ovarian cancers as opposed to normal epithelial cells [30].

As stated, previous work in this area has determined that exogenous LPA treatment on the human derived ovarian cancer cell line SKOV-3 can induce expression and secretion of uPA [23]. However, like many secreted proteases, activation of the uPA enzyme requires further cellular processing and therefore enzyme expression does not always correlate to enzyme activity [31]. To address this issue I have applied a proteomic strategy referred to as activity-based protein profiling (ABPP) that utilizes chemical probes to directly measure the levels of active uPA secreted from LPA stimulated cells.

Methodology

Culturing and LPA Treatment of SKOV-3 Cells

To analyze further the effect of LPA on uPA activation, I decided to use the human ovarian cancer cell line SKOV-3. For each experiment, 4 x 10^6 SKOV-3 cells, obtained from the National Cancer Institute’s Developmental Therapeutics Program, were cultured in 15 cm dishes with RPMI-1640 medium (Gibco) supplemented with 10% fetal calf serum (FCS) at 37 °C in a humidified atmosphere of 5% CO2 / 95% air. After a 24-hour incubation period, the cells received fresh RPMI-1640 containing LPA or vehicle (DMSO), but lacking in FCS. This is because FCS contains other proteins that can interfere with analysis, including serine protease inhibitors. The cells were then were incubated for an additional 24 hours in the new media at which point the secreted proteome and cells were collected for analysis by ABPP.

Preparation of Cellular Proteomes

Following incubation with LPA or vehicle control, the media from SKOV-3 cells were collected for analysis by ABPP. This media contains proteins secreted by the cells and is referred to as Conditioned Media (CM). The amount of protein in whole CM can vary from cell line to cell line and is relatively low in comparison to cellular proteomes (membrane and soluble proteomes): there is often only 100 ug protein in 20 mL of CM. Because these concentrations of protein are below the detection limits of ABPP it is necessary to concentrate the amount of protein present to a smaller volume in order to be able to visualize the proteins in-gel. A classical approach to achieve higher concentrations of protein involves precipitation of proteins with ammonium sulfate. This process can be quite time consuming, however, requiring several hours to precipitate proteins and multiple centrifugation steps to recover precipitated proteins. In addition, the ammonium sulfate process requires large volumes (at least 500 mL) of CM to get appreciable amounts of protein. Producing this much CM would be prohibitively expensive because the synthesized LPA I treated my cells with is expensive. To increase the throughput of my experiments as well as reduce the cost of materials used, I decided to make use of centrifugal filtration devices (Millipore), which use size exclusion membranes, to concentrate secreted proteins directly in the media. Concentrated secreted proteomes were applied to a desalting column (GE Healthcare) to remove components of the media that could interfere with determination of protein concentration (e.g. dyes). For other cellular proteomes, cells were washed twice in ice-cold PBS. Cell pellets were isolated by centrifugation at 1,400 x g for 3 minutes and dounce-homogenized in Tris buffer (50 mM Tris-HCl, pH 8.0). Membrane proteomes were isolated by centrifugation at 4 °C at 100,000 x g to provide a soluble fraction and a particulate fraction (pellet). The pellet was then washed and resuspended in Tris buffer by sonication to provide a membrane fraction. All proteome protein concentrations were determined by a protein assay kit (Bio-Rad) and analyzed by ABPP.

Activity-Based Protein Profiling (ABPP)

Traditional proteomic technologies and methodologies are aimed at measuring protein abundance, and therefore, like genomic approaches such as gene-chip analysis, provide only an indirect assessment of protein activity. To address this central problem a chemical strategy referred to as activity-based protein profiling (ABPP) has been developed [32, 33]. This approach utilizes activity-based probes (ABP’s) to profile the functional state of enzymes in whole-proteomes. ABP’s label active enzymes but not their inactive precursors (zymogens) or inhibitor-bound forms are composed of at least two key chemical elements, a reactive group that covalently modifies the active site of a
specific class of enzymes and a chemical tag for isolation or detection of probe labeled proteins (Figure 1). ABPP has several advantages over traditional techniques used to assess protein activity. It is generally more sensitive than Western Blot techniques. Compared to zymography, a common method for analyzing protease activities with a non-denaturing gel, ABPP characterizes a protein’s activity while it is in a more native environment where the impact of endogenous inhibitors is taken into account. Therefore, in order to look at the activity of the serine protease uPA, I used an ABP, fluorophosphonate tagged rhodamine (FP-R) (Figure 2A), that targets the serine hydrolase superfamily [34]. In addition to proteases like uPA, the serine hydrolase superfamily includes esterases, transacylases, and amidases.

This probe is composed of a fluorophosphonate reactive group, a well known serine hydrolase inhibitor, that forms a covalent bond with the serine nucleophile (Figure 2B) and rhodamine tag that can be detected with a flatbed scanner. Cancer cell proteomes were prepared as described above and labeled at 0.5 mg/mL with 2 uM FP-R for 60 minutes with CM proteomes and labeled at 1.0 mg/mL with 2 uM FP-R for 30 minutes with cell proteomes. After treatment with FP-R, labeling was quenched and a portion of each sample was treated with PNGaseF (New England Biolabs) following manufacturers protocols to provide a deglycosylated proteome. Samples were then treated with 1:3 of 4x reducing loading buffer and separated by SDS-PAGE (10% acrylamide). Labeled proteins were then visualized in-gel with a Hitachi FMBio IIe flatbed fluorescence scanner (MiraiBio). Active proteins labeled by the probe fluoresce upon analysis with the scanner and are viewed as discrete bands on a gel.

Materials
LPA was purchased from Avanti lipids and FP-R was synthesized by other lab members.

Figure 1. ABPP probes label active, but not inactive (e.g. inhibitor-bound, zymogen) enzymes in their native environments. Enzymes labeled are detected in-gel by the fluorescent tag of the probe.

Figure 2. A. Schematic of the fluorophosphonate-rhodamine (FP-R) probe that targets the serine hydrolase superfamily. FP-R is composed of a fluorophosphonate reactive group (green) and a rhodamine tag (red). B. Covalent interaction between FP-R and a serine nucleophile at the active site of a serine protease.
Results and Discussion

Initial ABPP analysis of SKOV-3 serine hydrolases was initiated by former and current lab members. Before performing studies on LPA effects on serine hydrolase activities I wanted to duplicate the results previously generated with my modified protocol that used spin concentrators instead of ammonium sulfate precipitation. Using the FP-R probe I varied the labeling protocol by adjusting labeling times and probe concentration to obtain optimal conditions for proteome analysis (visualization of low abundance proteins with minimal background). This resulted in using 2 uM FP-R for 60 minutes for analysis of secreted proteomes and 2 uM FP-R for 30 minutes for analysis of membrane and soluble proteomes. The primary reason for varied labeling conditions was due to the low amount of total protein in the secreted proteome.

Preliminary analysis of LPA effects on SKOV-3 cells were performed by treating cells with 20 uM of oleoyl LPA. I chose this form of LPA for initial experiments because it had previously been reported to affect uPA expression levels [23]. As a positive control, a variant of the human breast cancer cell line MDA-MB-231 was labeled by ABPP, but not subject to treatment with LPA or DMSO. This cell line has exceptionally high uPA expression and activity as confirmed by Western and ABPP, respectively. Active uPA appears as a single 27 kilodalton band in both breast and ovarian cancer cell lines in Figure 3. This experiment revealed that treatment of SKOV-3 cells with 20 uM oleoyl LPA induced the production of active uPA which can also be seen in the breast cancer cell lines.

After optimizing the protocol, my next experiment was to determine the effect of various forms of LPA (18:1, 18:0, 16:0 acyl, and 16:0 alkyl) on uPA activity. Interestingly, I observed a significant increase in uPA activity upon treatment with all forms of LPA (Figure 4).

I then analyzed other proteomic fractions from LPA treated SKOV-3 cells to look for changes in serine hydrolases within the membrane and soluble proteomic fractions. Treatment with 20 uM of various forms of LPA did not result in any visible changes in activity, however this aspect of my study could benefit from further analysis as will be described in my conclusions.

Per my experiments, it appeared that oleoyl LPA (LPA 18:1) resulted in the largest enhancement of uPA activity. To study this further, I treated SKOV-3 cells with a range of oleoyl LPA concentrations (0, 1, 5, 20, and 100 uM). Here, uPA activity is enhanced for concentrations 1-20 uM. However, when treated with 100 uM there is less active uPA observed than the 20 uM treated sample.

![Figure 3.](image-url)
Conclusions

My first result showed that oleoyl LPA is capable not only of upregulating uPA expression (as shown by other groups), but is also capable of enhancing uPA’s activity in the human ovarian cancer cell line SKOV-3. This result was first suggested by Pustilnik et al. with in-gel zymography experiments. It was exciting to see that forms other than oleoyl LPA (specifically all the forms I tested: palmitoyl, palmityl, and stearoyl) were able to enhance uPA activity. To date, this result has not been reported. Pustilnik et al. suggested in 1999 that Oleoyl LPA was the only form of the lipid able to upregulate and enhance uPA activity. My data likely points to a different conclusion because ABPP does not measure a protein’s abundance (versus Western assays used by Pustilnik et al.) and because ABPP is typically more sensitive than zymography when measuring protein activity. Furthermore, the mechanism of uPA activation by LPA occurs through GPCR signaling pathways. Although these LPA receptors have slight differences in EC50’s for different forms of LPA, they do not appear to have exclusive selectivity for any acyl or alkyl chains being investigated in this study.

It was bit surprising and perhaps disappointing that exogenous LPA did not invoke changes to other serine hydrolase activities in the membrane or cytosolic proteomes of SKOV-3 cells. Most likely, I didn’t see changes in the cell proteomes for uPA and other proteases at large because the activity of these enzymes in cells must be highly regulated, lest they damage the cell. Other serine hydrolase activities, besides those of proteases, known to...
be detected by the FP-R probe include esterases, transacylases, and amidases. These serine hydrolases, in theory, could have been altered by LPA. Because hydrolases other than uPA were not affected by LPA, though, it is likely that the diverse cellular responses to LPA (proliferation, etc.) are not mediated by serine hydrolases in the cellular membrane or cytosol portions of ovarian cancer cells.

To definitively determine the effect of exogenous LPA on intracellular serine hydrolases, however, further studies using more sensitive techniques like protein mass-spectrometry would be useful. Protein mass-spectrometry would allow for the detection of very low abundance protein activities for samples treated with and without LPA.

Puzzling at first were the results from the final experiments I conducted. After treating my SKOV-3 cells with oleoyl LPA at different concentrations, I noticed that uPA activity did not necessarily increase linearly with LPA concentration. That is, while treatment with 20 uM LPA resulted in more uPA activity compared to the 5 and 1 uM treatments, I saw greater uPA activity from cells treated with 20 uM LPA than from cells treated with 100 uM LPA. These data suggest that LPA has a paradoxical effect on uPA expression and activity. At lower doses, LPA stimulates both uPA expression and activity, while it appears that at higher doses uPA expression is still further induced (as put forth by Pustilnik et al. with Western Analysis showing concentration-expression dependence even at high concentrations), while the activity of this protease is diminished. This could be an effect of overwhelming the cellular machinery involved in processing of the mature active form of uPA. Another possibility is that at extremely high concentrations of LPA (100 uM), the lipid activates additional cellular machinery that participates in the inactivation of uPA.

Above all, these experiments underscore the need to further examine protease activity rather than inferring this parameter from indirect measurements of protein expression. From a biologist’s perspective, these findings suggest that uPA activity may contribute to LPA’s pro-cancerous effects in ovarian tumors, especially at lower (and likely more physiologically relevant) concentrations of the lipid.

References


