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Lysophosphatidic Acid Activates Telomerase in Ovarian Cancer Cells Through Hypoxia-Inducible Factor-1α and the PI3K Pathway

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ABSTRACT

Telomerase is reactivated in over 90% of tumors and plays critical roles in tumor progression. The mechanisms by which telomerase is up-regulated in cancer cells are poorly understood. Here we showed that a bioactive lipid, lysophophatidic acid (LPA), up-regulated the expression of human telomerase reverse transcriptase (hTERT) and telomerase activity in serous ovarian adenocarcinoma cell lines SKOV3, A2780, and HEY, but not in OCC1, a clear cell ovarian cancer cell line. This cell type specific effect of LPA on telomerase regulation may reflect distinctive genetic backgrounds in different histological subtype of ovarian cancer cells. Our data further suggest that the phosphatidylinositol 3-phosphate kinase (PI3K) pathway and hypoxia-inducible factor-1α (HIF-1α) are likely to be involved in LPA-induced hTERT expression. Targeting human telomerase by LPA is potentially involved in its role of promoting tumor progression. J. Cell. Biochem. 105: 1194–1201, 2008.

KEY WORDS: LPA; hTERT; TELOMERASE; HIF-1α; PI3K/Akt

Telomerase is an RNA-dependent DNA polymerase that synthesizes telomeric DNA and thus counteracts the "end-replication" problem occurring during semi-conservative DNA replication [Shay and Wright, 2005]. Telomerase comprises two essential components: the RNA component (*hTR*) that serves as a template for telomeric DNA synthesis and the catalytic protein with the human telomerase reverse transcriptase activity (hTERT) that adds the telomeric repeats onto the end of chromosome [Collins, 2006; Stewart and Weinberg, 2006]. The telomerase activity is turned off during embryonic development by transcriptional repression of *hTERT*, which is undetectable in most normal human somatic cells but present in over 90% of cancerous cells [Shay and Bacchetti, 1997]. Telomerase is required for the long-term proliferation potential of human stem cells and cancer cells. Earlier studies have demonstrated that ectopic expression of hTERT in normal human cells leads to extension of life-span and immortalization of many cell types [Bodnar et al., 1998; Harley, 2002]. Inhibition of telomerase in telomerase positive cancer cells induces cell death [Hahn et al., 1999a; Herbert et al., 1999; Zhang et al., 1999]. Moreover, it has been shown that expression of telomerase was required for the oncogenic transformation of several normal cell types [Hahn et al., 1999b; Drayton et al., 2003; Zongaro et al., 2005; Mizumoto et al., 2006]. Together, these observations support the fundamental roles of telomerase in tumorigenesis. The molecular mechanisms controlling telomerase up-regulation during cellular immortalization and tumorgenesis are beginning to be elucidated [Cong et al., 2002; Dong et al., 2005; Dwyer et al., 2007].

In ovarian tissues, telomerase is absent in normal ovarian surface epithelium and premalignant lesions, but up-regulated in over 95% of ovarian carcinomas [Shay and Bacchetti, 1997]. Concurrently, lysophophatidic acid (LPA), a bioactive lipid growth factor for ovarian cancer, is elevated in both plasma and ascites of ovarian cancer patients and promotes cancer cell growth, migration, invasion, and tumor metastasis [Xu et al., 1998, 2003; Ren et al., 2006]. LPA exhibits pleiotrophic biological activities via the interaction with specific G protein-coupled receptors, LPA_1/Edg-2,

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LPA_2/Edg-4, and LPA_3/Edg-7 [Moolenaar et al., 2004]. It has been shown that LPA promotes ovarian cancer cell proliferation by directly increasing the level of cyclin D1 [Hu et al., 2003], up-regulating VEGF, IL-6, and IL-8 expression, which facilitate tumor angiogenesis [Schwartz et al., 2001; Sivashanmugam et al., 2004; Chou et al., 2005; Sako et al., 2006]. In addition, LPA regulates matrix metalloproteinase and urokinase plasminogen activator activity in ovarian cancer cells, which contributes directly to cell invasion and may be involved in ovarian cancer metastasis [Pustilnik et al., 1999; Fishman et al., 2001].

A number of factors and pathways have been implicated in the regulation of hTERT expression and telomerase activity [Cong et al., 2002]. However, the regulation of telomerase expression and activity under pathological conditions is poorly understood. HIF-1 is a heterodimeric transcriptional factor composed of the basic helix-loop-helix-PAS protein HIF-1α and the arylhydrocarbon receptor nuclear translocator (ARNT or HIF-1β), play a critical role in tumor progression and angiogenesis by activation of hypoxia-inducible genes expression. HIF-1α is highly regulated, whereas HIF-1β is constitutively expressed [Semenza, 2003]. It has been reported that the minimum promoter of *hTERT* contains two HIF-1 responsive elements (HRE) required for *hTERT* up-regulation by HIF-1α in hypoxia condition [Nishi et al., 2004]. However, the mechanism of telomerase regulation by HIF-1α has not been fully elucidated, especially, the role of HIF-1α in activation of telomerase during tumorigenesis is not known.

Telomerase and LPA have been implicated in tumorigenesis, particularly in ovarian cancer [Xu et al., 1995; Fang et al., 2002; Sengupta et al., 2007], with both of them were elevated in ovarian cancer cells, when compared to normal ovarian surface epithelium cells. More recently, Bermudez et al. [2007] have shown that LPA up-regulates telomerase activity via a VEGF-dependent pathway and identified Sp-1 binding sites in hTERT promoter region for LPA's activity. In this study, we have investigated the regulation of telomerase expression by LPA in additional ovarian cancer cell lines and elucidated the new signaling molecules (phosphatidylinositol 3-phosphate kinase—PI3K and HIF-1α) involved in LPA-induced up-regulation of *hTERT* expression.

MATERIALS AND METHODS

REAGENTS AND ANTIBODIES

LPA was purchased from Avanti Polar Lipids (Alabaster, AL). LY294002, PD98059, and rapamycin were purchased from Calbiochem (San Diego, CA). Resveratrol was purchased from Sigma–Aldrich (St. Louis, MO). β-actin monoclonal antibody was from Cell Signaling (Beverly, CA) and HIF-1α monoclonal antibody was from BD (San Diego, CA). Total Akt and Phospho-Akt antibodies were obtained from (Cell Signaling). LipofectAMINE 2000 was obtained from Invitrogen (Carlsbad, CA).

CELL CULTURE

Ovarian cancer cell lines were maintained in RPMI 1640 (Hyclone, Logan) supplemented with 10% fetal bovine serum (Hyclone). For LPA treatment, cells were treated with 40 μM LPA dissolved in phosphate-buffered saline (PBS) or treated with PBS as control. For pharmacologic inhibitor treatment, cells were pretreated with inhibitors 10 mM LY294002, 100 μM PD98059, 10 mM rapamycin or 50 μM resveratrol dissolved in DMSO for 2 h prior to LPA treatment.

PLASMIDS

p3996del, p1009del, and p342 are *hTERT* promoter-luciferase reporter plasmids in which various lengths of DNA upstream of the initiating ATG codon of *hTERT* were cloned into pGL2-Enhancer (Promega, Madison, WI). Reporter plasmids p342mt1, p342mt2, and p342mt contain one or two mutations in the two putative binding sites of HIF-1α at nt −241 and −33, in which CACGTGGC were changed into TTTGTGGC [Nishi et al., 2004]. Site-directed mutagenesis was performed using the plasmid p342 with the following primer pairs: p342mt1, forward, 5′-CAG GAC CGC GCT TCC **TTT** GTG GCG GAG GGA CTG and reverse, 5′-CAG TCC CTC CGC CAC AAA GGA AGC GCG GTC CTG; p342mt2, forward, 5′-CTG CGT CCT GCT GCG **TTT** GTG GGA AGC CCT GGC and reverse, 5′-GCC AGG GCT TCC CAC AAA CGC AGC AGG ACG CAG. The mutated sequences in the p342 were confirmed by DNA sequencing and then subcloned into *Kpn*I and *Bgl*II sites of pGL2 enhancer plasmid to generate p342mt1, p342mt2, and p342mt.

ASSAY OF TELOMERASE ACTIVITY

Telomerase activity was assayed using *Telo*TAGGG Telomerase PCR–ELISA^{PLUS} kit (Roche, Indianapolis, IN) according to the manufacturer's protocol. To perform the telomerase PCR–ELISA within a linear range, all of cell extracts equivalent to 1,000 cells were used. Absorbance values were measured using the Tecan Genios Microplate Reader and telomerase activity was expressed as relative telomerase activity against negative control.

RT–PCR

Total RNA was collected using TRIzol reagent (Invitrogen). 2 μg of DNase I (Ambion, Austin) treated RNAs were reverse transcribed to single-strand cDNA using M-MLV Reverse Transcriptase (Promega) with 1 μg oligo(dT). Primer sequence of *GAPDH*, *hTERT*, and *HIF-1α* are the following: *GAPDH* forward primer 5-CGGAGTCAACGG-ATTTGGTCGTAT-3, *GAPDH* reverse primer 5-TGCTAAGCAGTTG-GTGGTGCAGGA-3; *hTERT* forward primer 5-AGAACGTTCCGC-AGAGAAAA-3, *hTERT* reverse primer 5-ATGTACGGCTGGA-GGTCTGT-3; *HIF-1α* forward primer 5-TCCATGTGACCATGAGG-AAA-3, *HIF-1α* reverse primer 5-TCCATGTGACCATGAGGAAA-3.

WESTERN BLOT AND IMMUNOFLUERSCENCE STAINING ANALYSIS

Cell lysates were prepared in RIPA lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% Sodium Deoxycholate, 0.1% SDS). Protein concentrations of cell lysates were determined using BCA assay kit in accordance to the manufacturer's instructions. One hundred micrograms of protein extracts were resolved on 12% SDS–PAGE and transferred to PVDF membranes (Bio-Rad, Hercules, CA). Immunoblotting was performed using HIF-1α antibodies (1:500), serine 473-phospho-Akt antibody (1:1,000), and total Akt antibody (1:1,000). β-actin (1:5,000, Sigma–Aldrich) was used as loading control. For immunofluorescence staining analysis, cells were grown on coverslips and treated with ±LPA for indicated time

periods. The coverslips were fixed with 4% cold ultrapure formaldehyde (Merck, Germany) and permeabilized with 0.5% Triton X-100 (Amresco) for 5 min. Then, the coverslips were blocked in 0.6% goat serum in PBS for 1 h and incubated with primary HIF-1α antibody (1:20) and secondary fluorescent antibodies (1:200) for 1 h at 37 °C respectively. After washed with PBS, coverslips were counterstained with 0.1 μg/ml 4,6-diamino-2-phenylindole (DAPI, Roche, Switzerland). Images are obtained with a laser scanning confocal microscope.

LUCIFERASE REPORTER ASSAY

HEY cells (6×10^4) were plated in 24-well culture dishes and transiently co-transfected with 0.7 μg of luciferase reporter and 0.1 μg of pCMV-β-gal plasmid using Lipofectamine™ 2000 reagent (Invitrogen). Cultures were replenished with medium containing 10% serum at 4 h post-transfection. Prior to collection, HEY cells were treated with or without LPA for 24 h. Luciferase activity was measured at 48 h post-transfection using the Luciferase Assay System (Promega) according to the manufacturers' instructions. Transcriptional activity was expressed as relative luciferase activity after normalization with β-galactosidase activity.

STATISTICAL ANALYSIS

Data were subjected to the Student's *t*-test for determination of statistical significance. $P < 0.05$ was considered significant.

RESULTS

LPA UP-REGULATED hTERT EXPRESSION IN OVARIAN CANCER CELLS

To further understand the role of LPA in regulating hTERT expression in more commonly used ovarian cancer cell lines, we tested the effect of LPA on *hTERT* mRNA levels in four ovarian cancer cell lines using RT-PCR. We found that LPA significantly up-regulated *hTERT* mRNA in ovarian cancer cell lines SKOV3, A2780, and HEY (all serous adnocarcinoma cell lines), but not in OCC1, a clear cell ovarian cancer cell line (Fig. 1A). LPA signaling is mainly mediated by its specific G-protein-coupled receptors [Moolenaar et al., 2004], we have found that all these four ovarian cancer cells lines expressed LPA receptors LPA_1, LPA_2, and LPA_3 (data not shown), suggesting that factor(s) other than LPA receptors is responsible for the lack of *hTERT* mRNA up-regulation in OCC1 cells.

The time course of LPA-induced telomerase expression was further examined in HEY, a representative ovarian serous adenocarcinoma cell line. We observed that LPA induced *hTERT* RNA expression, but not telomerase RNA (*hTR*), in a time dependent manner. Induction of *hTERT* transcription reached the highest level at 24 h and decreased after 36 h of LPA treatment (Fig. 1B). Correlated with the induction of *hTERT* mRNA level, telomerase activity was increased to maximum at 24 h and decreased after 36 h of LPA treatment (Fig. 1C).

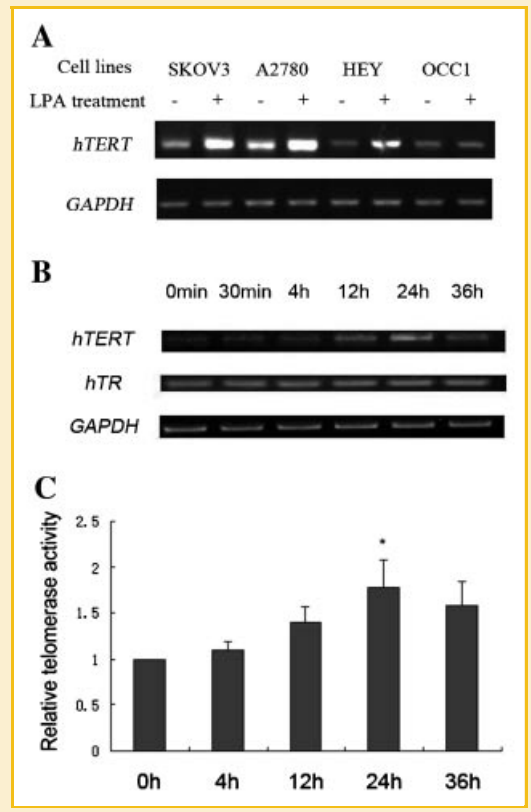

Fig. 1. LPA induced *hTERT* mRNA expression and telomerase activity. A: LPA up-regulated *hTERT* transcription in different ovarian cancer cell lines. The cells were treated with or without LPA for 24 h. Total RNA was isolated and RT-PCR was conducted using specific *hTERT* primers. B: LPA up-regulated *hTERT* transcription in time-dependent manner. The HEY cells (1×10^6) were treated with LPA for the indicated time periods. Total RNA was isolated, and RT-PCR was conducted using specific *hTERT* primers. C: Telomerase activity was measured in cultured HEY cells treated with LPA for the indicated time periods. Results are the mean ± SD (standard deviation) from three independent experiments in triplicates. Columns, mean; bars, SD; * denoted statistical significance, $P < 0.05$.

ACTIVATION OF hTERT PROMOTER ACTIVITY BY LPA REQUIRED HIF-1α

In an effort to understand the mechanisms by which LPA induced *hTERT* expression, we characterized the effect of LPA on the *hTERT* promoter activity in ovarian cancer cells. We observed that LPA was able to stimulate the luciferase activity using *hTERT* promoters containing the full-length 3996 bp, the 1009 bp, or the 342 bp sequences upstream from ATG (Fig. 2A,B), suggesting that the 342 bp sequence contains the responsive element(s) for LPA-induced *hTERT* up-regulation. It has been reported that the minimum promoter of *hTERT* contains two HIF-1α responsive elements (HRE) required for *hTERT* up-regulation by HIF-1α under hypoxia conditions [Nishi et al., 2004], and LPA induces VEGF expression through activation of HIF-1α in ovarian cancer cells [Lee et al., 2006]. We tested whether LPA induced *hTERT* transcription via HIF-1α. There are two hypoxia-responsive elements (HREs) in the 342 promoter region. We mutated each of these sites separately or in

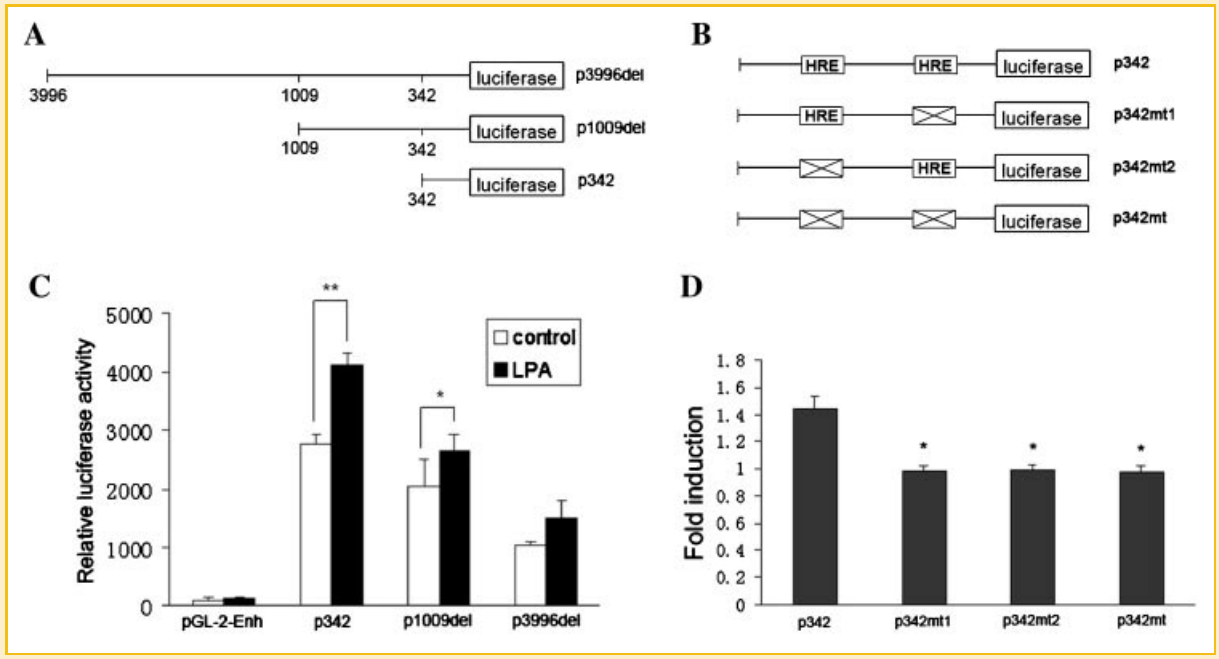

Fig. 2. LPA-induced telomerase activity required the HIF-1α transcription factor. A: Schematic diagrams of *hTERT* promoter reporter plasmids. B: HEY cells were transfected with *hTERT* promoter reporter constructs along with pCMV-β-galactosidase expression vector for the normalization of transfection efficiency. Cells were treated with or without LPA (40 μM) for 24 h. After 48 h of post-transfection, luciferase and β-galactosidase assays were performed. Reporter activity was normalized by dividing luciferase activity with β-galactosidase activity. Results were expressed as the mean ± SD from three independent experiments in triplicates. C: Schematic representations of the minimum *hTERT* promoter reporter constructs containing wild type or mutant HIF-1α responsive elements. D: HEY cells were transfected with p342, p342mt1, p342mt2, and p342mt as well as pCMV-β-galactosidase expression vector. Cells were treated with or without LPA (40 μM) for 24 h luciferase and β-galactosidase assays were performed after 48 h of post-transfection. Results are expressed as the fold-induction in presence of LPA from three independent experiments in triplicates. Columns, mean; bars, SD; * denoted statistical significance, $P < 0.05$, ** when $P < 0.01$.

combination [Nishi et al., 2004]. We observed that mutations in each or both of these HRE sites abolished the induction of *hTERT* promoter activity by LPA (Fig. 2C,D), suggesting that both of these sites are required for LPA's effect. We next examined the effects of LPA on HIF-1α expression. HEY cells were treated by LPA for different times as indicated in Figure 3 and expression of HIF-1α was examined by RT-PCR and Western blot analyses. Interestingly, LPA did not alter the mRNA levels of *HIF-1α* (Fig. 3A), but up-regulated HIF-1α protein levels from 30 min to 12 h (Fig. 3B), suggesting post-transcriptional regulation is involved. In addition, parallel to increased HIF-1α protein expression, we observed that HIF-1α was translocated to nucleus 4 h post of LPA treatment (Fig. 3C). Together, these observations suggest that HIF1α is likely to be required for LPA's transcription activity on *hTERT*.

It has been shown that resveratrol (3,5,4′-trihydroxystilbene) produced by plants inhibited HIF-1α expression by promoting its degradation [Park et al., 2007]. To further confirm the role of HIF-1α in LPA-induced *hTERT* up-regulation, we examined the effects of HIF-1α inhibitor resveratrol on LPA-induced *hTERT* up-regulation. The ovarian cancer cell lines SKOV3, A2780, HEY, and OCC1 were pretreated by resveratrol for 2 h, followed by LPA treatment. As shown in Figure 3D,E, LPA significantly increased the levels of HIF-1α protein, and resveratrol suppressed HIF-1α accumulation induced by LPA in the three serous ovarian cancer cell lines SKOV3, A2780, and HEY. Concomitantly, *hTERT* mRNA levels have increased by the treatment of LPA, and this induction was suppressed by the HIF-1α inhibitor resveratrol. However, no significant changes in either HIF-1α protein levels or *hTERT* expression in respond to LPA have been detected in OCC1 cells, a clear cell ovarian cancer cell line. The differences of LPA responsiveness in these cell lines may be related to the distinctive cellular contexts in different histological subtypes of ovarian cancer cells [Schwartz et al., 2001].

PI3K WAS INVOLVED IN LPA INDUCED hTERT EXPRESSION

Both PI3K and mitogen-activated protein kinase (MAPK) signaling pathway have been implicated in the regulation of HIF-1α expression [Zhou et al., 2004]. The PI3K pathway regulates HIF-1α protein level via mammalian target of rapamycin (mTOR)-dependent and -independent mechanisms [Pore et al., 2006]. To investigate the signaling pathway mediated HIF-1α expression by LPA in HEY ovarian cancer cells, we examined the effects of PI3K, MAPK, and mTOR inhibitors on LPA induced *hTERT* expression. HEY cells were pretreated by different inhibitors and *hTERT* expression was monitored by RT-PCR. As shown in Figure 4A, LPA-induced *hTERT* expression was completely attenuated by the PI3K inhibitor LY294002, but only slightly affected by the MAPK inhibitors PD98059 and the mTOR inhibitor rapamycin, suggesting that PI3K, but not MAPK or mTOR, was mainly involved in LPA induced *hTERT* expression in HEY cells.

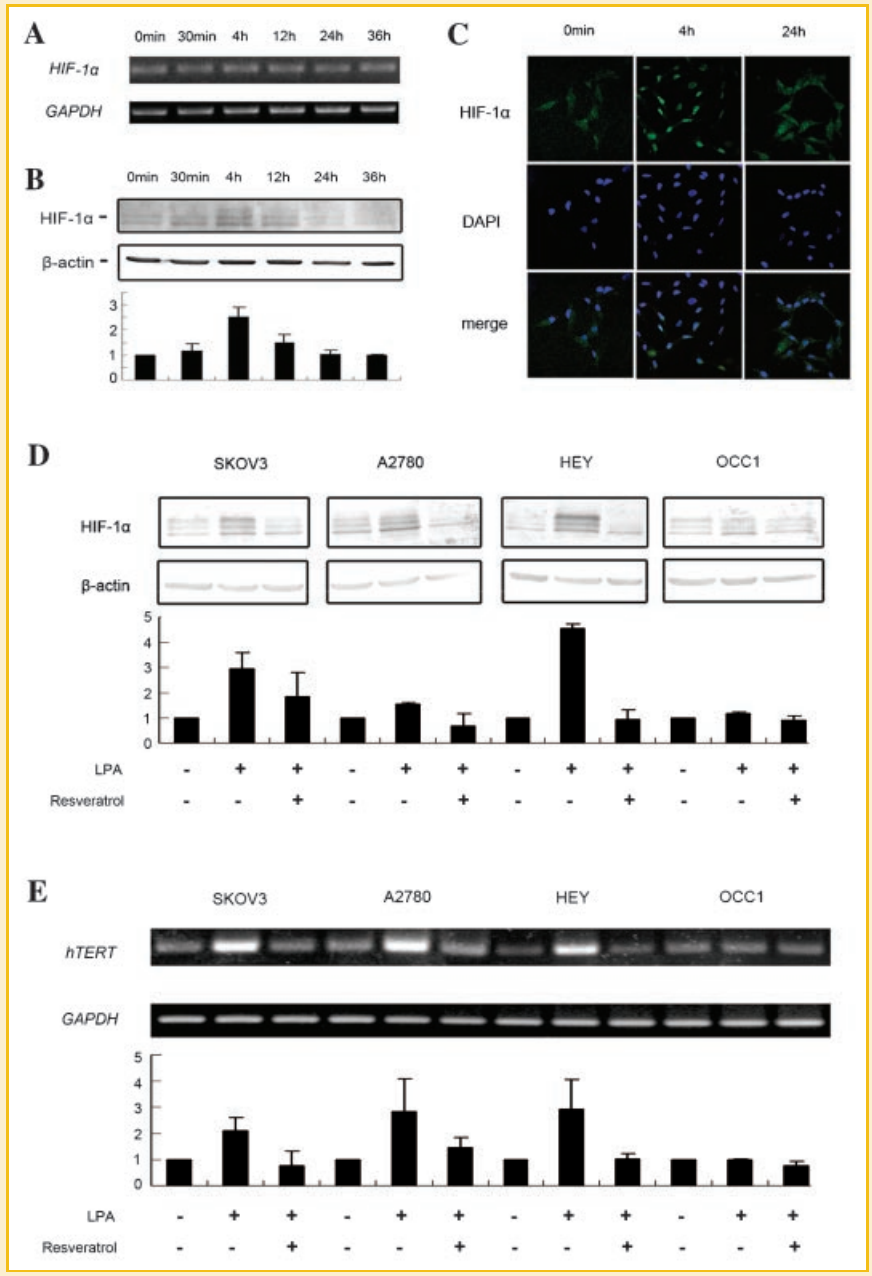

Fig. 3. LPA induced HIF-1α over-expression and nuclear translocation. A: effect of LPA on *HIF-1α* transcription. HEY cells were treated by LPA for indicated time periods. Total RNA was isolated and *HIF-1α* transcription was analyzed by RT-PCR with *HIF-1α* specific primers. B: effect of LPA on HIF-1α protein expression. HEY cells were incubated in the presence of LPA for the indicated time periods. HIF-1α protein level was determined by immunoblotting with anti-HIF-1α antibody. C: nuclear translocation of HIF-1α induced by LPA. The cells seeded in slides were treated with or without LPA (40 μM) for indicated time periods and cellular distribution of HIF-1α was analyzed by immunofluorescence staining with anti-HIF-1α antibody. D: resveratrol inhibited LPA-induced HIF-1α expression. Cells were incubated with resveratrol (50 μM) or PBS as control for 2 h prior to LPA treatment. Cells were collected after 4 h of LPA treatment and cell lysates were subjected to Western blotting analysis with the HIF-1α antibody, or with β-actin antibody for loading control. E: resveratrol blocked LPA-induced *hTERT* expression. Cells were treated similarly as in D, and were collected after 24 h of LPA treatment. Total RNAs were isolated and analyzed by RT-PCR as described in Figure 1A. Levels of HIF-1α and *hTERT* were quantified related to β-actin or *GAPDH* respectively by densitometric analysis (band intensity) in B,D,E, representative of three experiments with similar results. Columns, mean; bars, SD.

Akt is known as an important downstream target for PI3K and commonly activated in human cancer. There were a number of studies indicated that PI3K/Akt pathway involved in the regulation of HIF-1α [Pore et al., 2006]. We characterized the phosphorylation of Akt S473 in response to LPA. Consistent with previous report [Baudhuin et al., 2002], we found that LPA induced Akt S473 phosphorylation (LPA 40 μM and 30 min) in HEY ovarian cancer cells. Phosphorylation of Akt S473 was persistent and then decreased to the basal level 4 h after LPA treatment (Fig. 4B). The PI3K inhibitor LY294002 efficiently blocked Akt S473

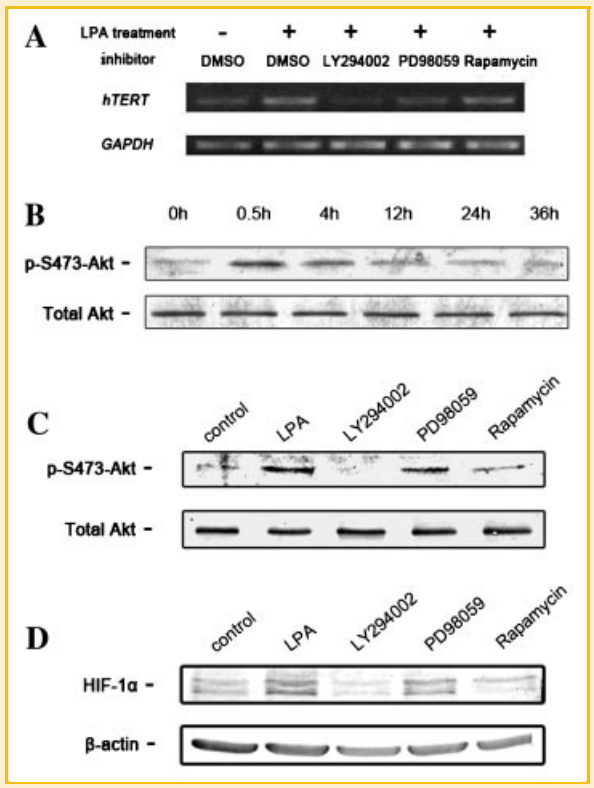

Fig. 4. PI3K pathway was involved in LPA induced HIF-1α over-expression and *hTERT* transactivation. A: effect of pharmacologic inhibitors on LPA-induced *hTERT* transcription. Cells were pretreated for 2 h with vehicle or inhibitors and then exposed to LPA for 24 h. *hTERT* and *GAPDH* expression were analyzed by RT-PCR, *GAPDH* was used as a loading control. B: effect of LPA on Akt phosphorylation. HEY cells were incubated by LPA for indicated time periods. Serine 473-phospho-Akt and total-Akt protein levels were analyzed by Western blotting with specific antibodies. C: effect of pharmacologic inhibitors on LPA induced Akt phosphorylation. Cells were pretreated for 2 h with vehicle or inhibitors and then exposed to LPA for 30 min, Serine 473-phospho-Akt and total-Akt protein levels were analyzed by Western blotting with specific antibodies. D: effect of pharmacologic inhibitors on LPA induced HIF-1α protein over-expression. Cells were pretreated for 2 h with vehicle or inhibitors and then exposed to LPA for 4 h, HIF-1α protein expression was analyzed by Western blotting with HIF-1α specific antibodies. Representative of three experiments with similar results.

phosphorylation and HIF-1α accumulation in response to LPA (Fig. 4C,D). Interestingly, although rapamycin did not affect *hTERT* expression level (Fig. 4A), it did moderately reduce pS473-Akt and HIF-1α levels (Fig. 4 C,D), suggesting that elevated HIF-1α may not always correlate with *hTERT* expression.

Collectively, our results suggested that LPA enhanced HIF-1α expression and nuclear translocation through the PI3K pathway, thereby induced *hTERT* expression and telomerase activity in ovarian cancer cells.

DISCUSSIONS

The importance of telomerase in tumorigenesis has been fully recognized [Shay and Bacchetti, 1997; Hahn et al., 1999b; Shay and Wright, 2005]. Thus, to understand how telomerase is up-regulated in cancer cells becomes very critical for developing new therapeutic strategies for cancer treatment. Similarly, the role of LPA in ovarian cancer has been extensively studied and its therapeutic potential has been realized [Xu et al., 1998, 2003; Mills and Moolenaar, 2003]. To link these two important molecules in ovarian cancer is very important and significant. We have provided convincing data to show that LPA regulates expression and activity of telomerase in multiple ovarian cancer cells lines, suggesting telomerase is an important molecule though which LPA exerts its oncogenic effects.

Accumulated information suggests that telomerase activity can be regulated at multiple levels by different factors in different cellular context [Cong et al., 2002]. However, the molecular mechanisms and pathways governing telomerase expression and activity are still elusive. Bermudez et al. [2007] has shown in ovarian carcinoma cell lines PA-1, SW626, and a telomerase-negative, non-tumorigenic SV40 large-T antigen transfected human ovarian surface epitithelial (IOSE) cell line, that LPA regulates telomerase via a transcriptional factor Sp1 and the ERK1/2 pathway. In this report, we have shown that LPA up-regulated *hTERT* expression and telomerase activity in HEY, SKOV3, and A2780 cells through activation of HIF-1α and PI3K. MAPK signaling pathway may not be involved in HEY cells, since the inhibitor of MAPK did not have an effect on LPA-induced *hTERT* expression. These results suggest LPA may employ distinct signaling pathways in different ovarian cancer cell lines. Since PI3K is an upstream activator of the MAPK signaling pathway in many different ovarian cancer cells, targeting PI3K may be more effective to reach the therapeutic effects in different ovarian cancer.

Ovarian cancer is a highly lethal type of gynecological disease. Based on the type of epithelial differentiation and morphological criteria, there are four major subtypes including serous, mucinous, endometrioid, clear cell. Serous tumors are the most common subtype representing about one-half of all ovarian cancer, the endometrioid adenocarcinomas account for 20–25% of ovarian cancer, whereas clear cell and mucinous adenocarcinomas are less common, which account for less than 10% of ovarian cancer cases [Schwartz et al., 2001; Soslow, 2008]. Recent studies indicate that the different histological types of ovarian cancer likely represent distinct disease entities [Feeley and Wells, 2001]. In support with this notion, it has been shown that clear cell ovarian cancer has a distinctive pattern of gene expression, which distinguishes it from other type of ovarian cancer [Schwartz et al., 2001]. In this report, we showed that LPA induced *hTERT* expression in ovarian serous adenocarcinomas cell lines SKOV3, A2870, and HEY, but not in OCC1, a clear cell ovarian cancer cell line. Consistently, we showed that LPA increased HIF-1α expression, this induction was blocked by HIF-1α inhibitor Resveratrol, which subsequently suppressed LPA-induced *hTERT* up-expression in ovarian serous adenocarcinomas cell lines SKOV3, A2870, and HEY. Although the effects of resveratrol on hTERT expression and cell proliferation remains to be investigated in ovarian cancer cells, resveratrol efficiently inhibited HIF-1α expression and subsequently blocked hTERT up-regulation induced by LPA, suggesting its potential therapeutic values for ovarian cancer treatment. Interestingly, we found that LPA has no effect on the expression of HIF-1α and *hTERT* in the clear cell

ovarian cancer cell line OCC1. This different effect of LPA on telomerase regulation may be associated with distinctive genetic backgrounds in different histological subtype of ovarian cancer cells. Recently, Pectasides et al. [2006] have reviewed the clinical feature of patients with ovarian clear cell carcinomas, they have found that the recurrences are more frequent and survival rates are lower in patients with clear cell carcinomas than those with advanced serous subtype of tumors, suggesting that the intrinsic chemoresistance may be associated with this subtype and novel treatment approaches should be adopted in clinical management for patients with ovarian clear cell carcinomas. Therefore the classification of histological subtype of ovarian carcinomas may have important biological and therapeutic implications.

It is of interesting to note that the *hTERT* promoter activity induced by LPA was decreased to the same degree when either one or two of the HIF-1α binding sites were mutated, suggesting that both of these sites are required for the activity and the two sites may have a cooperative effect. This is consistent with the report showing that transactivation of *hTERT* promoter by HIF-1α was abolished when one or two of HIF-1 binding sites were altered [Nishi et al., 2004]. In addition, we observed in the *hTERT* promoter reporter assay that the minimum promoter p342 has greater promoter activity than the full-length promoter p3996del or p1009del, suggesting that a negative regulatory element(s) may exist upstream of the minimum promoter.

We have shown that LPA not only increased expression of HIF-1α, but also its nuclear translocation. HIF-1α is mainly regulated post-transcriptionally through the von Hippel-Lindau protein (VHL) mediated ubiquitin-proteasome degradation [Ke and Costa, 2006]. We have shown that LPA increases the levels of HIF-1α protein rather rapidly (which 30 min), but not the RNA level. These data are fully consistent with post-transcriptional regulation of HIF-1α. HIF-1α is critical transcription factor for a number of important cancer-related genes in various cancer cells [Bárdos and Ashcroft, 2005]. Thus, its nuclear translocation is very important to its function. We have shown that LPA-induced cell migration and invasion are significantly enhanced under hypoxia conditions, where HIF-1α plays an important role [Kim et al., 2006]. The current work has added an another link between LPA and HIF-1α, suggesting that increased cellular responses to LPA under hypoxia conditions may be related to LPA's ability to up-regulate expression of HIF-1α and increase its nuclear translocation. While this remains to be tested directly, a network among LPA-PI3K-HIF-1α -telemerase has been emerging through our work, which may represents targets for ovarian cancer therapy.

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REFERENCES

Bárdos JI, Ashcroft M. 2005. Negative and positive regulation of HIF-1: A complex network. Biochim Biophys Acta 1755:107–120.

Baudhuin LM, Cristina KL, Lu J, Xu Y. 2002. Akt activation induced by lysophosphatidic acid and sphingosine-1-phosphate requires both mitogen-activated protein kinase kinase and p38 mitogen-activated protein kinase and is cell-line specific. Mol Pharmacol 62:660–671.

Bermudez Y, Yang H, Saunders BO, Cheng JQ, Nicosia SV, Kruk PA. 2007. VEGF- and LPA-induced telomerase in human ovarian cancer cells is Sp1-dependent. Gynecol Oncol 106:526–537.

Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu CP, Morin GB, Harley CB, Shay JW, Lichtsteiner S, Wright WE. 1998. Extension of life-span by introduction of telomerase into normal human cells. Science 279:349–352.

Chou CH, Wei LH, Kuo ML, Huang YJ, Lai KP, Chen CA, Hsieh CY. 2005. Up-regulation of interleukin-6 in human ovarian cancer cell via a Gi/PI3K-Akt/NF-kappaB pathway by lysophosphatidic acid, an ovarian cancer-activating factor. Carcinogenesis 26:45–52.

Collins K. 2006. The biogenesis and regulation of telomerase holoenzymes. Nat Rev Mol Cell Biol 7:484–494.

Cong YS, Wright WE, Shay JW. 2002. Human telomerase and its regulation. Microbiol Mol Biol Rev 66:407–425.

Dong CK, Masutomi K, Hahn WC. 2005. Telomerase: Regulation, function and transformation. Crit Rev Oncol Hematol 54:85–93.

Drayton S, Rowe J, Jones R, Vatcheva R, Cuthbert-Heavens D, Marshall J, Fried M, Peters G. 2003. Tumor suppressor p16INK4a determines sensitivity of human cells to transformation by cooperating cellular oncogenes. Cancer Cell 4:301–310.

Dwyer J, Li H, Xu D, Liu JP. 2007. Transcriptional regulation of telomerase activity: Roles of the Ets transcription factor family. Ann NY Acad Sci 1114:36–47.

Fang X, Schummer M, Mao M, Yu S, Tabassam FH, Swaby R, Hasegawa Y, Tanyi JL, LaPushin R, Eder A, Jaffe R, Erickson J, Mills GB. 2002. Lysophosphatidic acid is a bioactive mediator in ovarian cancer. Biochim Biophys Acta 1582:257–264.

Feeley KM, Wells M. 2001. Precursor lesions of ovarian epithelial malignancy. Histopathology 38:87–95.

Fishman DA, Liu Y, Ellerbroek SM, Stack MS. 2001. Lysophosphatidic acid promotes matrix metalloproteinase (MMP) activation and MMP-dependent invasion in ovarian cancer cells. Cancer Res 61:3194–3199.

Hahn WC, Stewart SA, Brooks MW, York SG, Eaton E, Kurachi A, Beijersbergen RL, Knoll JH, Meyerson M, Weinberg RA. 1999a. Inhibition of telomerase limits the growth of human cancer cells. Nat Med 5:1164–1170.

Hahn WC, Counter CM, Lundberg AS, Beijersbergen RL, Brooks MW, Weinberg RA. 1999b. Creation of human tumour cells with defined genetic elements. Nature 400:464–468.

Harley CB. 2002. Telomerase is not an oncogene. Oncogene 21:494–502.

Herbert B, Pitts AE, Baker SI, Hamilton SE, Wright WE, Shay JW, Corey DR. 1999. Inhibition of human telomerase in immortal human cells leads to progressive telomere shortening and cell death. Proc Natl Acad Sci USA 96: 14276–14281.

Hu YL, Albanese C, Pestell RG, Jaffe RB. 2003. Dual mechanisms for lysophosphatidic acid stimulation of human ovarian carcinoma cells. J Natl Cancer Inst 95:733–740.

Ke Q, Costa M. 2006. Hypoxia-inducible factor-1 (HIF-1). Mol Pharmacol 70:1469–1480.

Kim K, Sengupta S, Berk M, Kwak YG, Escobar PF, Belinson J, Mok SC, Xu Y. 2006. Hypoxia enhances lysophosphatidic acid responsiveness in ovarian cancer cells and lysophosphatidic acid induces ovarian tumor metastasis in vivo. Cancer Res 66:7983–7990.

Lee J, Park SY, Lee EK, Park CG, Chung HC, Rha SY, Kim YK, Bae GU, Kim BK, Han JW, Lee HY. 2006. Activation of hypoxia-inducible factor-1alpha is necessary for lysophosphatidic acid-induced vascular endothelial growth factor expression. Clin Cancer Res 12:6351–6358.

Mills GB, Moolenaar WH. 2003. The emerging role of lysophosphatidic acid in cancer. Nat Rev Cancer 3:582–591.

Mizumoto Y, Kyo S, Ohno S, Hashimoto M, Nakamura M, Maida Y, Sakaguchi J, Takakura M, Inoue M, Kiyono T. 2006. Creation of tumorigenic human endometrial epithelial cells with intact chromosomes by introducing defined genetic elements. Oncogene 25:5673–5682.

Moolenaar WH, van Meeteren LA, Giepmans BN. 2004. The ins and outs of lysophosphatidic acid signaling. Bioessays 26:870–881.

Nishi H, Nakada T, Kyo S, Inoue M, Shay JW, Isaka K. 2004. Hypoxia-inducible factor 1 mediates upregulation of telomerase (hTERT). Mol Cell Biol 24:6076–6083.

Park SY, Jeong KJ, Lee J, Yoon DS, Choi WS, Kim YK, Han JW, Kim YM, Kim BK, Lee HY. 2007. Hypoxia enhances LPA-induced HIF-1alpha and VEGF expression: Their inhibition by resveratrol. Cancer Lett 258:63–69.

Pectasides D, Pectasides E, Psyrri A, Economopoulos T. 2006. Treatment issues in clear cell carcinoma of the ovary: A different entity? Oncologist 11:1089–1094.

Pore N, Jiang Z, Shu HK, Bernhard E, Kao GD, Maity A. 2006. Akt1 activation can augment hypoxia-inducible factor-1alpha expression by increasing protein translation through a mammalian target of rapamycin-independent pathway. Mol Cancer Res 4:471–479.

Pustilnik TB, Estrella V, Wiener JR, Mao M, Eder A, Watt MA, Bast RC, Jr., Mills GB. 1999. Lysophosphatidic acid induces urokinase secretion by ovarian cancer cells. Clin Cancer Res 5:3704–3710.

Ren J, Xiao YJ, Singh LS, Zhao X, Zhao Z, Feng L, Rose TM, Prestwich GD, Xu Y. 2006. Lysophosphatidic acid is constitutively produced by human peritoneal mesothelial cells and enhances adhesion, migration, and invasion of ovarian cancer cells. Cancer Res 66:3006–3014.

Sako A, Kitayama J, Shida D, Suzuki R, Sakai T, Ohta H, Nagawa H. 2006. Lysophosphatidic acid (LPA)-induced vascular endothelial growth factor (VEGF) by mesothelial cells and quantification of host-derived VEGF in malignant ascites. J Surg Res 130:94–101.

Schwartz BM, Hong G, Morrison BH, Wu W, Baudhuin LM, Xiao YJ, Mok SC, Xu Y. 2001. Lysophospholipids increase interleukin-8 expression in ovarian cancer cells. Gynecol Oncol 81:291–300.

Semenza GL. 2003. Targeting HIF-1 for cancer therapy. Nat Rev Cancer 10:721–732.

Sengupta S, Kim KS, Berk MP, Oates R, Escobar P, Belinson J, Li W, Lindner DJ, Williams B, Xu Y. 2007. Lysophosphatidic acid downregulates tissue inhibitor of metalloproteinases, which are negatively involved in lysophosphatidic acid-induced cell invasion. Oncogene 26:2894–2901.

Shay JW, Bacchetti S. 1997. A survey of telomerase activity in human cancer. Eur J Cancer 33:787–791.

Shay JW, Wright WE. 2005. Senescence and immortalization: Role of telomeres and telomerase. Carcinogenesis 26:867–874.

Sivashanmugam P, Tang L, Daaka Y. 2004. Interleukin 6 mediates the lysophosphatidic acid-regulated cross-talk between stromal and epithelial prostate cancer cells. J Biol Chem 279:21154–21159.

Soslow RA. 2008. Histologic subtypes of ovarian carcinoma: An overview. Int J Gynecol Pathol 2:161–174.

Stewart SA, Weinberg RA. 2006. Telomeres: Cancer to human aging. Annu Rev Cell Dev Biol 22:531–557.

Xu Y, Fang XJ, Casey G, Mills GB. 1995. Lysophospholipids activate ovarian and breast cancer cells. Biochem J 309:933–940.

Xu Y, Shen Z, Wiper DW, Wu M, Morton E, Elson P, Kennedy AW, Belinson J, Markman M, Casey G. 1998. Lysophosphatidic acid as a potential biomarker for ovarian and other gynecologic cancers. JAMA 280:719–723.

Xu Y, Xiao YJ, Zhu K, Baudhuin LM, Lu J, Hong G, Kim KS, Cristina KL, Song L, S Williams F, Elson P, Markman M, Belinson J. 2003. Unfolding the pathophysiological role of bioactive lysophospholipids. Curr Drug Targets Immune Endocr Metabol Disord 3:23–32.

Zhang X, Mar V, Zhou W, Harrington L, Robinson MO. 1999. Telomere shortening and apoptosis in telomerase-inhibited human tumor cells. Genes Dev 13:2388–2399.

Zhou J, Schmid T, Frank R, Brune B. 2004. PI3K/Akt is required for heat shock proteins to protect hypoxia-inducible factor 1alpha from pVHL-independent degradation. J Biol Chem 279:13506–13513.

Zongaro S, de Stanchina E, Colombo T, D'Incalci M, Giulotto E, Mondello C. 2005. Stepwise neoplastic transformation of a telomerase immortalized fibroblast cell line. Cancer Res 65:11411–11418.