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Downstream molecular events in the altered profiles of lysophosphatidic acid-induced cAMP in senescent human diploid fibroblasts

Ik-Soon Jang[1]*, Ji-Heon Rhim[1]*, Sang Chul Park[1] and Eui-Ju Yeo[2,3]

[1]Department of Biochemistry and Molecular Biology
Aging and Apoptosis Research Center
Seoul National University College of Medicine
Seoul 110-799, Korea
[2]Department of Biochemistry, Gachon Medical School
Incheon 405-760, Korea
[3]Corresponding author: Tel, 82-32-460-3519;
Fax, 82-32-930-5088; E-mail, euiju@gachon.ac.kr
*These authors contributed equally to this work.

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Abbreviations: AC, adenylyl cyclase; COX, cyclooxygenase; CaMK, calmodulin kinase; CREB, cAMP-responsive element binding protein; EDGs, endothelial cell differentiation genes; ERK, extracellular signal-regulated kinase; LPA, lysophosphatidic acid; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MSK1, mitogen- and stress-activated kinase1; PKA, protein kinase A; PKC, protein kinase C

Abstract

Lysophosphatidic acid (LPA) is a phospholipid growth factor that acts through G-protein-coupled receptors. Previously, we demonstrated an altered profile of LPA-dependent cAMP content during the aging process of human diploid fibroblasts (HDFs). In attempts to define the molecular events associated with the age-dependent changes in cAMP profiles, we determined the protein kinase A (PKA) activity, phosphorylation of cAMP-response element binding protein (CREB), and the protein expression of CRE-regulatory genes, *c-fos* and *COX-2* in young and senescent HDFs. We observed in senescent cells, an increase in mRNA levels of the catalytic subunit α of PKA and of the major regulatory subunit Iα. Senescence-associated increase of cAMP after LPA treatment correlated well with increased CREB phosphorylation accompanying activation of PKA in senescent cells. In senescent cells, after LPA treatment, the expression of *c-fos* and *COX-2* decreased initially, followed by an increase. In young HDFs, CREB phosphorylation decreased following LPA treatment, and both c-fos and COX-2 protein levels increased rapidly. CRE-luciferase assay revealed higher basal CRE-dependent gene expression in young HDFs compared to senescent HDFs. However, LPA-dependent slope of luciferase increased more rapidly in senescent cells than in young cells, presumably due to an increase of LPA-induced CREB phosphorylation. CRE-dependent luciferase activation was abrogated in the presence of inhibitors of PKC, MEK1, p38MAPK, and PKA, in both young and senescent HDFs. We conclude that these kinase are coactivators of the expression of CRE-responsive genes in LPA-induced HDFs and that their changed activities during the aging process contribute to the final expression level of CRE-responsive genes.

Keywords: aging; cyclic AMP response element-binding protein; cyclic AMP-dependent protein kinases; cyclooxygenase 2; lysophosphatidic acid; proto-oncogene proteins c-fos

Introduction

Lysophosphatidic acid (LPA) is a phospholipid growth factor that elicits a variety of cellular responses, such as changes in cell shape, chemotaxis, proliferation, and differentiation, depending on the cell type (Moolenaar *et al.*, 1997; An *et al.*, 1998b; Moolenaar, 2000). The intracellular biochemical signaling events that mediate these effects of LPA include an increase in cytoplasmic calcium concentration, stimulation of phospholipases, activation of phosphatidylinositol 3-kinase and the Ras-Raf-MAP kinase cascade, and inhibition of adenylyl cyclase (AC) (Moolenaar *et al.*, 1997; Moolenaar, 2000).

Recently, the cell surface G-protein-coupled receptors for LPA were identified as products of a family of endothelial cell differentiation genes (EDGs) (Contos *et al.*, 2000; Fukushima *et al.*, 2001). The major members of this EDG family of proteins were shown to be EDG-2 (Hecht *et al.*, 1996), EDG-4 (An *et al.*, 1998a), and EDG-7 (Bandoh *et al.*, 1999), recently renamed as LPA1, LPA2, and LPA3, respectively. Additional LPA receptors are being discovered. The specific responses of LPA receptors are manifested only when the G-proteins

are coupled to an appropriate receptor. For example, LPA1 is coupled to pertussis toxin-sensitive Giα, LPA2 to both Giα and Gqα (An *et al.*, 1998a), and LPA3 to a pertussis toxin-insensitive G-protein, Gqα (Bandoh *et al.*, 1999). LPA1 and LPA2 inhibit all types of ACs through Gi activation, resulting in the reduction of cAMP (Taussig *et al.*, 1993).

cAMP binds to the regulatory subunits of protein kinase A (PKA), eliciting the release of the catalytic subunits which diffuse through the cytosol and phosphorylate various substrates (Clegg *et al.*, 2000; Hansson *et al.*, 2000; Skalhegg and Tasken, 2000). The cAMP-responsive element binding protein (CREB), a key regulator of gene expression, is a substrate of the activated PKA and becomes activated when phosphorylated on Ser133 (Yamamoto *et al.*, 1988; Gonzalez and Montminy, 1989). Phosphorylation of CREB at Ser133 was induced not only by PKA but also by a variety of other signaling cascades that include protein kinase C (PKC) (Gonzalez *et al.*, 1989; Cardenas *et al.*, 2004), calmodulin kinase (CaMK) (Wu *et al.*, 2001), ERK1/2 (Roberson *et al.*, 1999; Shaywitz and Greenberg, 1999; Wu *et al.*, 2001), p38 mitogen-activated protein kinase (p38MAPK) (Togo, 2004), and mitogen- and stress-activated kinase1 MSK1 (Lee *et al.*, 2003; Schuck *et al.*, 2003).

Phosphorylated CREB drives the transcription of a large number of genes having CRE through interaction with its nuclear partner CREB binding protein (Chrivia *et al.*, 1993). One of these CRE-regulated genes is *c-fos*, a member of the AP1 family of transcription factors that play a key role in normal and aberrant cell growth. Activation of ERK1/2 is required for *c-fos* expression (Cook *et al.*, 1999). LPA also stimulates CREB and subsequently induces c-fos via MAPK signaling components, ERK1/2, p38MAPK, and MSK1, in Rat-2 fibroblasts (Lee *et al.*, 2003) and in mouse embryonic stem cells (Schuck *et al.*, 2003). Cyclooxygenase (COX), the key rate-limiting enzyme in the prostaglandin synthetic pathway, is another CRE-regulated gene. Two isoforms of COX have been identified: a constitutive COX-1 and an inducible COX-2. Activation of ERK1/2 and phosphorylation of CREB are also upstream steps in COX-2 induction (Hahn *et al.*, 2002).

We observed in a previous study, that production of LPA-induced cAMP was altered during the aging of human diploid fibroblasts (HDFs) (Jang *et al.*, 2003). In contrast to increased cAMP production in senescent HDFs, LPA inhibited cAMP production in young HDFs. LPA-induced cAMP accumulation correlated well with the stimulation of AC. Age-related quantitative and qualitative alterations in LPA receptors and downstream signaling molecules including inhibitory Gi proteins and PKC-dependent AC isoforms contribute to cAMP accumulation by LPA treatment in senescent cells (Jang *et al.*, 2003).

In this study, we have shown that LPA differentially modulates the activation of PKA and phosphorylation of CREB, and cause differential expression of CRE-regulated genes such as *c-fos* and *COX-2*, in young and senescent fibroblasts. We also confirmed the involvement of other protein kinases in the expression of CRE-regulated genes by using *in vivo* CRE *cis*-reporting system with luciferase activity and protein kinase inhibitors: bisindolylmaleimide (bIM) for PKC, PD98059 and U0126 for ERK1/2, SB203580 for p38MAPK, H89 for MSK1 as well as PKA.

Materials and Methods

Materials

The following reagents were used in this study: Dulbecco's modified Eagle's medium, LPA and a monoclonal anti-beta actin antibody, from Sigma (St. Louis, MO), fetal bovine serum (FBS), penicillin, streptomycin, and superscript II reverse transcriptase kit, from Gibco/BRL Life Technologies, Inc. (Carlsbad, CA), PCR reagents including *Taq* polymerase, from Takara Shiga (Japan); [γ-^{32}P]ATP, from Amersham Pharmacia Biotech (Buckinghamshire, UK), PathDetect *in vivo* CRE cis-reporting system with luciferase activity, from Stratagene (La Jolla, CA), polyclonal antibodies against total CREB, and COX-2, from Santa Cruz Biotechnology, Inc (Santa Cruz, CA), a monoclonal antibody against phosphorylated CREB, from Cell Signaling Technology (Beverly, MA), an anti-c-fos monoclonal antibody (Clone G54-9.9), from BD Bioscience Pharmingen (Mountain View, CA), PD98059, U0126, SB203580, and H89, from Calbiochem (San Diego, CA).

Cell culture

Primary human fibroblasts were isolated from newborn foreskins as described previously (Boyce and Ham, 1983), and maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and antibiotics. Intracellular components of young cells from the early stage of culture, with population doubling (PD) of less than 25, were compared to those of senescent cells with PD 65-70 cultures. Senescent cells were characterized by morphology, enhanced β-galactosidase activity, and a reduced rate of proliferation (Yeo *et al.*, 2000a). Prior to LPA treatment, the cells were grown for 2-3 days to 60-70% sub-confluency in the culture medium, and then serum-starved to quiescence by incubation with serum free medium containing 0.1% BSA (SFM) for 2 days.

Semi-quantitative RT-PCR

Total RNA was extracted from young and senescent cells using an acid guanidinium thiocyanate phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). In order to compare the amounts of mRNA in young and senescent cells, semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed, as described previously (Nicoletti and Sassy-Prigent, 1996). A series of mixtures of RNA from young and senescent cells was prepared as indicated, so that each mixture had the same total amount of RNA (2 μg) in a constant volume (12 μl). The RT reaction was carried out in a final volume of 20 μl using superscript II reverse transcriptase according to the manufacturer's protocol, and 4 μl of the final RT product mix was then PCR amplified. The primer sets used were: 5'-AGG CAA AAC AGA TTC AG-3' and 5'-CTC CTT CCC CAA CAC TG-3' for PKA RIα; 5'-GAA GGT GAT TCC CAA GGA CTA CAA-3' and 5'-TCC CGG TCG ATC CCC CAG AG-3' for RIβ; 5'-CGA CGC CAA AGG GGA CAG-3' and 5'-GGG GCA CAG ACT CAA TAA ATG-3' for RIIα; 5'-CTG AAC GCC TGA AAG TAG TAG-3' and 5'-GCA GTG GGT TCA ACA ATA TCC-3' for RIIβ; 5'-AGT CCC GCT CAG AAC ACA G-3' and 5'-GAT AAT CTC AGG GGC CAG G-3' for PKA Cα; 5'-TAA TGC CGG ACT TGA AC-3' and 5'-TCT GCC TTT AAC TCT TT-3' for PKA Cβ; and 5'-TAC GCC GAC CAG CCC ATC CAG-3' and 5'-AAC CAC AGG GGC ACA AGC ACA-3' for PKA Cγ.

Measurement of PKA activity

LPA-treated cells were harvested and homogenized in an ice-cold homogenization buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 2 mM EGTA, and protease inhibitors), centrifuged at 20,000 × *g* at 4°C for 20 min, and PKA assays were performed as described previously (Yang *et al.*, 1999). The kinase reaction mixture contained 10 μl of substrate cocktail (500 μM kemptide and 10 μM cAMP), 10 μl of inhibitor cocktail (20 μM PKC inhibitor peptide and 20 μM compound R24571), 10 μl of tissue homogenate, and 10 μl of a mixture containing 0.5 mM ATP, 75 mM $MgCl_2$ and 10 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol). After incubation for 10 min at 30°C, 25 μl of the mixture was blotted onto a P81 paper square. The paper was washed once with 0.75% phosphoric acid and the radioactivity incorporated into Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) was measured using a scintillation counter (Model Tri-Carb 1600 CA, Packard Instrument Company), in triplicate. PKI-inhibitable kinase activity was calculated as percentage of total PKA activity.

CRE binding luciferase assay

Quiescent cells were transfected with PathDetect *in vivo* CRE cis-reporting system with luciferase activity and a plasmid pcDNA with β-galactosidase activity as a control for transfection efficiency. Cells were pretreated with 10 μM of inhibitors of PKC, MEK-1, and p38MAPK, and PKA/MSK1 for 1 h and treated with 30 μM of LPA for the indicated times. Nuclear extracts were assayed for CRE binding luciferase activity as described in the manufacturer's instruction.

Western blot analysis

Expression levels of signaling proteins were examined by Western blot analysis, as described previously (Yeo *et al.*, 2000b). Total cell lysates were prepared in a lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 10 mM NaF, 1 mM DTT, 1 mM PMSF, 25 μg/ml leupeptin, 25 μg/ml aprotinin, 5 mM benzamidine, and 1% Igepal CA630. Protein concentrations of the lysates were determined using a BCA protein assay kit, as described by the manufacturer. Cell lysates containing equal amounts of protein were resolved by SDS-polyacrylamide gel electrophoresis and transferred onto Immobilon PVDF membranes. Blots were blocked with a solution containing 5% non-fat dried milk and 0.1% Tween 20, and treated with antibodies in the blocking solution overnight. Blots were then washed and further incubated with horseradish peroxidase-conjugated anti-rabbit IgGs (1:5,000). The immune complexes were visualized using an enhanced chemiluminescence (ECL) system, as described by the manufacturer.

Statistical analysis

The Graph-Pad Prism (GraphPad, San Diego, CA) was used for statistical analysis. Student's *t*-test was used to assess differences between LPA-treated and untreated (data at time 0) groups for the indicated times. Data are presented as the mean ± standard deviation and *P* values of less than 0.05, 0.01, and 0.001 were marked as *, **, and ***, respectively, in some figures. *P* values of less than 0.05 were considered to be significant.

Results

Altered expression of catalytic and regulatory subunits of PKA in senescent cells

Previously, we reported that levels of cellular components of cAMP signaling systems variously

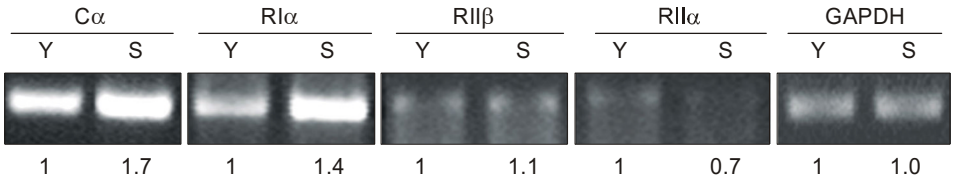

Figure 1. mRNA levels of PKA catalytic and regulatory subunits in young and senescent HDFs. Total RNA was isolated from young (PD 23, Y) and senescent cells (PD 70, S) using an acid guanidinium thiocyanate phenol-chloroform extraction method and the mRNA levels were compared by semi-quantitative RT-PCR. RT-PCR products of the PKA catalytic subunit (Cα) and the regulatory subunits (RIα, RIβ, RIIα) were separated on 1.2% agarose gel, photographed. The density of each band was analyzed using a densitometer and their relative expressions in senescent HDFs were compared to those in young HDFs. This experiment was performed three times and similar results were observed. The result of a typical experiment is shown.

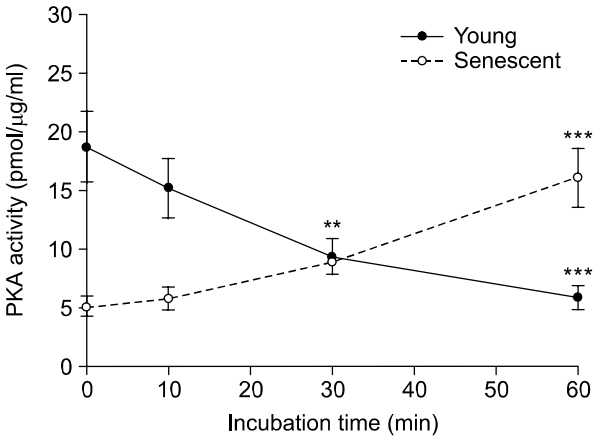

Figure 2. LPA-induced PKA activation profiles in young and senescent HDFs. Subconfluent young (PD 20) and senescent (PD 67) fibroblasts were serum starved for 2 days and treated with 30 μM of LPA for the indicated times. The PKA activities in the total cell lysates were measured as described in "Materials and Methods". The radioactivity incorporated into the kinase substrate was measured using a beta-counter, and the enzyme activity was expressed as pmol/μg/min. This experiment was performed three times and similar results were observed, and the result of a typical experiment is shown. The results shown represent the means ± standard deviation of a representative experiment. $^{**}P < 0.05$ and $^{***}P < 0.001$ in comparison with the control values (at time 0) in each group.

change in HDFs. In this report, we examined the changes in catalytic and regulatory subunits of PKA. We detected specific mRNA transcripts of the human PKA catalytic subunits Cα and Cβ, regulatory subunits RIα, RIβ, RIIα, RIIβ, and GAPDH as a control, by semi-quantitative RT-PCR with specific primers (Figure 1). Young HDFs contained mainly catalytic subunit Cα and regulatory subunit RIα, and to a minor degree RIβ and RIIα. We found that the mRNA levels of major catalytic subunit Cα and the regulatory subunit RIα increased in the aged cells, whereas the levels of RIIα mRNA decreased slightly and that of minor regulatory subunit RIβ did not change (Figure 1).

Altered LPA-induced PKA activation in senescent HDFs

Cellular cAMP decreased in a time- and dose-dependent manner after incubation with LPA in young fibroblasts, whereas cAMP increased in senescent fibroblasts (Jang *et al.*, 2003). To test whether PKA activation by LPA also altered with age, we determined the PKA activity after LPA treatment. As shown in Figure 2, basal PKA activity is much lower in senescent cells than in young cells. Total PKA activity decreased in a time-dependent manner in LPA-treated young cells, but increased in senescent cells.

Altered LPA-induced CREB phosphorylation in senescent HDFs

Once PKA is activated, phosphorylation of CREB, one of substrates of PKA, increases inside the nucleus. We measured the phosphorylation level of CREB by immunoblot analysis with a phospho-specific antibody that recognizes CREB on Ser133. As shown in Figure 3, basal CREB phosphorylation is much higher in young cells than in senescent cells. LPA, at a dose of 30 μM, reduced phosphorylation of CREB at Ser133 after 30 min in young fibroblasts, but stimulated it in senescent fibroblasts in a time-dependent manner. This CREB phosphorylation pattern correlated well with the pattern of cAMP accumulation and the PKA activity profile. These results suggest that the senescence-associated increase of cAMP levels after LPA treatment causes increased CREB phosphorylation.

LPA-induced early gene expression in young and senescent HDFs

Transcription of early genes such as *c-fos* increases after p-CREB activation of a CRE box located upstream. We tested the effect of CREB phosphorylation on CRE-regulated early response transcription

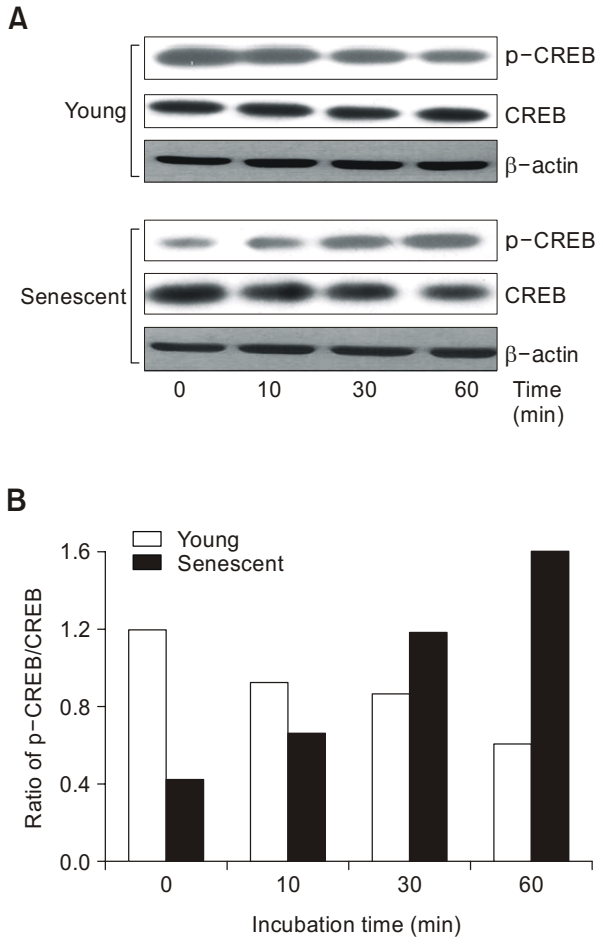

Figure 3. LPA-induced CREB phosphorylation in young and senescent HDFs. Subconfluent young (PD 20) and senescent (PD 67) fibroblasts were serum starved for 2 days and treated with 30 μM of LPA for the indicated times. The lysates containing the same amount of protein (45 μg) were analyzed by immunoblotting using polyclonal anti-CREB antibodies, monoclonal anti-phospho-CREB and anti-β-actin antibodies (A). The density of each band was analyzed using a densitometer and the relative density of phosphorylated CREB (p-CREB) to that of total CREB were calculated and plotted (B). The experiment was performed three times with similar results.

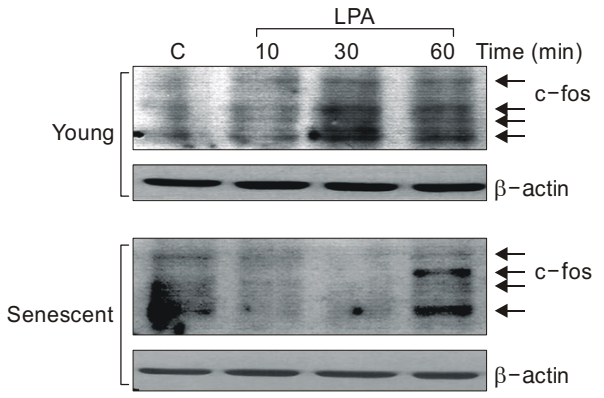

Figure 4. LPA-induced c-fos expression in young and senescent HDFs. Subconfluent young and senescent fibroblasts were treated with 30 μM of LPA for the indicated times. The lysates containing the same amount of protein (45 μg) were analyzed by immunoblotting using polyclonal anti-c-fos antibodies. Four isoforms of c-fos were indicated by arrows. The content of β-actin was also monitored as a control. The experiment was performed three times with similar results.

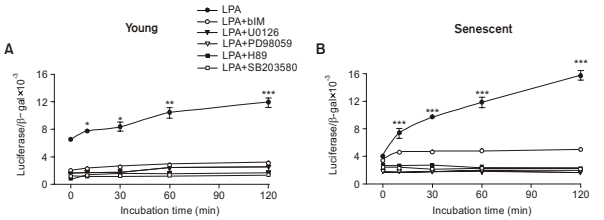

Figure 5. LPA-induced COX-2 expression in young and senescent HDFs. Subconfluent young and senescent fibroblasts were treated with 30 μM of LPA for the indicated times. The lysates containing the same amount of protein (45 μg) were analyzed by immunoblotting using polyclonal anti-COX-2 antibodies. The content of a constitutive isoform COX-1 and β-actin was also monitored as controls. The experiment was performed three times with similar results.

factor c-fos after LPA treatment. Many isoforms of c-fos (e.g., 55 kDa, 57 kDa, 60 kDa, and 62 kDa) have been described, and they could also undergo posttranslational modification (Curran *et al.*, 1985). The purified mouse anti-fos monoclonal antibody recognized c-fos as multiple bands in the area of 50-65 kDa in young and senescent HDFs (Figure 4). Results from three experiments show that 30 μM of LPA significantly increased c-fos protein levels within 30 min in young HDFs. In senescent cells, the expression of c-fos decreased initially until 30 min but increased at 1 h after LPA treatment. We also examined the expression of the key enzyme in prostaglandin synthesis: COX-2, which was shown to be induced by LPA in the other cell systems (Ershov and Bazan, 1999; Hahn *et al.*, 2002; Symowicz *et al.*, 2005). A constitutive isoform COX-1 was also examined as a control. In young HDFs, the inducible isoform COX-2 protein level increased rapidly within 10 min but COX-1 was unaltered (Figure 5). The expression of COX-2 decreased initially until 30 min but increased 1 h after LPA treatment in senescent HDFs.

Effect of kinase inhibitors on LPA-induced CRE-luciferase activity in young and senescent HDFs

In order to confirm the changes in CRE-dependent gene expression induced by LPA, we measured the luciferase activity in HDFs transfected with Path-

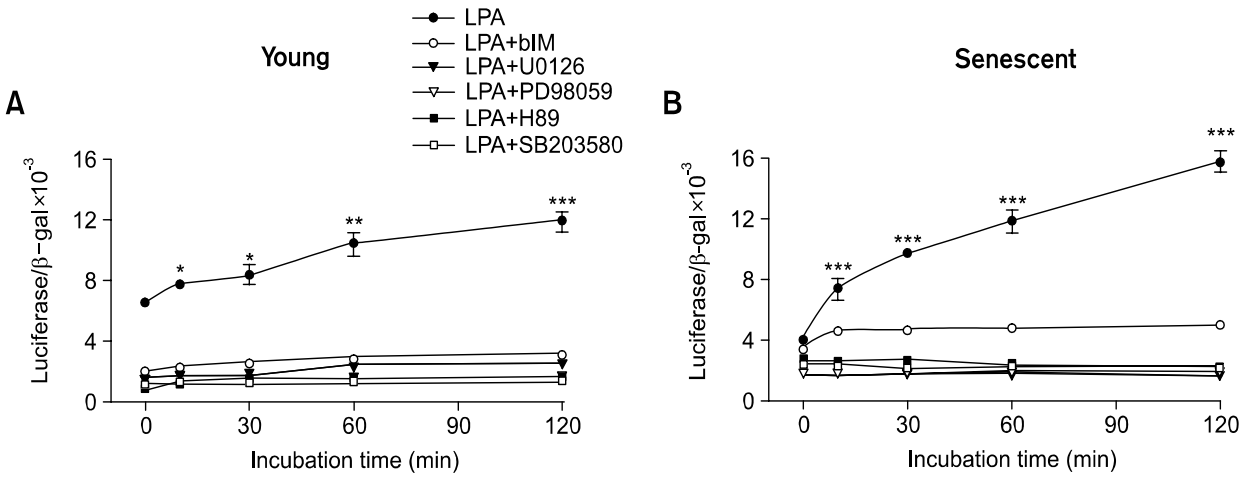

Figure 6. Effect of protein kinase inhibitors on CRE-luciferase activity. Quiescent young (A) and senescent fibroblasts (B) were transfected with PathDetect *in vivo* CRE cis-reporting system with luciferase activity and a plasmid pcDNA with β-galactosidase activity as a control for transfection efficiency. Cells were pretreated with 10 μM of inhibitors to PKC (bisindolylmaleimide, bIM), MEK-1 (U0126 and PD98059), p38MAPK (SB203580), and PKA (H89) for 1 h and then treated with 30 μM of LPA for the indicated times. The activities of luciferase and β-galactosidase were measured in the cell nuclear extract as described in Materials and Methods. The results shown represent the means ± standard deviations of a representative experiment. $^{*}P < 0.01$, $^{**}P < 0.05$ and $^{***}P < 0.001$ in comparison with the control values (at time 0) in LPA group.

Detect *in vivo* CRE cis-reporting system and a plasmid pcDNA with β-galactosidase activity as a control for transfection efficiency. The luciferase assay revealed a high level of CRE-dependent gene expression in the absence of LPA, and LPA treatment slowly increased CRE-dependent gene expression in young HDFs (Figure 6A, -•-). In senescent cells, though the basal luciferase activity was weaker, LPA-stimulated luciferase activity increased more rapidly compared to young cells, indicating an increase of CRE-dependent gene expression (Figure 6B, -•-).

The observed discrepancy between PKA-associated CREB phosphorylation and CRE-dependent gene expression suggests an involvement of other protein kinases. In addition to PKA, PKC, CaMK, ERK1/2 and p38 MAPK have been implicated in CREB phosphorylation and its activation. LPA-stimulated expression of luciferase under the control of the CRE box was examined after pretreatment of the cells with PKC inhibitor, bisindolylmaleimide (Toullec *et al.*, 1991), MAPK/ERK kinase (MEK) inhibitor, PD98059 (Alessi *et al.*, 1995), and U0126 (Duncia *et al.*, 1998), and p38MAPK inhibitor, SB203580 (Clerk and Sugden, 1998), as well as PKA inhibitor, H89 (Engh *et al.*, 1996). Pretreatment of cells with the protein kinase inhibitors for 1 h inhibited the CRE-luciferase activity induced by LPA both in young and senescent HDFs (Figure 6A and 6B). Our data suggest that the cooperative effect of the above kinases may be required for the expression of CRE-responsive genes.

Discussion

Correlation of cAMP status with LPA-induced PKA activation and CREB phosphorylation in young and senescent HDFs

In our previous study, we demonstrated an altered profile of LPA-induced cAMP accumulation in the senescent state (Jang *et al.*, 2003). In this study, we found that the mRNA levels of both the major catalytic subunit Cα, and of the regulatory subunit RIα, also increased in senescent fibroblasts (Figure 1). Our results are in agreement with the findings of Liu *et al*. (Liu *et al.*, 1986), who showed increased levels of PKA in extracts of senescent IMR-90 lung fibroblasts compared to young cells. Blumenthal *et al*. (Blumenthal *et al.*, 1993) also demonstrated a higher PKA activity in parallel with increased PKA protein content in senescent cells, than both in replicating or quiescent cells. Since the regulatory subunit, RIα, also increased in senescent HDFs, it would bind to the catalytic subunits of PKA, resulting in lower PKA activity (Figure 2) and lower CREB phosphorylation (Figure 3) in the absence of LPA. The increased level of PKA holoenzyme may allow the senescent HDFs to respond to LPA sensitively. We also found that PKA profiles after LPA treatment are similar to those of cAMP reported previously (Jang *et al.*, 2003). Total PKA activity, following LPA treatment, decreases in a time-dependent manner in young cells, but increases in senescent cells (Figure 2).

Though it was suggested that a diminished expression of CREB might contribute to the altered cAMP-mediated regulation of gene expression with senescence (Dimri and Campisi, 1994; Chin *et al.*, 1996), we observed that the protein content of CREB was not altered, but its phosphorylation was enhanced, after LPA treatment in senescent cells (Figure 3). Therefore it is unlikely that the altered expression of CREB protein is responsible for the increased cAMP signaling of senescent cells. Our data clearly suggest that activation of cAMP signaling may give rise to robust CREB phosphorylation via PKA activation in senescent HDFs.

Discrepancy of CREB phosphorylation and CRE-regulated Gene expression by LPA

It was previously reported that LPA rapidly stimulated phosphorylation of CREB at Ser133 in a time- and dose-dependent manner in Rat-2 fibroblasts (Lee *et al.*, 2003). LPA was also shown to increase TCF and CREB phosphorylation and subsequent activation of the *c-fos* promoter, resulting in an increase of *c-fos* mRNA about two fold at a concentration of 100 μM in C6 glioma cells (Segura *et al.*, 2005), and about 60 fold increase at a concentration of 20 μM in mouse embryonic stem cells (Schuck *et al.*, 2003). LPA was also shown to increase *COX-2* mRNA expression in retinal pigment epithelium (RPE) cells (Ershov and Bazan, 1999), in renal mesangial cells (Hahn *et al.*, 2002), and in ovarian cancer cells (Symowicz *et al.*, 2005). LPA was shown to contribute, in part, to the development, progression, and metastasis of ovarian cancer by inducing the expression of genes including *COX-2* (Symowicz *et al.*, 2005). However, unlike other cell systems, young HDFs showed a time-dependent decrease in CREB phosphorylation after treatment with 30 μM LPA (Figure 3). Moreover, the CRE-regulated gene expression did not correlate well with CREB phosphorylation in young HDFs. CRE-regulated gene products, *c-fos* and *COX-2*, increased in young HDFs (Figure 4 and Figure 5, respectively). In contrast, a time-dependent increase in CREB phosphorylation was observed in senescent HDFs (Figure 3) and the induction profiles of *c-fos* (Figure 4) and *COX-2* (Figure 5) are different from those of young cells-decreasing initially (at 30 min) but increasing later. This discrepancy between CREB phosphorylation and CRE-regulated gene expression suggest the possible operation of other mechanisms of age-dependent transcriptional control than the PKA system, depending on the type and age of the cell.

Protein kinases involved in CREB phosphorylation and CRE-regulated gene expression

Sequence analysis of CREB cDNA predicts a cluster of PKA, PKC and casein kinase II consensus recognition sites near the N terminus of the protein (Gonzalez *et al.*, 1989). The proximity of these potential phosphorylation sites indicates that they may interact either positively or negatively to regulate CREB bioactivity (Gonzalez *et al.*, 1989). Indeed, involvement of several protein kinases including PKA, PKC, CaMK and MAPK signaling components on CREB phosphorylation and CRE-regulated gene expression has been reported.

It was suggested that MAPK cascade couples PKA and PKC to CREB phosphorylation in area CA1 of rat hippocampus (Roberson *et al.*, 1999). Activation of ERK1/2 is required for *c-fos* expression (Cook *et al.*, 1999) and is an upstream step in COX-2 induction (Hahn *et al.*, 2002). LPA also stimulates CREB *via* MAPK signaling components, ERK1/2 and p38MAPK, in Rat-2 fibroblasts (Lee *et al.*, 2003). The likely agent of coupling MAPK and CREB phosphorylation was suggested to be MSK1 (Schuck *et al.*, 2003), which is activated by both ERK1/2 and p38MAPK and is required for induction of *c-fos* by LPA in mouse embryonic stem cells (Schuck *et al.*, 2003) and Rat-2 fibroblasts (Lee *et al.*, 2003). Phenylephrine stimulates phosphorylation and DNA binding activity of CREB in adult rat ventricular myocytes through multiple signaling pathways involving ERK1/2, p38MAPK, MSK1 and PKA (Markou *et al.*, 2004). Forskolin-induced CREB phosphorylation and activation in NIH 3T3 cells was shown to be mediated directly by PKA and by a delayed PKA-dependent p38/MSK-1 pathway (Delghandi *et al.*, 2005). In our study, we also found that inhibition of MAPK signaling components (ERK1/2 with PD98059 and U0126, of p38MAPK with SB203580) efficiently blocked LPA-induced CRE-luciferase activity in both young and senescent HDFs (Figure 6). These data suggest that MAPK signaling components are required for CRE-regulated gene expression by LPA in HDFs.

Mitogenic doses of LPA induce sustained MAPK activation and DNA synthesis in Rat-1 fibroblasts (Cook *et al.*, 1999). However, activation of the MAPK cascade alone is not sufficient to induce *c-fos* expression, which requires cooperation with other signals such as Ca^{2+} mobilization (Cook *et al.*, 1999). In fact, the induction of *c-fos* observed in response to LPA was strongly inhibited by buffering the intracellular [Ca^{2+}] (Cook *et al.*, 1999). Calcium release from intracellular stores promotes calcium wave propagation into the nucleus, which is critical for CREB-mediated transcription by synaptic NMDA receptors (Hardingham *et al.*, 2001). Studies on the role of Ca^{2+}/cAMP-dependent signal transduction

and transcription factor CREB in SK-N-MC cells also demonstrated that the NPY Y(1) receptor induces the expression of CRE containing target genes through the CaM kinase-CREB pathway, and inhibits them when cellular cAMP levels are elevated in SK-N-MC cells (Sheriff *et al.*, 2002). Wu *et al.*, who focused on the kinetic contributions of various signaling pathways to the overall time course of CREB phosphorylation, reported that phosphorylated CREB formation was dominated by the CaMK pathway up to 10 min, and by the MAPK pathway at 60 min (Wu *et al.*, 2001). The two pathways acted in concert at 30 min. It was also demonstrated that PKC mediates the stimulation of CREB-dependent gene expression through p38MAPK pathway upon membrane disruption (Togo, 2004).

Previously, we reported that LPA-triggered cytosolic Ca^{2+} signals were partially reduced but conserved during the aging process (Yeo *et al.*, 2002). Ca^{2+}-dependent PKC α/β are activated with different time courses both in young and senescent HDFs (data in press). We now find that inhibition of PKC with bisindolylmaileimide efficiently blocked LPA-induced CRE-luciferase activity in both young and senescent HDFs (Figure 6). Activation of Ca^{2+} signaling in senescent cells will initially affect Ca^{2+}-dependent protein kinases such as PKCα/β and CaMK, and will in turn interact with other protein kinases such as ERK1/2, p38MAPK and MSK1 as well as PKA for the expression of CRE-regulated genes.

The *c-fos* promoter contains several regulatory *cis*-elements, namely a serum response element (SRE) and AP-1-like site, in addition to a CRE. These promoter elements are interdependent and thus connected by protein complexes (Robertson *et al.*, 1995). SRF with TCF phosphorylation is required for SRE-regulated c-fos induction (Treisman, 1995) and CREB and ATF1 phosphorylation and subsequent recruitment of the co-activator CREB binding protein (CBP) are required for CRE-regulated c-fos induction. The above mentioned protein kinases are also involved directly or indirectly in TCF phosphorylation for SRE activation, and ATF1 phosphorylation for CRE activation in addition to CREB phosphorylation.

COX-2 was shown to be induced by LPA in the several cell systems (Ershov and Bazan, 1999; Hahn *et al.*, 2002; Symowicz *et al.*, 2005). We also observed LPA-induced *COX-2* induction especially in young HDFs. The *COX-2* induction mechanism is also complicated by the promoter with several regulatory elements in addition to CRE, which include nuclear factor kappaB (NF-κB), CCAAT/enhancer-binding protein (C/EBP), NF-IL-6, Pea-1, myb, xenobiotic-response element, Pea-3/SP-1 (GenBank #D28235). LPS was shown to induce activation of JNK-mediated AP-1 and ERK/p38 kinase-mediated CREB or C/EBPdelta, which bind the core elements of *COX-2* promoter (Uto *et al.*, 2005). UVB induces *COX-2* expression via activations of p38MAPK and JNK, and thus DNA binding activity of AP-1 transcription factor in human keratinocytes HaCaT cells (Cho *et al.*, 2005). It was suggested that the age-associated and ceramide-induced increase in *COX-2* transcription is mediated through higher NF-κB activation, because of a greater I κB degradation in old macrophages (Wu *et al.*, 2003). Aging can also modulate differentially these upstream kinases and transcription factors after LPA treatment, which might explain the discrepancy between LPA-induced CREB phosphorylation and *COX-2* gene expression in the two age groups of HDFs.

Taken together, our study found a senescence-associated increase of cAMP after LPA treatment, which correlates well with increased CREB phosphorylation accompanying activation of PKA in senescent cells. However, CREB phosphorylation does not correlate well with the CRE-responsive gene expression. Most of CRE-responsive genes such as *c-fos* and *COX-2* contain a complex mix of promoters which interact with a variety of transcription factors in addition to CREB. These transcription factors could be modulated by various upstream protein kinases whose activities are also altered during aging. Further studies are needed to clarify the influence of age-related alteration of cAMP signaling at the transcriptional level.

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