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γ-Linolenic Acid Methyl Ester from *Rhizopus oryzae* KSD-815 Isolated from *Nuruk* Induces Apoptosis in Prostate Cancer LNCaP Cells

Sang-Jin Lee[1,2], Sungyoul Hong[2], and Gye-Won Kim[1]*

[1]*Research Laboratories, Kooksoondang Brewery Co., Ltd., Seongnam 462-120, Republic of Korea*
[2]*Department of Genetic Engineering, Sungkyunkwan University, Suwon 440-746, Republic of Korea*

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Gamma-linolenic acid methyl ester (methyl GLA) was purified from the cultures of *Rhizopus oryzae* KSD-815, which is a filamentous fungus and has been used to make Korean traditional alcoholic beverage. In the present study, molecular mechanisms by which methyl GLA induces apoptosis in human prostate cancer cells, LNCaP, were investigated. It was found that methyl GLA mediated-apoptosis was provoked by down-regulation of mammalian target of rapamycin (mTOR) pathway, resulting from activation of AMP-activated protein kinase (AMPK) pathway and inactivation of Akt. On the other hand, a marked increase of p38 mitogen-activated protein kinase (MAPK) phosphorylation was observed within 30 min after methyl GLA treatment. Although p38 MAPK specific inhibitor, SB203580, completely blocked caspase-9 activation but not caspase-8, p38 MAPK activation was not affected by the broad caspase inhibitor, *Z*-VAD-*FMK*, which nevertheless prevented methyl GLA-provoked apoptosis. Interestingly, interference of methyl GLA-mediated p38 MAPK with SB203580 attenuated inactivation of mTOR, independent of AMPK pathway. Results indicate that methyl GLA can independently utilize two different signaling pathways for inducing the apoptosis cascade, and p38 MAPK activation is a specific regulatory role in methyl GLA-provoked apoptosis, enhancing the action mechanisms by fermentation metabolites to regulate cell death.

Key words: apoptosis, γ-linolenic acid methyl ester, mTOR pathway, *Nuruk*, P38 MAPK, prostate cancer, *Rhizopus oryzae* KSD-815

The mammalian target of rapamycin (mTOR) is a highly conserved serine/threonine kinase and its activation requires positive signals from both nutrients and growth factors [Fingar and Blenis, 2004]. The mTOR complex 1 (mTORC1) is nutrient sensitive, acutely inhibited by rapamycin, and functions as a master regulator of cell growth, angiogenesis, and metabolism [Sabatini, 2006]. Although precise mechanisms are unknown, mTOR-mediated alterations in protein synthesis, aberrant cell cycle signaling, and inhibition of apoptosis may all play causal roles [Hay and Sonenberg, 2004]. Phosphatidylinositol-3-kinase (PI3K)/Akt has been implicated in the activation of the mTOR protein kinase. One critical target of Akt that regulates mTOR is the tumor suppressor protein, tuberous sclerosis complex 2 (TSC2). The LKB1/AMP-activated protein kinase (AMPK) pathway is linked to tumor growth and proliferation through the regulation of the mTOR pathway [Shaw *et al*., 2004]. AMPK directly phosphorylates TSC2, negatively regulating mTOR activity to control tumor growth and proliferation [Inoki *et al*., 2003]. Regulation of TSC2 by AMPK is independent of the Akt pathway, which phosphorylates and inactivates TSC2 [Inoki *et al*., 2002]. Because the regulatory pathway of mTOR activity is often deregulated in human cancer cells, mTOR has been considered for use in the target of cancer treatment and prevention, and the ability of mTOR inhibitors to attenuate actions of pro-growth, pro-proliferation, and pro-survival has generated much interest. Based on its regulation of mTOR, the activation of the LKB1/AMPK pathway could be a mechanism to prevent the formation and progression of cancer [Guertin and Sabatini, 2005].

Mitogen-activated protein kinases (MAPKs) transduce signals from cell membrane to nucleus and are a family of serine/threonine kinases. MAPKs control fundamental cellular processes, including cell growth, proliferation, differentiation, migration, and apoptosis [Bradham and

*Corresponding author
Phone: +82-31-739-5381; Fax: +82-31-739-5382
E-mail: kgw@ksdb.co.kr

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McClay, 2006; Dhillon *et al*., 2007]. Recently, evidence has emerged that p38 MAPK, one of the MAPKs, play a crucial role in both the promotion and inhibition of apoptosis [Galan *et al*., 2000; Horowitz *et al*., 2004]. The regulation of cell survival by p38 MAPK is probably cell-specific and generally dependent on the stimulus, which will ultimately lead to different profiles of enzyme activation. The tumor suppressive effects of p38 MAPK appear to be mediated in several different ways such as the activation of p53-induced apoptosis and a negative regulator of cell cycle progression [Shimada *et al*., 2003]. The molecular mechanisms that determine whether p38 MAPK signaling either promotes or inhibits cell proliferation and survival have not been elucidated.

Gamma-linolenic acid (GLA) is an n-6 polyunsaturated fatty acid (PUFA) and essential for the formation of metabolites from precursor essential fatty acid. GLA was reported to induce lipid peroxidation in tumor cells and lead to altered mitochondrial metabolism and ultrastructure, cytochrome c release, caspase activation, and apoptosis [Colquhoun and Schumacher, 2001]. Studies on the C6 rat glioma and primary culture model have shown GLA inhibits cell proliferation, induces apoptosis, and directly affects both tumor cell cycle progression and angiogenesis [Miyake *et al*., 2009]. In addition, GLA suppresses the expression of the oncogenes *ras*, and *bcl-2*, and enhances the activity of p53. Tumoricidal action of GLA was seen irrespective of the form (free acid, ethyl or methyl ester) in which GLA was added to the tumor cells [Das, 2004]. However, the cellular mechanism and targets of GLA in cancer cells, including prostate cancer, remain unknown.

Methyl GLA, a γ-linolenic acid methyl ester, was purified from the cultures of *Rhizopus oryzae* KSD-815 isolated from *Nuruk*, which has been used to make traditional fermented alcoholic beverages in Korea. In the present study, the capability of methyl GLA to cause cell death in human prostate cancer cells, LNCaP, and the pathways involved in the molecular mechanisms of the methyl GLA-mediated apoptotic system were assessed.

Materials and Methods

Reagents. Propidium iodide (PI), [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT), 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI), and SB203580 were purchased from Sigma (St. Louis, MO). *Z*-VAD-*FMK* and *Z*-ITED-*FMK* were obtained from CalBiochem (La Jolla, CA). Antibodies against phospho-AMPK (Thr^{172}), pan-AMPKa, phospho-PI3K (Tyr^{458}/Tyr^{199}), PI3K, phospho-Akt (Ser^{473}), Akt, phospho-mTOR (Ser^{2448}), mTOR, phospho-ACCa (Ser^{79}), ACCa, cleaved form of caspase-3 and poly-ADP ribose polymerase (PARP), full-length-caspase-3, and PARP were obtained from Cell Signaling Technology (Beverly, MA). LKB1, caspase-8, caspase-9, phospho-p38 (Thr^{180}/Tyr^{182}), p38, phospho-p70S6K (Thr^{389}), and p70S6K were obtained from Millipore (Bedford, MA). TSC2 and a-tubulin were from Santa Cruz Biotechnology (Santa Cruz, CA). Fetal bovine serum (FBS) and cell culture media were from Gibco-BRL (Grand Island, NY).

Cell culture and viability assay. The human prostate cancer cell line, LNCaP, was kindly provided by Prof. Park, J.S. (Chungnam National University, Daejeon, Korea). LNCaP cells were cultured in RPMI 1640 supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL Streptomycin at 37°C and 5% CO_2. Cell viability was quantified using MTT colorimetric assay. Briefly, cells were seeded in a 96-well plate (2×10^4 cells/well) overnight and treated with methyl GLA for 24 h. MTT solution was added to the wells, and cells were incubated for 4 h at 37°C. The optical density was measured at 540 nm.

Extraction of *Rhizopus oryzae* KSD-815 and isolation of γ-linolenic acid methyl ester. The dried and powdered *Nuruk* (8 kg) were extracted with 80% aqueous methanol (MeOH) three times at room temperature. The extracts were successively partitioned with water, ethyl acetate (EtOAc) and *n*-butanol. The EtOAc extract was suspended in 80% MeOH and partitioned with *n*-hexane. From the *n*-hexane fraction, a compound was isolated through repeated silica gel and ODS column chromatographies. The chemical structure of the compound was determined as methyl GLA (**1**) on the basis of the interpretation from NMR (Varian, Palo Alto, CA) and GC/MS (JMS-700, JEOL, Tokyo, Japan), and confirmed by comparing the molecular ion peaks $[M]^+$ and fragmentation ion peaks with those of the Wiley Library in the GC/MS experiment and literature data.

GC/MS analysis. A sector mass spectrometer was interfaced with Hewlett-Packard 6890 gas chromatograph (Santa Clara, CA) equipped with an on-column injector (J&W Scientific, Folsom, CA). A DB-5 capillary column (30 m×0.32 mm i.d., film thickness 0.25 mm) was used. The injector and transfer line temperatures were kept at 230 and 270°C, respectively. The carrier gas helium was used at a constant flow rate of 1 mL/min. The GC oven program was started at 100°C (hold time 3 min), and raised 10°C/min to 200°C, and finally 5°C/min to 250°C (hold time 5 min). Mass spectra (*m*/*z* 30-400) were recorded at a rate of five scans per second with ionization energy of 70 eV. The temperature of the ion source was 250°C. Methyl GLA was identified by comparing *m*/*z* values on the mass spectra with those in the library of Wiley/NBS, and the retention time (11.51 min) on the

chromatogram was compared with that of authentic methyl GLA.

DAPI staining. LNCaP cells were plated in Lab-Tek II chamber slides (Nalge Nunc International, Naperville, IL) at 1.0×10^5 cells per chamber, incubated overnight, and exposed to methyl GLA. After 24-h treatment, DAPI was added to the culture medium of the living cells and changes in nuclear morphology were detected by fluorescence microscopy.

DNA fragmentation assay. Approximately 1×10^6 cells were lysed in buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, and 0.5% Triton X-100). The cell lysate was incubated with proteinase K for 60 min at 37°C and cleared by centrifugation at 13,000×*g* for 20 min. DNA in the supernatant was extracted with an equal volume of a mixture of phenol:chloroform:isoamylalcohol (25:24:1) at room temperature for 15 min, and precipitated with an equal volume of isopropyl-alcohol and 1/10 volume of 5 M sodium chloride overnight at −20°C. The precipitates were spun down at 13,000×*g* for 20 min and washed once with ice-cold 70% ethanol. The air-dried DNA pellets were resuspended in TE buffer containing 5 mg/mL of RNase and incubated for 10 min at 65°C. Fragmented DNA was analyzed on 1.5% agarose gels in the presence of 0.5 mg/mL ethidium bromide.

Annexin V/PI staining assay. Annexin V/PI double staining was carried out with the Annexin V-FITC Apoptosis Detection kit (BD Bioscience, San Diego, CA) per the manufacturer's protocol, and samples were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) and CellQuest 2.0 analysis software.

Western blotting analysis. LNCaP cells were harvested and lysed with a 25 mM Tris-HCl buffer containing protease inhibitors (1 mM PMSF, 5 μg/mL aprotinin, 5 μg/mL pepstatin A, 5 μg/mL leupeptin, and 1.0 mM EDTA). Protein concentrations were quantified using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Protein (20-30 μg) was subjected to 7.5 to 10% SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore).

Blots evaluated with the SNAP i.d.™ Protein Detection System were processed as recommended in the user guide (Millipore). Briefly, after the blot holders containing the blots were placed in the SNAP i.d. system, a blocking buffer (0.1% non-fat dried milk) was added, and the vacuum was immediately activated. Primary antibodies diluted in the blocking buffer were added to the blot holders and incubated for 10 min at room temperature. The vacuum was initiated, and the blots were washed three times with TBS/T (20 mM Tris, 500 mM NaCl, and 0.1% Tween 20, pH 7.5). After the vacuum was turned off, the blots were incubated for an additional 10 min at room temperature with horseradish peroxidase (HRP)-labeled secondary antibodies. The vacuum was activated once again, and the blots were washed three times with TBS/T. For chemilunimescence detection, the probed blots were incubated for 5 min with Immobilon Western HRP substrate (Millipore).

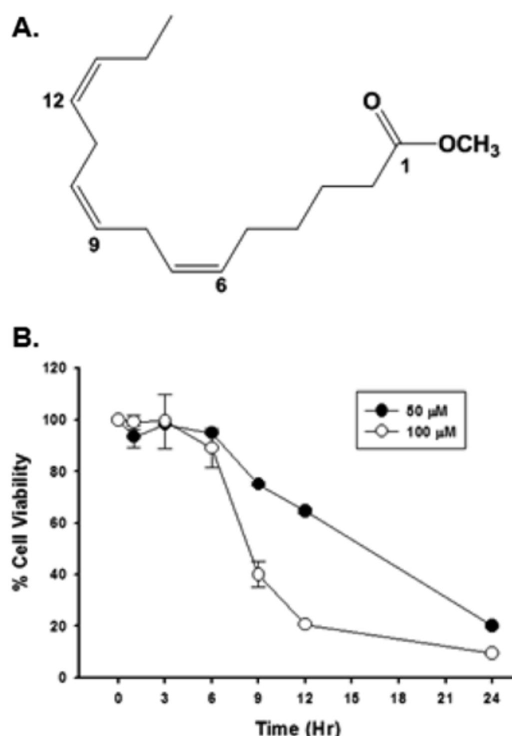

Fig. 1. Methyl GLA purified from *Rhizopus oryzae* inhibits human prostate cancer cell growth. (A) Chemical structure of methyl GLA. (B) LNCaP cells were seeded at 2×10^4 cells per well in a 96-well plate, and methyl GLA was added. Viability was measured by MTT assay after 24 h. The results are the means±SD of three independent experiments.

Results

Identification and anti-proliferative effect of methyl GLA on prostate cancer cells. Methyl GLA was obtained as a yellow oil with physical properties of EI/MS *m/z*: 292 $[M]^+$, 243, 194, 175, 150, 107, 93, 87, 97, 67, 55, 41, 29; ^{1}H-NMR (400 MHz, $CDCl_3$, δ_H): 5.21 (6H, m, olefine-H), 3.63 (3H, s, OCH_3), 2.80 (2H, dd, *J*=6.4, 6.8 Hz), 2.78 (2H, dd, *J*=6.6, 6.4 Hz), 2.30 (2H, t, *J*=8.4 Hz), 2.07 (2H, m), 2.03 (2H, dt, *J*=6.6, 6.7 Hz), 1.41 (2H, m), 1.36 (2H, m), 1.30 (2H, m), 1.29 (2H, m), 1.27 (2H, m) (methylenes), 0.94 (3H, t, *J*=7.8 Hz, terminal methyl); ^{13}C-NMR (100 MHz, $CDCl_3$, δ_C): 174.9 (ester), 131.6, 130.6, 128.7, 128.6, 127.2, 126.4 (olefins), 51.5 (OCH_3), 34.9, 29.5, 29.4, 29.4, 29.3, 27.4, 25.6, 25.5, 24.9, 20.7

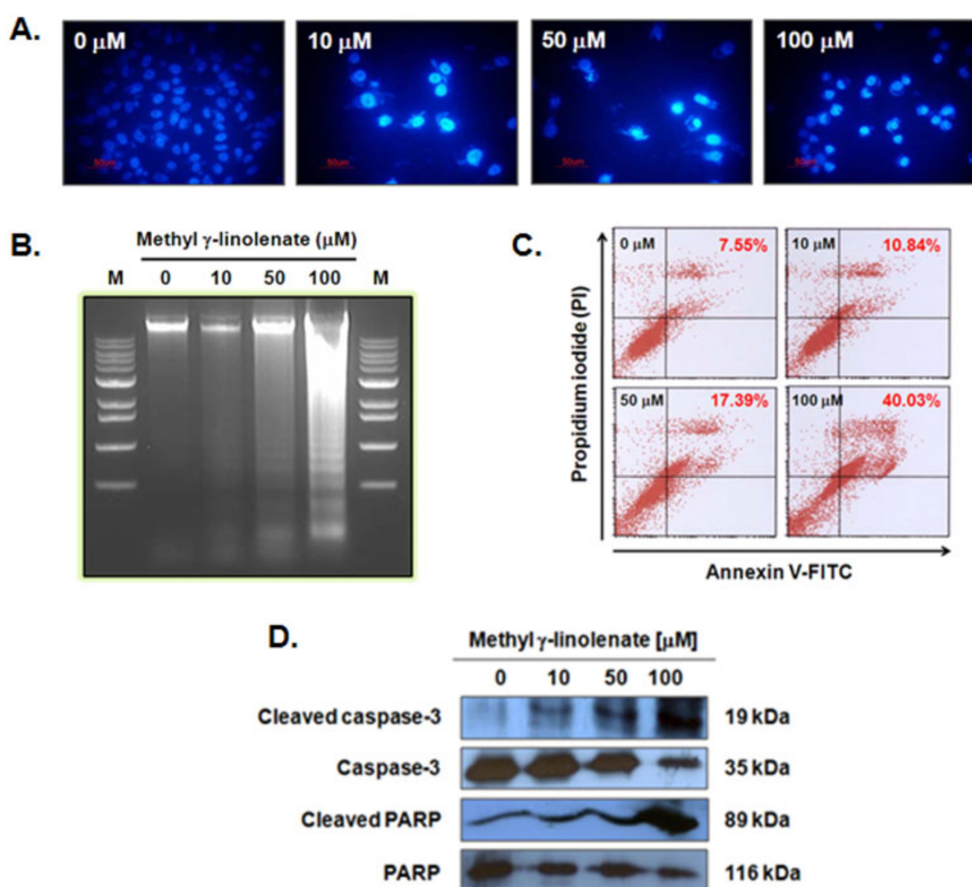

Fig. 2. Methyl GLA induces caspase-3-mediated apoptosis. (A) Fluorescence microscopic examination of untreated or methyl GLA-treated at the indicated concentrations for 24 h followed by DAPI staining. (B) LNCaP cells were treated with methyl GLA for 24 h. Total DNA was extracted and visualized under UV light. (C) Flow cytometry analysis of LNCaP cells treated with methyl GLA and labeled with Annexin V. The percentage of cells with dual-positive labeling for Annexin V and PI is indicated for each concentration. (D) LNCaP cells were treated with methyl GLA for 24 h. Cells were harvested, lysed, and subjected to Western blot analysis using anti-caspase-3, cleaved caspase-3, PARP or cleaved PARP antibodies. The results are the means±SD of three independent experiments.

(methylenes), 14.4 (terminal methyl). The structure of methyl GLA was confirmed by comparison of the spectroscopic data with the previously published data (Fig. 1A) [Gunstone, 1993].

To confirm the effect of methyl GLA on cell proliferation, the effect of methyl GLA on the growth of LNCaP cells first assessed using the MTT assay. Fig. 1B shows that there is a dose- and time-dependent decreases of cell viability. EC_{50} values of methyl GLA were 50 and 100 μM at 16 and 8 h, respectively.

Methyl GLA induces caspase-mediated apoptosis. In order to determine whether methyl GLA induces apoptosis in LNCaP cells, DAPI staining and DNA fragmentation assay were performed. The control cells displayed intact nuclear structure, whereas methyl GLA induced chromatin condensation and formation of apoptotic bodies (Fig. 2A). LNCaP cells treated with methyl GLA presented a characteristic ladder pattern of internucleosomal fragmentation in a dose-dependent manner (Fig. 2B). When an Annexin V/PI staining assay was performed, the cell population showed 40.03% of the dual positive region in 100 μM methyl GLA (Fig. 2C). Subsequently, the cleavage of caspase-3 and the subsequent proteolytic cleavage of PARP in this cell line were investigated. As revealed by Western blot analysis, methyl GLA induced a significant increase in the cleaved form of caspase-3 and a dose-dependent cleavage of PARP (Fig. 2D). These results show that apoptosis is implicated in the methyl GLA-induced inhibition of proliferation.

Methyl GLA-induced AMPK activation contributes to down-regulation of mTOR pathway. Because PI3K/Akt is one of the most important pathways regulating cell proliferation [Shaw *et al*., 2004], the effects of methyl GLA on the PI3K/Akt pathway were examined. Results showed methyl GLA treatment significantly reduced the PI3K activity and the phosphorylation of Akt in LNCaP

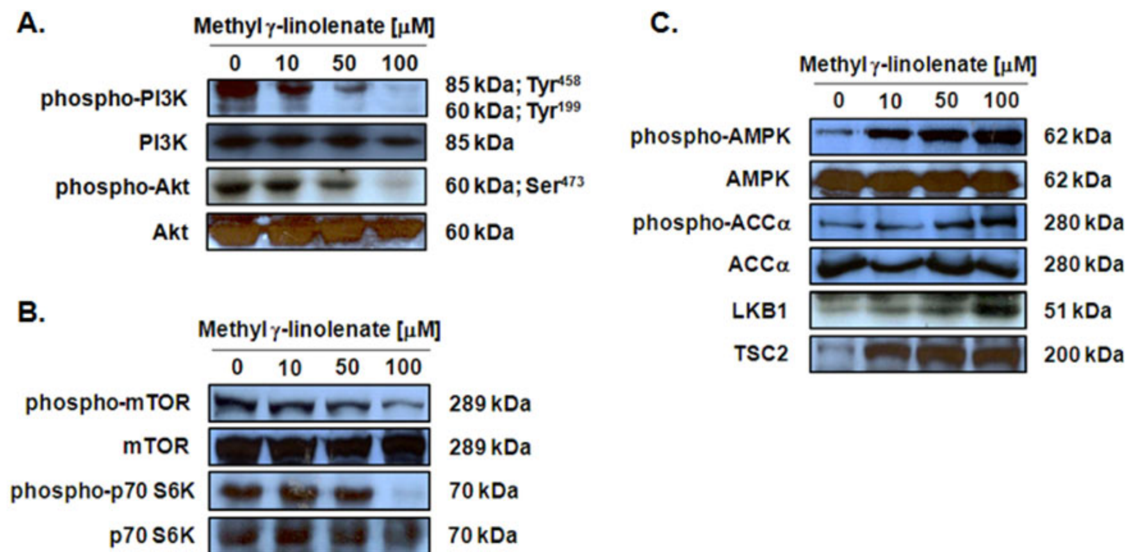

Fig. 3. Activation of AMPK by methyl GLA induces inactivation of mTOR pathway. LNCaP cells were treated with methyl GLA for 24 h. Cells were harvested, lysed, and subjected to Western blot analysis. (A) Western blotting was performed using antibodies specific for phosphorylated and total PI3K or Akt. (B) Phosphorylation of mTOR and p70S6K as well as total levels of mTOR and p70S6K were assessed. (C) Western blotting was performed using antibodies specific for phosphorylated and native AMPK, upstream kinase of AMPK (LKB1), a downstream substrate of AMPK (TSC2) or phosphorylated and native ACCa.

cells (Fig. 3A). Akt has been reported to activate the mTOR pathway by directly phosphorylating and inactivating TSC2 [Inoki *et al*., 2002; Li *et al*., 2004]. Thus, phosphorylation of mTOR and its downstream substrate, p70S6K, were analyzed, and as expected, methyl GLA down-regulated the phosphorylation of mTOR and p70S6K (Fig. 3B).

Inoki *et al*. [2003] reported that activated AMPK by energy starvation phosphorylates TSC2 and increases the activity of TSC1/TSC2 complex to inhibit mTOR pathway. To investigate if AMPK activation is responsible for the anti-proliferative effect observed by methyl GLA, the activation of the AMPK pathway was evaluated by Western blot analysis (Fig. 3C). Methyl GLA markedly activated AMPK in LNCaP cells, as was evidenced by the phosphorylation level at Thr^{172} in the active site of AMPK and Ser^{79} of acetyl-CoA carboxylase a (ACCa), a well characterized AMPK cellular target. Activation of AMPK was induced by the increased expression of LKB1, and up-regulated a down-stream substrate, TSC2, indicating that methyl GLA enhances the interaction with the endogenous AMPK pathway, and the attenuation of the Akt/mTOR pathway may be one of the mechanisms by which AMPK negatively regulates cell proliferation.

Methyl GLA-induced inactivation of mTOR is caspase-dependent. To determine whether caspase activities are required for the inactivation of mTOR pathway during anti-proliferative process triggered by methyl GLA, the effect of a broad spectrum caspase inhibitor, *Z*-VAD-*FMK*, on the methyl GLA-induced inactivation of mTOR was examined by Western blot analysis with specific antibody. *Z*-VAD-*FMK* prevented methyl GLA-induced inactivation of mTOR (Fig. 4). This result strongly suggests that inactivation of mTOR pathway by methyl GLA is dependent on caspase activation, and that caspase activation might indirectly regulate mTOR pathway in methyl GLA-suppressed cell proliferation.

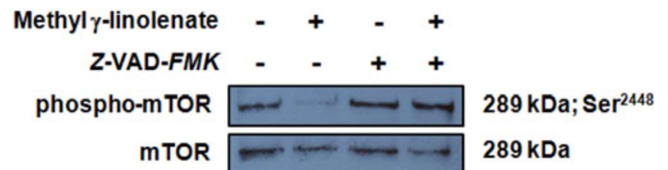

Fig. 4. Methyl GLA induces caspase-dependent inactivation of mTOR. LNCaP cells were treated with methyl GLA for 2 h in the presence or absence of 20 μM of *Z*-VAD-*FMK*. Phosphorylated mTOR was analyzed by Western blot analysis. The data represent a typical experiment conducted two times with similar results.

Activation of p38 MAPK is independent of caspase activity. To determine the role of p38 MAPK in methyl GLA-induced apoptosis, the activation status of p38 MAPK was analyzed with antibody specific to the phosphorylated form. Treatment with methyl GLA resulted in a dramatic increase of the phosphorylated form of p38 MAPK that was apparent at 30 min, peaked at 2 h after methyl GLA treatment, and disappeared after

Fig. 5. Methyl GLA induces activation of p38 MAPK, independent of caspase. (A) LNCaP cells were treated with 100 μM methyl GLA for the time periods indicated. Western blot analysis was carried out using antibodies against phosphorylated or total form of p38 MAPK. (B) LNCaP cells were treated with methyl GLA for 2 h in the presence or absence of 20 μM of *Z*-VAD-*FMK*. Expression of phosphorylated or total p38 MAPK was analyzed by Western blot analysis. The data represent a typical experiment conducted two times with similar results.

4 (Fig. 5A). In contrast, the level of the phosphorylated form of ERK did not alter over the time course examined (data not shown). These results suggest that methyl GLA can selectively induce activation of p38 MAPK during the apoptotic process.

Activation of p38 MAPK has been reported to occur prior to caspase activation during the apoptotic process induced by certain stimuli [Galan *et al*., 2000]. To determine whether caspase activities are required for the p38 MAPK activation in methyl GLA-mediated apoptosis, the effect of *Z*-VAD-*FMK* on the p38 MAPK activation by methyl GLA was examined by Western blot analysis. Results showed methyl GLA-induced p38 MAPK activation was not inhibited by *Z*-VAD-*FMK* (Fig. 5B). However, *Z*-VAD-*FMK* completely blocked the methyl GLA-induced caspase activation and apoptotic cell death (data not shown). These results strongly suggest that induction of p38 MAPK activity by methyl GLA is independent of caspase activation, and it is likely to be upstream events of caspase activation in methyl GLA-induced apoptosis cascade.

Activation of p38 MAPK is required for methyl GLA-mediated apoptosis. Using p38 MAPK inhibitor, SB203580, the effect of p38 MAPK inhibition on apoptotic cell death induced by methyl GLA was investigated, as measured by apoptosis and DNA fragmentation assay.

Fig. 6. Inhibition of p38 MAPK suppresses methyl GLA-induced apoptosis. LNCaP cells were treated with 100 μM methyl GLA for 2 h in the presence or absence of 20 μM SB203580, that was pre-incubated for 1 h. (A) Cell viability was measured by MTT assay. (B) Total DNA was extracted and visualized under UV light. (C) Western blot analysis carried out using antibodies against phosphorylated or total form of p38 MAPK, caspase-3, -8, and -9. (D) LNCaP cells were treated with methyl GLA for 8 h in the presence or absence of 20 μM *Z*-ITED-*FMK* pre-incubated for 1 h. LNCaP cells were harvested, lysed, and subjected to Western blot analysis using anti-caspases 8 and 9.

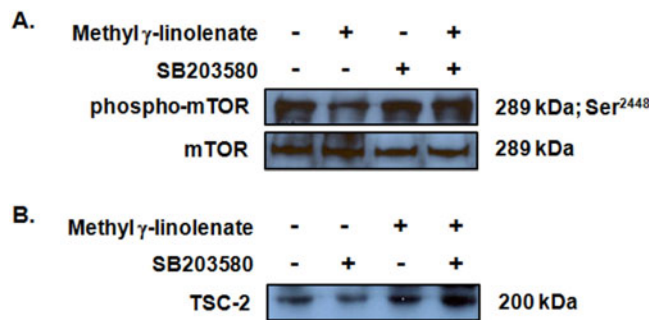

Fig. 7. Activation of p38 MAPK down-regulates mTOR pathway but not up-regulation of TSC-2 during methyl GLA-mediated cell death. LNCaP cells were treated with methyl GLA for 2 h in the presence or absence of 20 μM SB203580. Western blot analysis was performed using antibodies against phosphorylated or total form of mTOR (A) and TSC-2 (B).

Pre-incubation with SB203580 markedly inhibited apoptotic cell death and DNA fragmentation (Fig. 6A and B). These results indicate that p38 MAPK is involved in the apoptotic process induced by methyl GLA. Thus, the effect of p38 MAPK inhibition on the caspase activation was examined. Treatment with SB203580 did not alter the caspase-8 cleavage, but completely blocked caspase-9 activation (Fig. 6C). Caspase-8 inhibitor, *Z*-ITED-*FMK*, completely blocked methyl GLA-induced caspase-8 activation, but did not suppress caspase-9 activation (Fig. 6D). These results suggest that methyl GLA induces caspase-8-independent caspase-9 activation and activation of p38 MAPK is required for caspase-9.

Inhibition of p38 MAPK attenuates inactivation of mTOR pathway. To determine whether regulation of mTOR pathway are required for p38 MAPK activation in methyl GLA-induced apoptosis, the effect of SB203580 on the inactivation of mTOR pathway induced by methyl GLA was examined. Results showed treatment with methyl GLA induced significant down-regulation of mTOR (Fig. 7A). In contrast, inhibition of p38 MAPK pathway by SB203580 did not suppress phosphorylation of mTOR. As revealed by Western blot analysis, inhibition of p38 MAPK pathway did not affect the activation of TSC-2 expression (Fig. 7B). Taken together, these results indicate that activation of p38 MAPK signaling pathway is critical in the regulation of mTOR pathway during methyl GLA-mediated apoptosis, independent of AMPK pathway.

Discussion

GLA enhances free radical generation and lipid peroxidation, resulting in the induction of apoptosis in cancer cells without harming normal cells [Colquhoun and Schumacher, 2001; Das, 2004]. Moreover, GLA inhibits tumor cell proliferation, cycle progression, and angiogenesis, thereby enhancing their tumoricidal potential as compared to other essential fatty acids [Miyake *et al*., 2009]. Methyl GLA has been known as one of irrespective of the forms (free acid, ethyl or methyl ester) in which GLAs were added to the tumor cells [Das, 2004]. The methyl ester form of GLA isolated from the cultures of *Rhizopus oryzae* KSD-815 suppresses cell proliferation and induces apoptosis in prostate cancer cells (Figs. 1 and 2). In a preliminary experiment, cytotoxicity of methyl GLA was observed to be similar to that of GLA (data not shown). Hence, the role of methyl GLA in molecular mechanisms of apoptosis in human prostate cancer cells, LNCaP was investigated.

The one of stress-activated protein kinase, p38 MAPK, represents two independent parallel MAPK pathways that are believed to play important roles in apoptosis [Galan *et al*., 2000]. Activity of p38 MAPK promotes cancer cell growth and survival, and is correlated with the invasiveness of several cancer cells lines. P38 MAPK has been reported to activate the pro-survival PI3K/Akt pathway in fibroblast [Horowitz *et al*., 2004]. Conversely, Dhillon *et al*. [2007] reported that p38 MAPK is positively implicated in the induction of apoptosis in response to various stimuli. Consistent with these findings, in the present study, p38 MAPK was found to play an important role in methyl GLA-induced apoptosis. Inhibition of p38 MAPK by SB203580 attenuated methyl GLA-mediated apoptotic process. The rapid phosphorylation of p38 MAPK after methyl GLA treatment suggests that activation of p38 MAPK plays a key role in the early events of methyl GLA-induced apoptosis (Fig. 5A). Additionally, p38 MAPK was shown to cause cell cycle arrest and to regulate the cell cycle through modulation of p53 tumor suppressor proteins [Dhillon *et al*., 2007]. In our ongoing research, methyl GLA-activated p38 MAPK was found to contribute to the attenuation of mTOR pathway through up-regulation of p21 and p53, and consequent arrest of cell cycle and suppression of cell proliferation.

Though the apoptotic signal pathways that initiate the cell death program can originate from a number of sources, the signaling pathway ultimately leads to the activation of a family of cysteine proteases known as caspases. Caspases can cleave not only their apoptosis-related substances, but also a variety of signal transduction components including upstream kinases in the MAPK cascades [Dhillon *et al*., 2007]. It is suggested that the caspase-mediated activation of these kinases may enhance apoptosis and caspase may regulate the activation of p38 MAPK under certain circumstances. Certain caspases could act as the upstream regulator of p38 MAPK

cascades and caspase cascades are distinct in receptor-mediated and chemical-induced apoptosis [Horowitz *et al*., 2004]. However, the present study demonstrated that the activation of p38 MAPK by methyl GLA is insensitive to the broad caspase inhibitor, *Z*-VAD-*FMK*, suggesting that the activation of p38 MAPK during methyl GLA-induced apoptosis is either upstream or independent of the caspase cascade (Fig. 5B). Pre-incubation of a specific p38 MAPK inhibitor, SB203580, exerted effective inhibition of both p38 MAPK and caspase-9 activation, as well as protected cell from methyl GLA-induced apoptosis (Fig. 6). Furthermore, translocation of the proapoptotic protein, Bax, to mitochondria in response to methyl GLA was blocked (data not shown), is subjected to regulation by p38 MAPK. Recently published studies suggested the induction of apoptosis through inhibition of extracellular regulated kinase (ERK) pathway activity by proapoptotic signaling mediated by p38 MAPK pathway [Bradham and McClay, 2006; Dhillon *et al*., 2007]. Activation of p38 MAPK causes rapid inactivation of ERK pathway through protein phosphatase 2A (PP2A) activation, leading to caspase-mediated cleavage of the signaling proteins (Raf, MEK, and ERK) representing the end-stage removal of survival signaling. Activation of p38 MAPK exerts two critical events that lead to caspase-8- and mitochondria-mediated cell death, suppressing ERK activity [Park *et al*., 2003]. This investigation is further supported by observations made in the present study on the role of p38 MAPK in other apoptotic-regulatory cascades. These results suggest that p38 MAPK is involved in the induction of apoptosis and that activation of p38 MAPK acts as a step prior to caspase activation to complete of the methyl GLA-mediated apoptosis [Galan *et al*., 2000].

Suppression of cancer cell proliferation is mediated by several tumor suppressor proteins associated with AMPK pathway, including LKB1 [Hardie, 2004; Shaw *et al*., 2004] and TSC-2 [Inoki *et al*., 2003]. AMPK phosphorylation triggers TSC-2 activation at sites distinct from Akt sites, and mutations of AMPK sites abrogate the ability of AMPK to inhibit mTOR pathway [Hardie, 2004]. TSC-2 forms an inhibitory complex with TSC-1 that binds to and inhibits mTOR, leading to the negative regulation of cell growth and proliferation [Inoki *et al*., 2002; Guertin and Sabatini, 2005]. In our research, the treatment of LNCaP cells with methyl GLA was shown to inhibit mTOR pathway (Fig. 3C). These findings demonstrate that the mTOR pathway is the major target of methyl GLA in the regulation of cell proliferation in LNCaP cells. PI3K/Akt pathway plays a critical role in cell proliferation in prostate cancer, and hyperactivation of Akt were found in cellular models of prostate cancer, such LNCaP cells, as well as in prostate cancer specimens. Recent evidence indicates that constitutive activation of the PI3K pathway and its down-stream effectors occurs in a high proportion of prostate cancers [Thomas *et al*., 2004]. In fact, androgen ablation for prostate cancer reinforces the PI3K/Akt pathway and impedes its inhibition, thus contributing to increased resistance to the induction of apoptosis. Results of the present study show that methyl GLA dramatically suppresses cell proliferation and inhibits PI3K activity and Akt phosphorylation (Fig. 3A), which, in turn, phosphorylates and inactivates TSC-2 to positively regulate mTOR pathway. Furthermore, methyl GLA down-regulates mTOR and its substrate (Fig. 3B). The discovery that Akt directly phosphorylates mTOR at Ser^{2448}, whereas AMPK phosphorylates mTOR at Thr^{2446} suggests that there is an additional mechanism by which Akt and AMPK may antagonistically regulate the mTOR pathway [Cheng *et al*., 2004]. Therefore, methyl GLA could inhibit Akt phosphorylation to regulate cell proliferation, independent of AMPK pathway, indicating that methyl GLA may have a concurrent route for regulating the TSC-2/mTOR pathway.

In summary, the results of our research indicate that methyl GLA utilizes two distinct signaling pathways to induce apoptosis in human prostate cancer cells, LNCaP. In order to down-regulate mTOR pathway, methyl GLA provokes activation of AMPK pathway and independently down-regulates Akt, inducing TSC-2 activation. In addition, methyl GLA-activated p38 MAPK contributes to the attenuation of mTOR pathway through caspase-9 activation, independent of AMPK pathway. Elucidating the molecular mechanisms of *Rhizopus oryzae* KSD-815 metabolites in cell death is critical for both the understanding of cell death events and the development of combination therapy using drugs with other complementary targets. Methyl GLA is expected to enhance the efficacy of tumor treatment. Further study is warranted to clarify the relationship between AMPK pathway and p38 MAPK and definite molecular mechanism of p38 MAPK regulating mTOR pathway in methyl GLA-provoked apoptosis.

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