이학 석사 학위논문

Autophagy and PERK-eIF2α pathway protect ovarian cancer cells from metformin-induced apoptosis

난소암 세포주에서 Metformin에 의한 자가포식작용과 미접힘 단백질 반응의 기전 연구

2014년 8월

서울대학교 대학원

의과대학 협동과정 종양생물학 전공

문 희 선

난소암 세포주에서 Metformin 에 의한 자가포식작용과 미접힘 단백질 반응의 기전 연구

지도교수 송 용 상

이 논문을 이학 석사 학위논문으로 제출함

2014 년 4 월

서울대학교 대학원

의학과 협동과정 종양생물학 전공

문 희 선

문희선의 석사 학위논문을 인준함

2014 년 6 월

위 원 장 (인)

부위원장 (인)

위 원 (인)

Autophagy and PERK–eIF2α pathway protect ovarian cancer cells from metformin–induced apoptosis

by

Hee–sun Moon

A Thesis Submitted to the Interdisciplinary Graduate Program in Partial Fulfillment of the Requirements for the Degree of Master of Science in Cancer Biology at the Seoul National University

June 2014

Approved by Thesis Committee:

Professor _____ Chairman

Professor _____ Vice chairman

Professor _____

ABSTRACT

Autophagy and PERK-eIF2α pathway protect ovarian cancer cells from metformin-induced apoptosis

Metformin, an oral biguanide for the treatment of type II diabetes, has been shown to have anticancer effect in ovarian cancer. Metformin induces starvation, causing endoplasmic reticulum (ER) stress and autophagy. The unfolded protein response (UPR) signaling by ER stress and autophagy acted as a survival or a death mechanism depending on types of malignancies. In this study, we found that metformin-induced apoptosis was relieved by autophagy and PERK/eIF2α pathway selectively in ovarian cancer cells, but not in normal cells, such as 'normal' ovarian surface epithelial cells (OSE) and peripheral blood mononuclear cells (PBMC). Metformin induced autophagy, was verified by molecular markers, LC3B and ATG12-ATG5 for autophagosome formation at an early stage, and p62 along with increase of acidic vacuoles stained by acridine orange to detect degradation of the autophagosome at a

late stage. Interestingly, metformin induced interdependent activation of autophagy and unfolded protein response (UPR), especially PERK/eIF2α pathway. Interaction of autophagy with PERK/eIF2α pathway played a protective role in ovarian cancer cells against metformin-induced apoptosis, demonstrated by assay using small molecular inhibitors. Finally, metformin with pharmacologic inhibitors had no effects on OSE and PBMC. In conclusion, these results suggest that inhibition of interaction between autophagy and PERK could enhance selective anticancer effect of metformin in ovarian cancer cells.

Keywords: Ovarian cancer cells, Metformin, Apoptosis, Autophagy, PERK

Student number: 2012-21767

CONTENTS

LIST OF FIGURS

LIST OF ABBREVIATONS

LC3B: microtubule-associated proteins 1A/1B light chain 3B

ATG12-ATG5: conjugation of autophagy-related protein (ATG) 12 to ATG 5

p62: sequestosome-1 (SQSTM1)

PERK: protein kinase RNA-like endoplasmic reticulum kinase

eIF2α: eukaryotic initiation factor 2 alpha

ATF4: activating transcription factor 4

3-MA: 3-methyladenine

BafA1: bafilomycinA1

OSE: normal ovarian surface epithelial cells

PBMC: peripheral blood mononuclear cells

INTRODUCTION

Ovarian cancer, the most lethal malignancy of women [1], is often diagnosed at advanced stages. Although platinum-based chemotherapy and cytoreductive surgery are currently standard treatment for advanced stage ovarian cancer, most patients develop recurrence and resistance to chemotherapy. As a result, the median survival for patients with ovarian cancer has not improved [2]. Therefore, a new therapeutic strategy is needed to overcome the chemo-resistance.

Metformin, derived from French lilac, is known to mimic the condition of nutrient starvation by blocking the electron transport chain complex Ⅰ in mitochondria, and reducing the ATP/AMP ratio [3-5]. Although metformin is globally used to treat type Ⅱ diabetes, many studies have reported that metformin decreased the risk of ovarian cancer and, increased progression-free survival in ovarian cancer patients [6-8]. According to 'Clinical Trial.gov', a phase Ⅱ clinical trial for evaluation of metformin is ongoing in patients with stage ⅡC/Ⅲ/Ⅳ ovarian, fallopian tube, and primary

peritoneal cancer by the University of the Michigan Cancer Center. Many experimental studies demonstrated that metformin has anti-proliferative and cell death-inducing effects on ovarian cancer cells or ovarian cancer stem cells [9-12]. Recently, several reports have shown that metformin enhances effects of cisplatin or carboplatin, and also combination of metformin with LY294002 or phenethylisothiocynate (PEITC) has been shown to overcome the chemo-resistance of ovarian cancer [13-15].

During the metabolic stresses, such as nutrient depletion and hypoxia, autophagy and the unfolded protein response (UPR) signaling are essential for cellular homeostasis. Autophagy, a catabolic self-digestive process, is stimulated to provide energy sources for cells under nutrient depletion, as well as eliminate the damaged organelles and aggregated protein. In early-stage of autophagy, vesicle nucleation and elongation are promoted by conversion of LC3 Ⅰ to LC3 Ⅱ, conjugation of ATG12 to ATG5, and Beclin-1 activation to form a double-membraned vesicle called the autophagosomes. In late-stage of autophagy, p62 (SQSTM1) binds to ubiquitinated protein aggregates, and transports them to

the autophagosomes. The autophagosomes are fused with lysosomes, and these autophagic vesicles with p62 are degraded under the acidic condition [16,17]. The UPR signaling is induced by branches of endoplasmic reticulum (ER) stress sensor, such as PKR-like ER stress kinase (PERK), inositol-requiring transmembrane kinase (IRE1) and activating transcription factor 6 (ATF6), leading to reduction of abnormal protein and the regulation of autophagy [18,19].

However, autophagy and UPR signaling have shown both protective "yin" and pro-apoptotic "yang" in many types of malignancies [20]. According to previous study, metformin induces autophagy [21], but the exact role of autophagy is unclear. The UPR in response to metformin is also not well described in cancer cell. Therefore, we investigated the effects of metformin-induced autophagy and UPR signaling in ovarian cancer cells.

MATERIALS AND METHODS

1. Cell lines and Cell culture

The epithelial ovarian cancer cell lines, PA-1 (wild type-p53) and OVCAR-3 (mutant type-p53), are available from the American Type Culture Collection (Rockville, MD), and were used in all experiments. PA-1 was maintained in MEM medium (WelGENE, Seoul, Korea) and OVCAR-3 was cultured in RPMI 1640 medium (WelGENE, Seoul, Korea). The media were supplemented with 10% Fetal Bovine Serum (FBS) (Gibco-BRL, Gaithersberg, MD) and 1% Penicillin-Streptomycin (Invitrogen, Carlsbard, CA). These cells were seeded in cell culture dish (SPL, Seoul, Korea), and incubated at 37℃ with 5% CO_2.

2. Primary culture of Human ovarian surface epithelial cells

The Seoul National University Hospital Institutional Review Board approved all experiments, using normal tissue from patients (IRB

No. C-1307-008-502). Ovarian tissues isolated from three female patients were washed by Dubecco's Phosphate-Buffered Saline (PBS) (Gibco-BRL, Gaithersberg, MD) twice and unnecessary parts, such as vessel and medulla, were maximally eliminated with sterilized scissors and forceps. Only surface part (cortex) was treated by 2.4 U/ml Dispase (Gibco-BRL Gaithersberg, MD) in a Petri dish, and incubated at 4℃ overnight. Surface part was scratched by blazer and forceps. Epithelial cells were separated and collected for centrifugation at 1500rpm and 4℃ for 4min. The cells were incubated in MDCB105 (Sigma-Aldrich, St. Louis, MO) : M199 (Sigma-Aldrich, St. Louis, MO) complete medium in cell culture dish at 37℃ with 5% CO_2. A subculture was carried out until passage number 5.

3. Isolation of Primary human peripheral blood mononuclear cells from buffy coats

Buffy coats from five donors were washed by PBS. Peripheral blood mononuclear cells (PBMC) were isolated by ficoll density separation using Ficoll-Paque™ (GE Healthcare, NJ). PBMC were

washed by PBS twice, and counted by trypan blue staining. 2 x 10^6 cells/ml were cultured in RPMI 1640 medium with 10% FBS and 1% Penicillin-Streptomycin.

4. Reagents and Antibodies

1,1-Dimethylbiguanide hydrochloride (Metformin, Sigma-Aldrich, St. Louis, MO) was dissolved in distilled water at 1 M concentration. The solution was stored at -20℃, and diluted in medium before use. The primary antibodies, purchased from Cell Signaling Technology (Danvers, MA), were anti-LC3B (1:750), anti-Beclin-1 (1:1000), anti-ATG12-ATG5 (1:1000), anti-P-eIF2α (1:3000), and anti-cleaved caspase 3 (1:1000). Antibodies available from Santa Cruz Biotechnology (Santa Cruz, CA) were anti-P-PERK (1:3000), anti-PARP (1:1000), anti-p62 (SQSTM1, 1:1000), and anti-ATF4 (CREB-2, 1:500). The PERK inhibitor Ⅰ, GSK2606414 [22] (EMD Millipore, Billerica, MA) was soluble in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO), and was stored at 100 μM. The autophagy inhibitors, 3-methyladenine (3-MA) and bafilomycinA1 (BafA1), and α-tubulin antibody were from Sigma-Aldrich. 3-

MA was soluble in distilled water, and stored at 200 mM. BafA1 was melted in DMSO at 10 μM. Concentrations to be used are 0.5 μM GSK2606414, 5 mM 3-MA, and 100 nM BafA1.

5. MTT assay

The cells were seeded in 96-well plates (SPL, Seoul, Korea), and incubated for 24h. The cells were treated by control medium or metformin of 5, 10, 20, and 40 mM concentration for 24h and 48h. Cell proliferation was assessed by 0.2 mg/ml MTT (Thiazolyl blue tetrazolium bromide, Amresco, Solon, OH) dissolved in PBS. The MTT solution was added, and incubated at 37℃. After 3h, the MTT solution was removed, and 0.1 ml DMSO was added in each well to dissolve the purple formazan at room temperature for 30min. The absorbance was measured by the Multiskan spectrum (Thermo Scientific, Hudson, NH) at 540 nm.

6. Flow cytometry analysis

The cells with or without metformin were washed by PBS and

trypsinized. These cells were spin down in a FACS tube (BD Falcon, CA) by centrifuging at 4℃ for 5min. To analyze cell cycle, cells were fixed in 70% ethanol at −20℃ overnight. 0.2 mg/ml RNaseA (Sigma−Aldrich, St. Louis, MO) and propidium iodide (Invitrogen, Carlsbad, CA) were added. To examine apoptosis, cells were stained by using the Annexin V−FITC Apoptosis Detection Kit I (BD Bioscience, CA) according to the manufacturer's instructions. The fluorescent intensity was measured by a FACS Calibur flow cytometry, using the Cell Quest software (BD Bioscience, CA).

7. Western blotting

The 2X lysis buffer contained 1 M NaCl, 1 M Tris−HCl (pH 7.4), 0.1 M EDTA (pH 8.0), and 0.1 M EGTA. Cells were lysed by a mixture of 2X lysis buffer, 10% TritonX−100, sodium deoxycholate, 0.1 M Na_3VO_4, 0.1 M phenyl methyl sulfonyl fluoride, and EDTA free. Protein concentrations were determined by using the Pierce BCA Protein Assay Kit (Thermo Scientific, Hudson, NH). Protein extracts were stained by 5X SDS−PAGE loading buffer, fractionated on 10% SDS polyacrylamide gels, and transferred to

nitrocellulose membranes (BIO-RAD, Benicia, CA). Membranes were blocked by 5% skim milk with TBS-T (Tris-Buffered Saline with 0.1% Tween 20) at room temperature, and incubated with primary antibody at 4℃ overnight. Bands were confirmed by horseradish peroxidase-conjugated secondary antibody, diluted in 5% skim milk with TBS-T, and enhanced by WESTSAVE^{TM} (Ab frontier, Seoul, Korea). α-tubulin was used to ensure an equal protein volume.

8. Confocal microscopy imaging

The cells were incubated in a cover glass bottom dish for 24h. Acridine orange (Sigma-Aldrich St. Louis, MO) was used to detect the acidic vesicles of organelles. Cells were stained by 1 μg/ml acridine orange in complete medium (phenol red-free RPMI 1640, Gibco-BRL, Gaithersberg, MD) at 37℃ for 15min, and washed with PBS. Conformation of acidic vesicles with red color was observed by confocal microscope.

9. Statistical analysis

All results were shown as mean ± SEM. The values display percentage compare to control. Statistical significance was considered by P value<0.5 between groups analyzed by one-way ANOVA with Scheffe test in SPSS software.

RESULTS

Metformin selectively inhibits growth of ovarian cancer cells

To demonstrate the anti-proliferative effect of metformin on ovarian cancer cells, we applied various dose (5, 10, 20, and 40 mM) of metformin for 24h and 48h on PA-1 and OVCAR-3. Metformin suppressed cell viabilities in time- and dose-dependent manners. The half maximal inhibitory concentration (IC_{50}) values are 24.86 mM for PA-1 and 24.29 mM for OVCAR-3 with metformin for 24h, and are 12.30 mM for PA-1 and 15.23 mM for OVCAR-3 at 48h exposure (Figure 1A). As shown in Figure 1B, metformin changed shape of PA-1 and OVCAR-3 cells compare to control. Moreover, we cultured normal ovarian surface epithelial cells (OSE) freshly isolated from patients and peripheral blood mononuclear cells (PBMC) from buffy coats of donor to verify selective anti-proliferative effect of metformin. The dose-dependent treatment of metformin mildly inhibited growth of OSE and had no significant effect on PBMC viability (Figure 1C).

Metformin induces apoptosis and autophagy in ovarian cancer cells

To examine induction of cell death by metformin, we analyzed cell cycle and apoptosis by flow cytometry. In response to 20 mM metformin, fraction of Sub-G1 phase and Annexin V-PI positive cells were increased in a time-dependent manner (Figure 2A and C). Also, metformin induced more expression of cleaved PARP than control cells at 48h (Figure 2B).

To investigate induction of autophagy by metformin, we measured expression levels of autophagy markers by Western blotting, and stained the metformin-treated cells by using acridine orange to detect the formation of autophagic vacuoles. As a result, metformin increased conversion of LC3B I to LC3B II and ATG12-ATG5 expression level, and decreased p62. However, the expression level of Beclin-1 was not changed (Figure 2D). The expanded cytoplasm and increased red colored vesicles were observed in metformin-treated cells (Figure 2E). These results indicated that metformin promoted apoptosis and autophagy

Metformin stimulates interdependent action between PERK signaling and autophagy

The PERK signaling promotes autophagy [23]. We explored whether PERK signaling is involved in induction of autophagy. Metformin induced phosphorylation of PERK and eIF2α, and increased the expression of ATF4 detected by Western blotting (Figure 3A). When the phosphorylation of PERK was inhibited by PERK inhibitor I (GSK2606414), phosphorylated eIF2α and LC3B conversion by metformin were decreased (Figure 3B). 3-methyladenine (3-MA) inhibited LC3B conversion, while bafilomycinA1 (BafA1) accumulated LC3B protein. 3-MA is a blocker the class Ⅲ PI3K, which is critical for vesicle expansion, whereas BafA1 prevents fusion of lysosomes with autophagosomes and acidification by inhibiting vacuolar type H^+-ATPase (V-ATPase), and results in accumulation of LC3 [24]. We found that autophagy inhibition by 3-MA or BafA1 suppressed metformin-induced phosphorylation of PERK/eIF2α (Figure 3C). Collectively these results suggested that PERK phosphorylation mediated autophagy and autophagy also promoted PERK phosphorylation in

metformin-treated cells.

Autophagy and PERK pathway protect ovarian cancer cells against antitumor effects of metformin

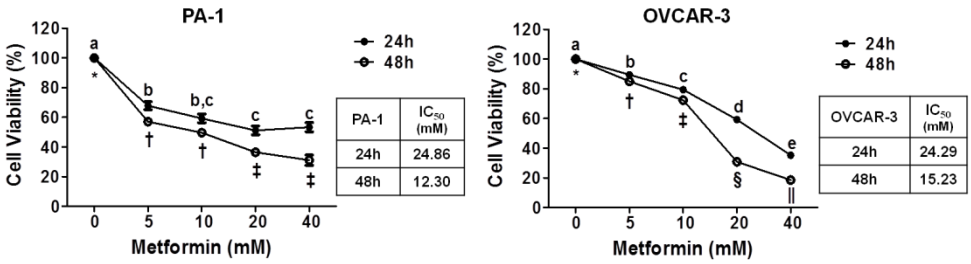
To determine a role of PERK and autophagy in growth of ovarian cancer cells and apoptosis, we conducted MTT assay by using pharmacologic inhibitors (GSK2606414, 3-Ma, and BafA1). As shown in Figure 4A and 4B, pharmacologic inhibitors exacerbated the growth inhibitory effect of metformin. In addition, metformin with GSK2606414, 3-MA, or BafA1 enhanced levels of cleaved caspase3 and cleaved PARP than metformin alone in PA-1 and OVCAR-3 cells (Figure 4C and 4D). These results indicate that autophagy induction and PERK phosphorylation suppresses the growth inhibition and apoptosis induced by metformin.

Inhibition of autophagy and PERK with metformin has no effects on survival of normal cells

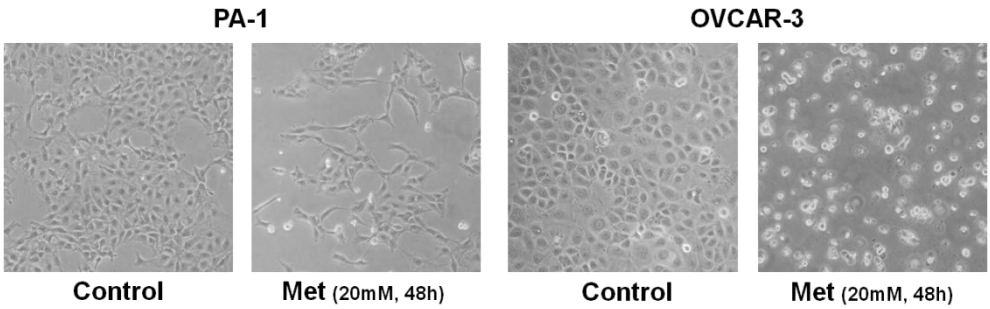
To demonstrate the efficacy of metformin with pharmacologic inhibitors for cancer cells exclusively, we pretreated GSK2606414, 3-MA, or BafA1 for 1h, followed by treatment of metformin for 24h. Metformin with GSK2606414 as well as with 3-MA or BafA1 has no effect on growth of PBMC (Figure 5A and 5B). Not only the cell viability of OSE were not decreased, but also apoptosis was not triggered, given that cleaved form of PARP was not induced in OSE with metformin combined with 3-MA or BafA1 (Figure 5C and 5D). Finally, these results specify that metformin with reduction of autophagy or PERK activity by pharmacologic inhibitors has no effect on survival of normal cells.

FIGURES

A


PA-1
Cell Viability (%)
Metformin (mM)
24h
48h
PA-1 | IC50 (mM)
24h | 24.86
48h | 12.30
OVCAR-3
Cell Viability (%)
Metformin (mM)
24h
48h
OVCAR-3 | IC50 (mM)
24h | 24.29
48h | 15.23

B


PA-1
OVCAR-3
Control
Met (20mM, 48h)
Control
Met (20mM, 48h)

C

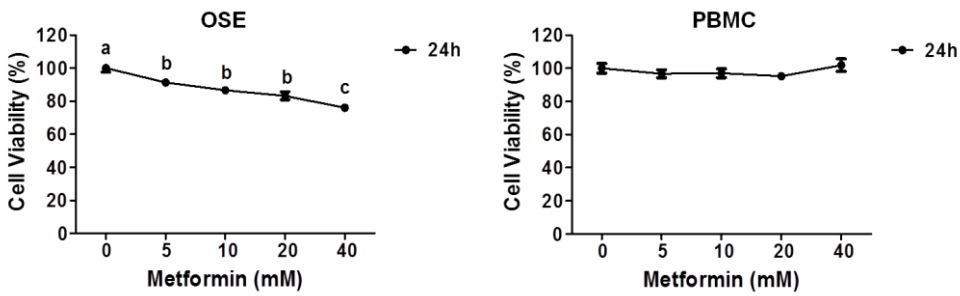

OSE
Cell Viability (%)
Metformin (mM)
24h
PBMC
Cell Viability (%)
Metformin (mM)
24h

Figure 1. Selective inhibition of cancer cell growth by metformin

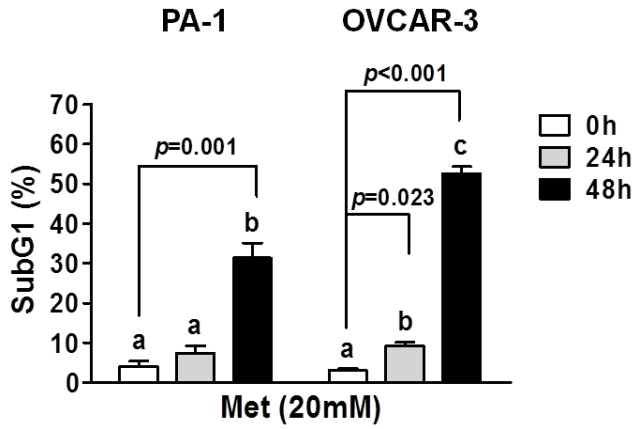
A. PA-1 and OVCAR-3 cells were treated with metformin (5, 10, 20, and 40 mM) at the indicated times (24h and 48h). The cell viability was estimated by MTT assay. The values were divided significantly ($p<0.05$) into groups (a, b, c, d, e at 24h, and ∗, †, ‡, §, ‖ at 48h). IC_{50} values were evaluated.

B. After treatment of 20 mM metformin (Met) for 48h, morphologic change of cells was compared with control cell by the phase-contrast microscope.

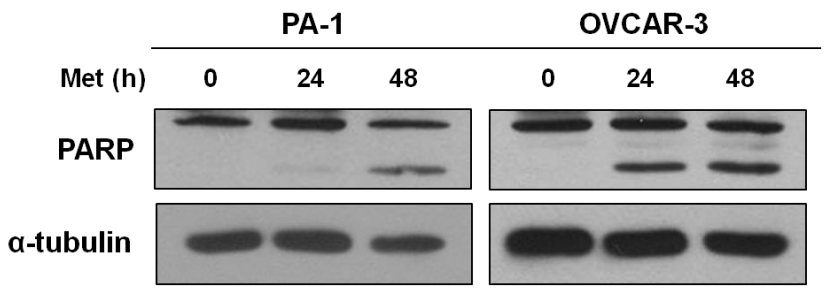
C. Normal ovarian surface epithelial cells (OSE) and peripheral blood mononuclear cells (PBMC) were treated with different doses of metformin for 24h. The cell viability of OSE was examined by MTT assay and cell numbers of PBMC were counted by trypan blue staining.

All results were shown as mean ± SEM of the three independent experiments.

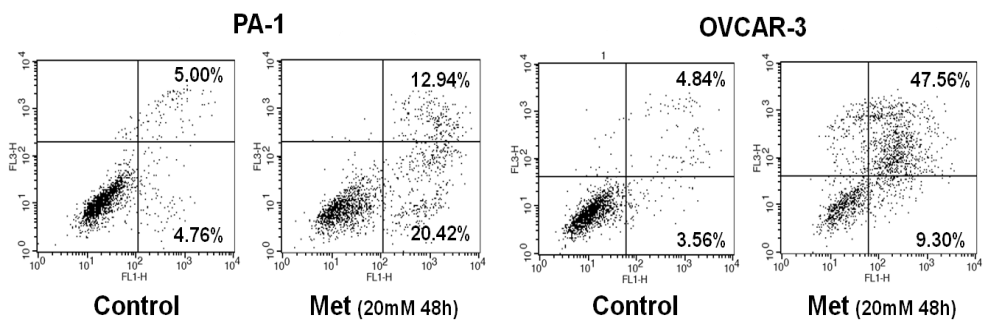
A


PA-1
OVCAR-3
p<0.001
p=0.001
p=0.023
0h
24h
48h
SubG1 (%)
70
60
50
40
30
20
10
0
a
a
b
a
b
c
Met (20mM)

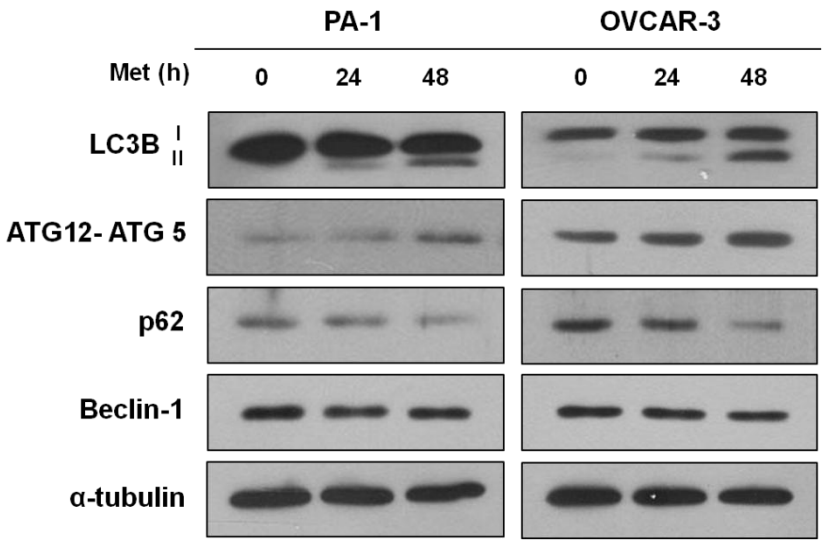
B


PA-1
OVCAR-3
Met (h)
0
24
48
0
24
48
PARP
α-tubulin

C


PA-1
OVCAR-3
5.00%
4.76%
12.94%
20.42%
4.84%
3.56%
47.56%
9.30%
FL3-H
FL1-H
Control
Met (20mM 48h)
Control
Met (20mM 48h)

D


PA-1
OVCAR-3
Met (h)
0
24
48
0
24
48
LC3B I
II
ATG12- ATG 5
p62
Beclin-1
α-tubulin

E

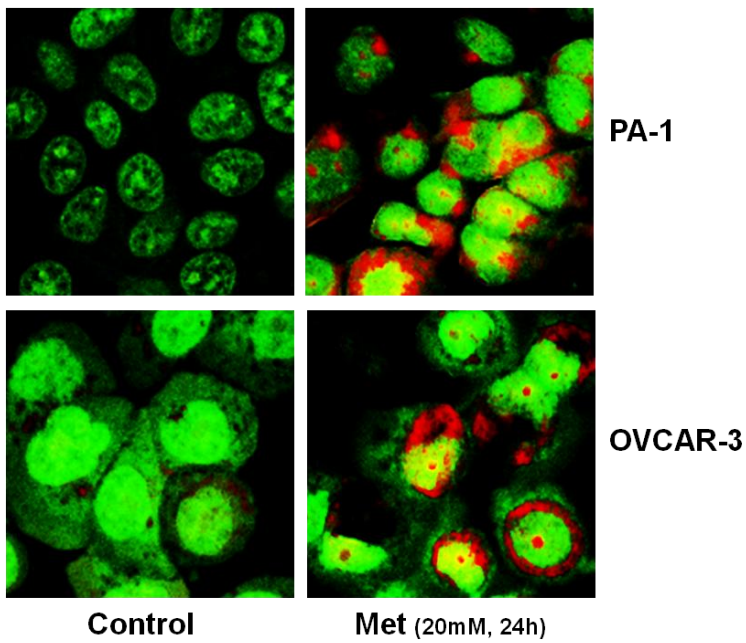

PA-1
OVCAR-3
Control
Met (20mM, 24h)

Figure 2. Induction of apoptosis and autophagy by metformin in ovarian cancer cells

PA-1 and OVCAR-3 cells were treated with 20 mM metformin (Met) for 24h and 48h.

A. To evaluate induction of apoptosis, the cells in sub G1 phase were sorted by flow cytometry. The percentage of cell debris in sub G1 was shown in a time dependent manner.

B. Expression level of cleaved-PARP was detected by Western blotting.

C. After treatment of Met for 48h, the population of apoptotic cells, stained by FITC annexin V and propidium iodide (PI), was analyzed by flow cytometry. The horizontal axis is annexin V-positive and the vertical axis is PI-positive.

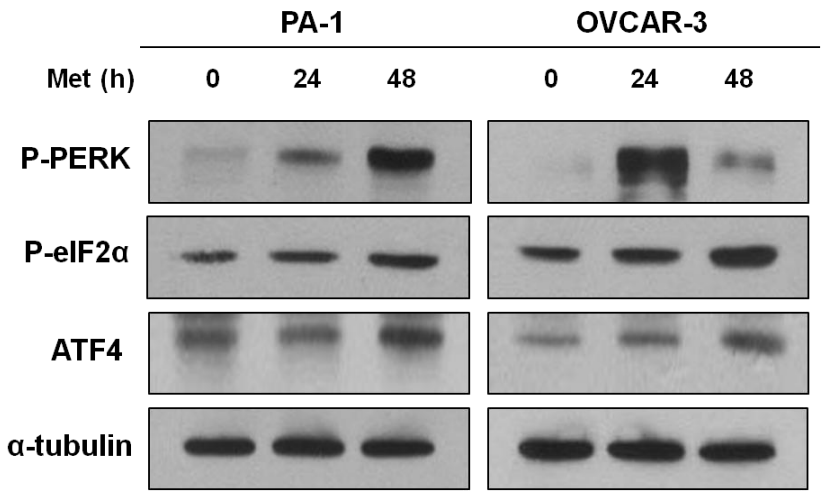
D. To confirm induction of autophagy, increased conversion of LC3B I to LC3B II, and expression levels of ATG12-ATG5 and p62 with no alteration of Beclin-1 expression were measured by Western blotting.

E. We checked the acidic vesicles, formed at a late-stage of

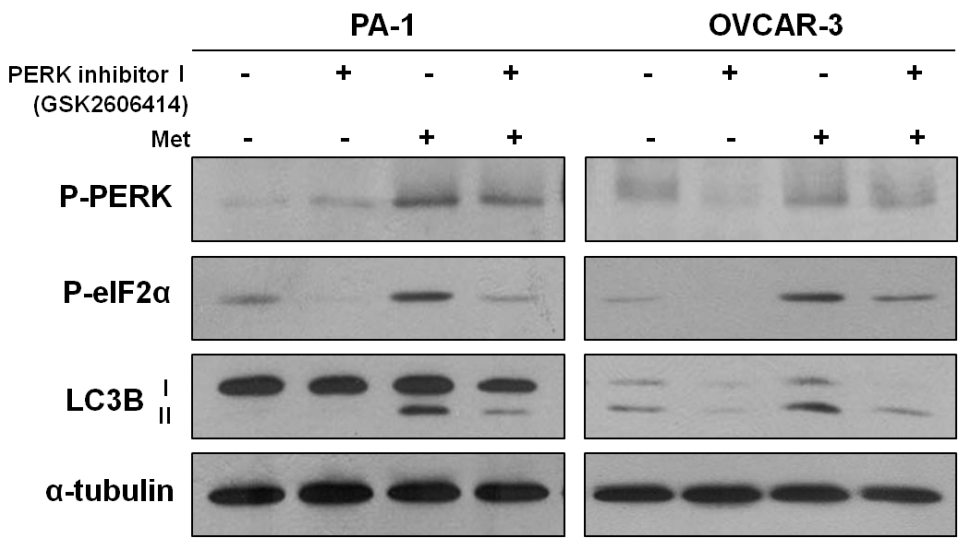
autophagy. The cells were cultured in a cover glass bottom dish and treated with Met for 24h. The cells were stained by 1 μg/ml acridine orange (AO) in phenol-free complete medium for 15min. Conformation of acidic vesicles with red color in Met-treated cells was compared with vesicles of control cells by confocal microscope.

All results were shown as mean ± SEM of the three independent experiments.

A


PA-1
OVCAR-3
Met (h)
0
24
48
0
24
48
P-PERK
P-eIF2α
ATF4
α-tubulin

B


PA-1
OVCAR-3
PERK inhibitor I
(GSK2606414)
Met
P-PERK
P-eIF2α
LC3B I
II
α-tubulin

C

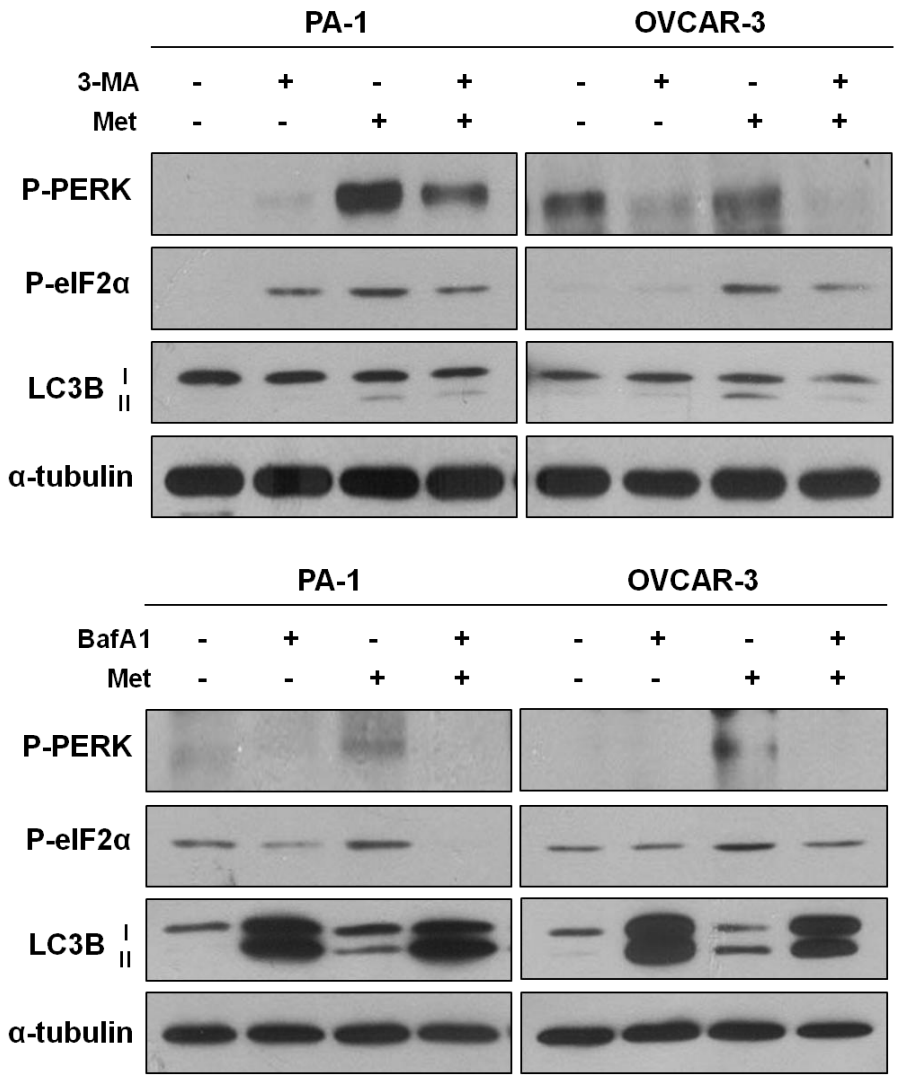

PA-1
OVCAR-3
3-MA - + - + - + - +
Met - - + + - - + +
P-PERK
P-eIF2α
LC3B I II
α-tubulin
PA-1
OVCAR-3
BafA1 - + - + - + - +
Met - - + + - - + +
P-PERK
P-eIF2α
LC3B I II
α-tubulin

Figure 3. Interdependent activation of PERK/eIF2α/ATF4 pathway and autophagy by metformin

We investigated interaction between PERK signaling and autophagy.

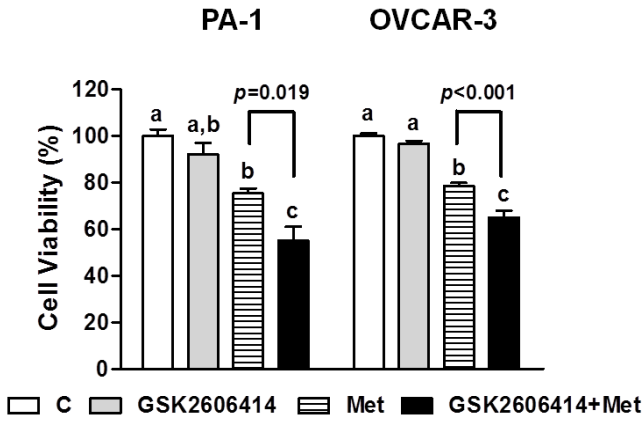
A. PA-1 and OVCAR-3 cells were treated with or without 20 mM metformin (Met) for 24h and 48h. Expression levels of phosphor-PERK, phosphor-eIF2α, and ATF4 were evaluated by Western blotting.

B. After pretreatment with 0.5 μM PERK inhibitor I (GSK2606414) for 1h, Met was supplemented for 48h. Western blotting shows abolition of PERK/eIF2α activation and LC3B conversion.

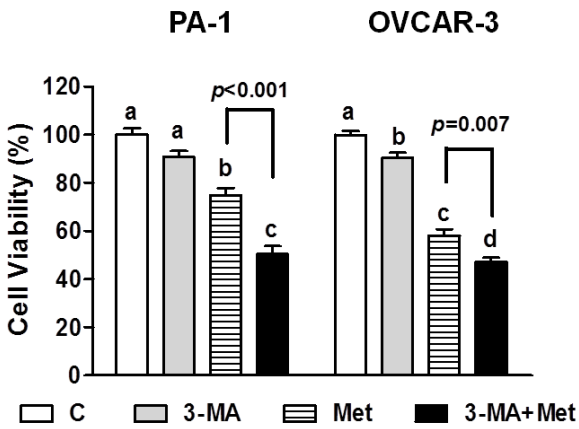
C. We identified whether the PERK activation set up before the autophagy induction. After pretreatment of 5 mM 3-methyladenine (3-MA) and 100 nM bafilomycinA1 (BafA1) for 1h, Met was applied for 48h. Decrease of phosphorylated PERK/eIF2α and changes of LC3B conversion were measured by Western blotting.

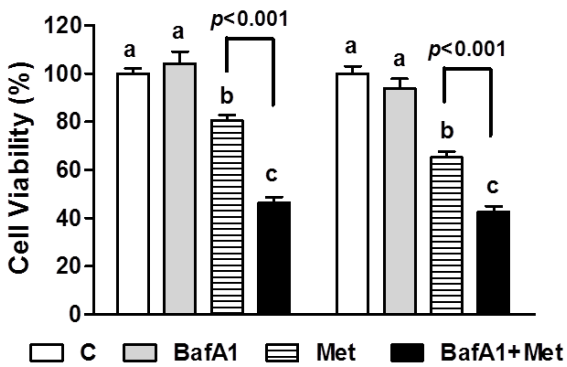
All results were conducted in three independent experiments.

A

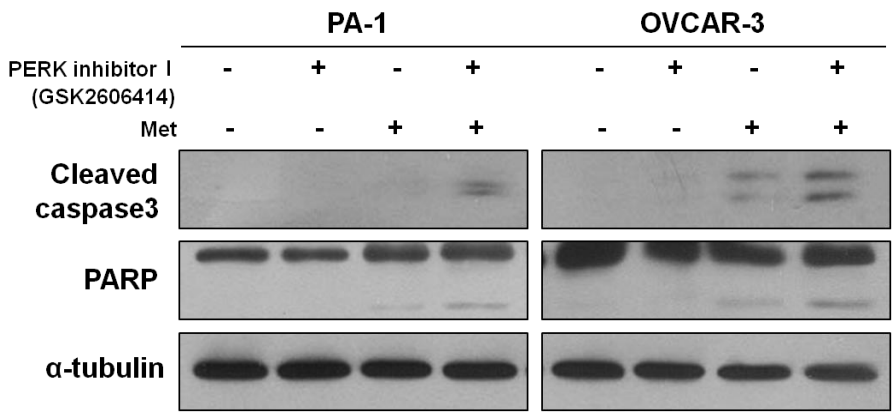

PA-1
OVCAR-3
Cell Viability (%)
120
100
80
60
40
20
0
p=0.019
p<0.001
a
a,b
b
c
a
a
b
c
C
GSK2606414
Met
GSK2606414+Met

B


PA-1
OVCAR-3
Cell Viability (%)
120
100
80
60
40
20
0
p<0.001
p=0.007
a
a
b
c
a
b
c
d
C
3-MA
Met
3-MA+Met


Cell Viability (%)
120
100
80
60
40
20
0
p<0.001
p<0.001
a
a
b
c
a
a
b
c
C
BafA1
Met
BafA1+Met

C


PA-1
OVCAR-3
PERK inhibitor I (GSK2606414)
Met
Cleaved caspase3
PARP
α-tubulin

D

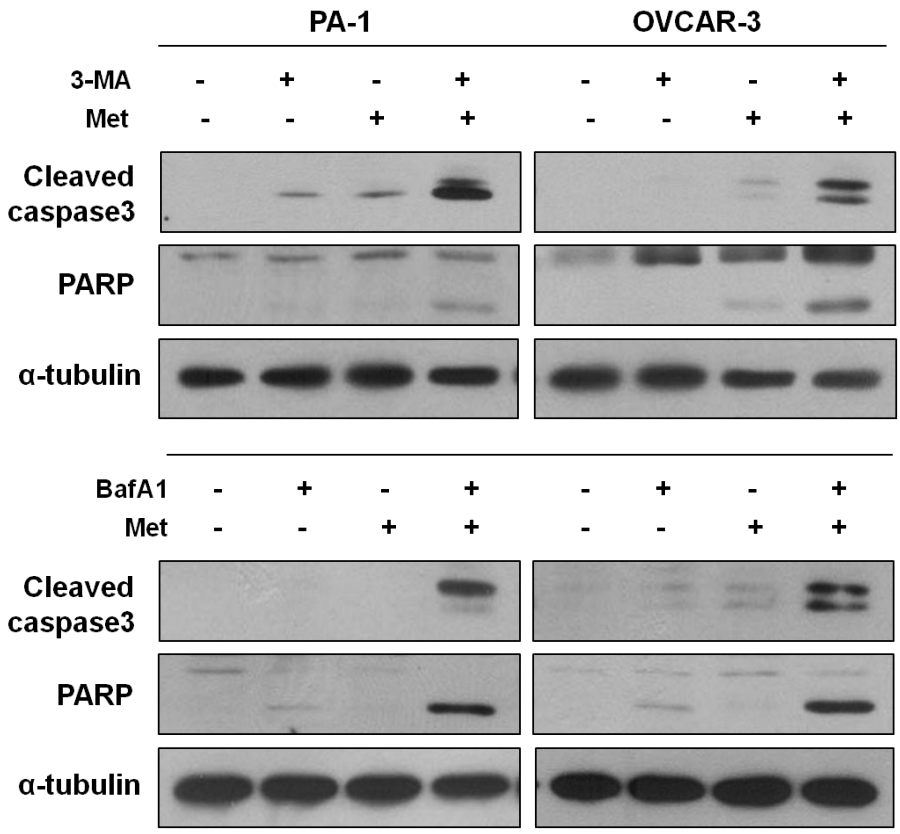

PA-1
OVCAR-3
3-MA
Met
Cleaved caspase3
PARP
α-tubulin
BafA1
Met
Cleaved caspase3
PARP
α-tubulin

Figure 4. Autophagy and PERK by metformin to prevent metformin-induced apoptosis

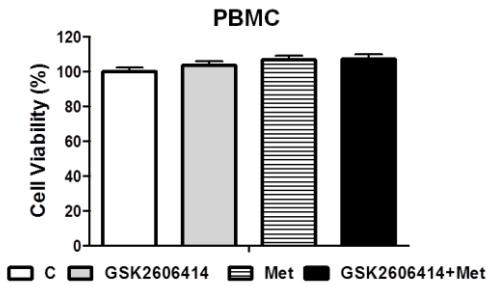
We examined the effect of autophagy and PERK signaling on cell growth and apoptosis. After 0.5 μM GSK2606414, 5 mM 3-MA, or 100 nM BafA1 was pretreated for 1h, 20 mM metformin (Met) was applied for 24h.

A, B. Inhibition of PERK and autophagy more decreased the cell viability (MTT assay), and

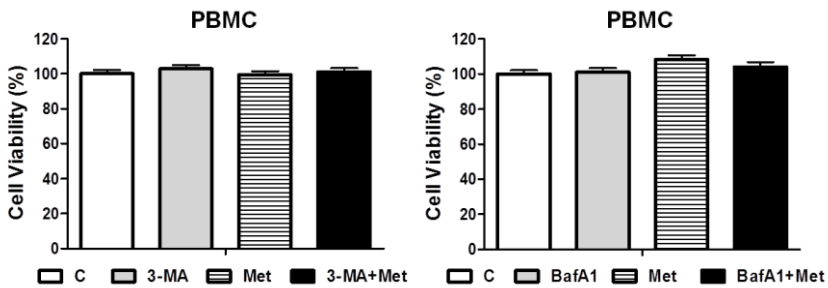
C, D. more enhanced expression levels of cleaved caspase3 and PARP (Western blotting) than Met treatment alone.

Thus, activation of autohpagy and PERK pathway prevents growth inhibition and apoptosis induction by metformin. All results were shown as mean ± SEM of the three independent experiments.

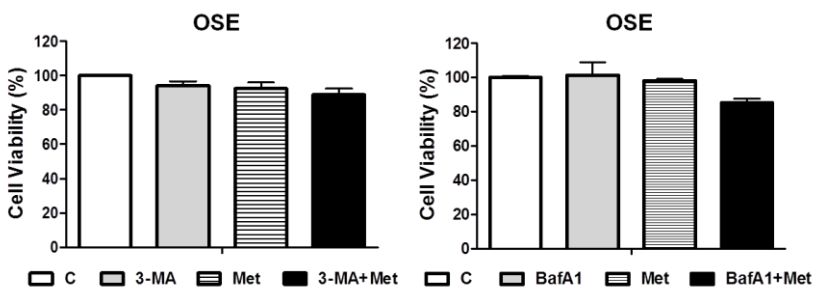
A


PBMC
Cell Viability (%)
120
100
80
60
40
20
0
C
GSK2606414
Met
GSK2606414+Met

B


PBMC
Cell Viability (%)
120
100
80
60
40
20
0
C
3-MA
Met
3-MA+Met
PBMC
Cell Viability (%)
120
100
80
60
40
20
0
C
BafA1
Met
BafA1+Met

C


OSE
Cell Viability (%)
120
100
80
60
40
20
0
C
3-MA
Met
3-MA+Met
OSE
Cell Viability (%)
120
100
80
60
40
20
0
C
BafA1
Met
BafA1+Met

D

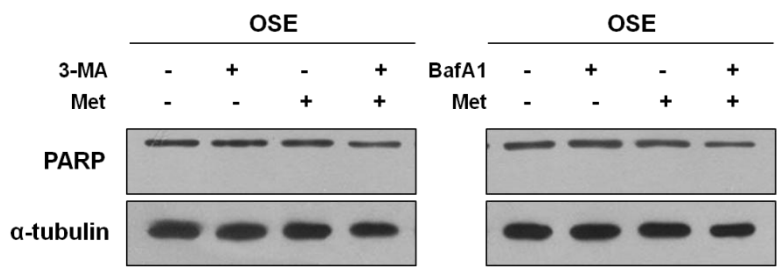

OSE
OSE
3-MA - + - +
Met - - + +
BafA1 - + - +
Met - - + +
PARP
α-tubulin

Figure 5. No cytotoxic effect of metformin combined with inhibitors of autophagy and PERK on normal cells, such as OSE and PBMC

The GSK2606414, 3-MA, or BafA1 was pretreated for 1h, before co-treatment with 20 mM metformin (Met) for 24h.

A, B. The growth of PBMC was estimated by trypan blue staining.

C. After co-treatment of Met with 3-MA or BafA1 for 24h, the viability of OSE was confirmed by MTT assay, and

D. PARP cleavage was visualized by Western blotting.

All results were shown as mean ± SEM of the three independent experiments.

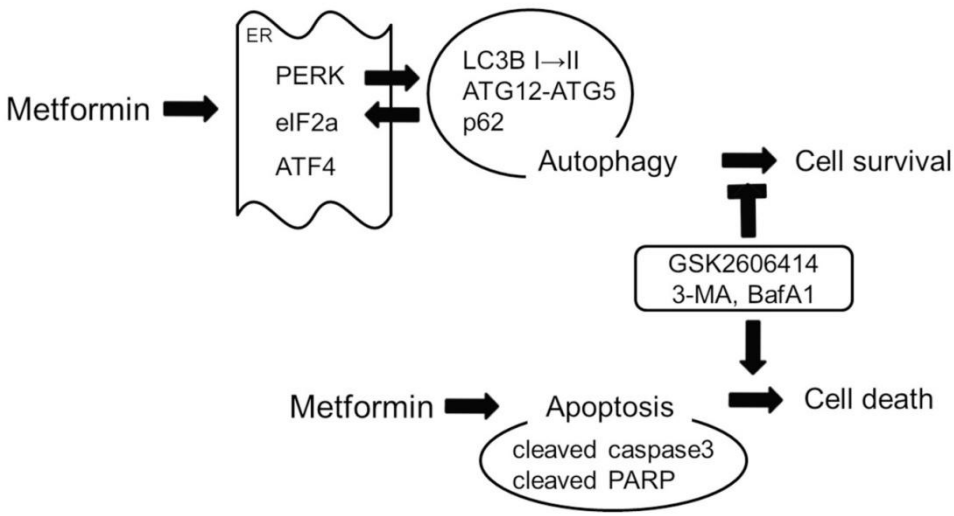
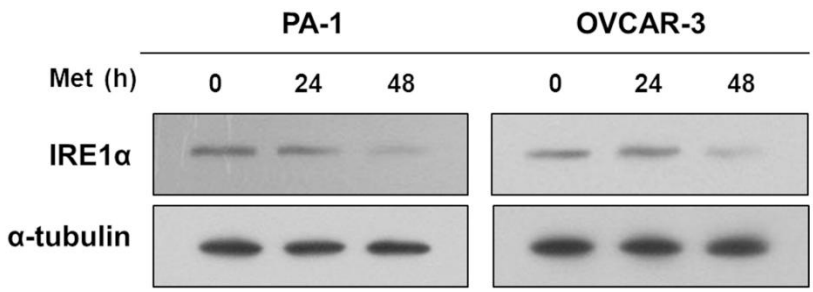


Figure 6. The effect of metformin on regulation of autophagy and PERK pathway, and its therapeutic implication in ovarian cancer cells



Supplementary Figure 1. PA-1 and OVCAR-3 cells were treated with or without 20 mM metformin (Met) for 24h and 48h. Expression level of IRE1α is confirmed by Western blotting. All results were conducted in three independent experiments.

DISCUSSION

The objective of our study is to verify the anti-cancer effects of metformin on ovarian cancer cells, and to determine the molecular mechanism of metformin on regulation of autophagy and UPR signaling. We found that metformin exclusively targets ovarian cancer cells, and interaction between autophagy and PERK phosphorylation leads to the suppression of growth inhibition and apoptosis by metformin for cellular homeostasis. Also, we suggest that inhibition of autophagy or PERK by pharmacologic inhibitors could enhance apoptosis induced by metformin in a cancer-specific manner.

Metformin inhibits proliferation of various ovarian cancer cell lines [9,10], and induces apoptosis with increase of caspase3/7 activity [11]. In our study, anti-tumor effects of metformin were also seen in terms of decrease in ovarian cancer cell growth and induction of apoptotic cell death with PARP cleavage (Figure 1 and 2A-C). The growth inhibitory effect of metformin is not significant in normal prostatic epithelial cells (RWPE-1) and the non-transformed

human mammary epithelial cell line (MCF10A) compared to cancer cells [25,26]. Likewise, we demonstrated that metformin more significantly inhibits growth of ovarian cancer cells than OSE and PBMC (Figure 1). Eric D. Segal *et al.,* have shown that the organic cation transporter 1 (OCT1) is important for efficacy of metformin in ovarian cancer cells [27]. OCTs are essential for cellular uptake and distribution of metformin, and expression level of three OCT family members (OCT1, 2, and 3) were higher in cancer cells than in normal cells. Thus, action of metformin may be affected by expression and activity of OCTs [28,29].

Under nutrient depletion by stressor such as metformin reducing ATP production, activation of autophagy and unfolded protein response (UPR) are crucial mechanism for cell survival and homeostasis, but the effect and the mechanism of metformin on autophagy and UPR is poorly understood. Thus, we investigate the role and correlation between autophagy and UPR induced by metformin, First, we confirmed that metformin activates autophagy, verified by increase of LC3B conversion and expression level of ATG12–ATG5, and decrease of p62 expression (Figure 2D and E),

consistent with previous reports that metformin induces autophagy in esophageal squamous cell carcinoma, lymphoma cell, and melanoma [21,30,31]. The UPR signaling is induced by three ER stress sensor, PKR-like ER stress kinase (PERK), inositol-requiring transmembrane kinase (IRE1) and activating transcription factor 6 (ATF6) [18]. In particular, the PERK and IRE1 have been implicated in regulation of autophagy. PERK/eIF2α pathway is required for LC3 conversion dependent on ATG5-ATG12-ATG6 complex to prevent the ER stress-mediated cell death [32]. PERK-dependent autophagy protects cancer cells in hypoxic condition through transcription of the LC3B and ATG5 [23]. IRE1 is linked to autophagosome formation in regulation of autophagy rather than PERK, saving cancer cells through maintenance of energy homeostasis [33]. To determine the activation of UPR signaling by metformin, we measured the expression level of phosphorylated PERK and IRE1α. As a result, metformin only activated PERK and its downstream eIF2α and ATF4 (Figure 3A), whereas expression level of IRE1α was decreased (Supplementary figure 1). Thus, we explored correlation between autophagy and PERK in metformin-treated ovarian cancer cells using autophagy inhibitor (3-MA,

BafA1) and PERK inhibitor I (GSK2606414). PERK inhibition suppressed conversion of LC3B I to II (Figure 3B), and inhibition of autophagy also suppressed PERK/eIF2α phosphorylation (Figure 3C). These results indicate interdependent activation between autophagy and PERK signaling.

The role of autophagy and UPR signaling is controversial issue depending on cancer type and the kind of stressors, since several results have reported that excessive induction of autophagy acts as a programmed cell death mechanism or activates apoptotic signals, leading to cell death [34], and the UPR signaling also has dual roles depending on intensity of stress [20]. According to previous findings, autophagy by metformin is for survival in esophageal squamous cell carcinoma, but death in lymphoma cell and melanoma [21,30,31]. ER stress-mediated UPR contributes to survival of leukemic cells, but the induction of apoptosis in lung and gastric cancer [35-37]. Therefore, it is important to understand the exact role of metformin in autophagy and UPR in ovarian cancer cells. We found that inhibition of autophagy and PERK enhanced the growth inhibition and apoptosis by metformin (Figure 4). Thus, interaction

between autophagy and PERK signaling is important for protection against metformin stress in ovarian cancer cells.

The protective role of autophagy and PERK has been shown to contribute to the chemo-resistance of cancer cell [38,39]. Therefore, targeting autophagy and PERK is a novel therapeutic strategy, providing new chances to sensitize cancer cells to chemotherapy [38,40,41]. The 3-methyladenine enhances cisplatin-, paclitaxel-, and resveratrol-induced apoptosis [42-44]. The bafilomycinA1 also potentiates sensitivity of radiation, of sulforaphane and apigenin [45-47]. Likewise, we measured that metformin-induced apoptosis was potentiated by 3-methyladenine and bafilomycinA1 (Figure 4D). Also, metformin with autophagy inhibitors for PBMC and OSE did not affect the cell growth and apoptosis (Figure 5B-D). Moreover, when we treated metformin with GSK2606414, apoptosis by metformin was enhanced in ovarian cancer cells, but no effect on OSE and PBMC (Figure 4C and 5A).

The interaction of the autophagy and PERK pathway protects

ovarian cancer cells against death by metformin. Finally, inhibition of autophagy and PERK by pharmacologic inhibitors enhanced the cytotoxicity of metformin, whereas there were no effects on normal cells (Figure 6). Taken together, metformin showed the selective effect on ovarian cancer cells as an anticancer agent targeting only cancer cell. Also, our study suggests that when the anticancer therapy combined with metformin is designed, the interaction between autophagy and UPR signaling should be considered. Moreover, metformin with autophagy or PERK inhibitors could serve as one of the therapeutic methods to control ovarian cancer.

REFERENCES

1. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. CA: a cancer journal for clinicians 2013;63(1):11-30.
2. Foley OW, Rauh-Hain JA, del Carmen MG. Recurrent epithelial ovarian cancer: an update on treatment. Oncology (Williston Park, NY) 2013;27(4):288-294, 298.
3. Viollet B, Guigas B, Sanz Garcia N, Leclerc J, Foretz M, Andreelli F. Cellular and molecular mechanisms of metformin: an overview. Clinical science 2012;122(6):253-270.
4. Pernicova I, Korbonits M. Metformin-mode of action and clinical implications for diabetes and cancer. Nature reviews Endocrinology 2014;10(3):143-156.
5. El-Mir MY, Nogueira V, Fontaine E, Averet N, Rigoulet M, Leverve X. Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I. The Journal of biological chemistry 2000;275(1):223-228.
6. Bodmer M, Becker C, Meier C, Jick SS, Meier CR. Use of metformin and the risk of ovarian cancer: a case-control analysis. Gynecologic oncology 2011;123(2):200-204.

7. Romero IL, McCormick A, McEwen KA et al. Relationship of type II diabetes and metformin use to ovarian cancer progression, survival, and chemosensitivity. Obstetrics and gynecology 2012;119(1):61-67.

8. Kumar S, Meuter A, Thapa P et al. Metformin intake is associated with better survival in ovarian cancer: a case-control study. Cancer 2013;119(3):555-562.

9. Gotlieb WH, Saumet J, Beauchamp MC et al. In vitro metformin anti-neoplastic activity in epithelial ovarian cancer. Gynecologic oncology 2008;110(2):246-250.

10. Rattan R, Giri S, Hartmann LC, Shridhar V. Metformin attenuates ovarian cancer cell growth in an AMP-kinase dispensable manner. Journal of cellular and molecular medicine 2011;15(1):166-178.

11. Yasmeen A, Beauchamp MC, Piura E, Segal E, Pollak M, Gotlieb WH. Induction of apoptosis by metformin in epithelial ovarian cancer: involvement of the Bcl-2 family proteins. Gynecologic oncology 2011;121(3):492-498.

12. Shank JJ, Yang K, Ghannam J et al. Metformin targets ovarian cancer stem cells in vitro and in vivo. Gynecologic oncology

2012;127(2):390-397.

13. Rattan R, Graham RP, Maguire JL, Giri S, Shridhar V. Metformin suppresses ovarian cancer growth and metastasis with enhancement of cisplatin cytotoxicity in vivo. Neoplasia 2011;13(5):483-491.

14. Chan DK, Miskimins WK. Metformin and phenethyl isothiocyanate combined treatment in vitro is cytotoxic to ovarian cancer cultures. Journal of ovarian research 2012;5(1):19.

15. Li C, Liu VW, Chan DW, Yao KM, Ngan HY. LY294002 and metformin cooperatively enhance the inhibition of growth and the induction of apoptosis of ovarian cancer cells. International journal of gynecological cancer : official journal of the International Gynecological Cancer Society 2012;22(1):15-22.

16. Maiuri MC, Zalckvar E, Kimchi A, Kroemer G. Self-eating and self-killing: crosstalk between autophagy and apoptosis. Nature reviews Molecular cell biology 2007;8(9):741-752.

17. Bjorkoy G, Lamark T, Brech A et al. p62/SQSTM1 forms protein aggregates degraded by autophagy and has a

protective effect on huntingtin-induced cell death. The Journal of cell biology 2005;171(4):603-614.

18. Ma Y, Hendershot LM. The role of the unfolded protein response in tumour development: friend or foe? Nature reviews Cancer 2004;4(12):966-977.

19. Hoyer-Hansen M, Jaattela M. Connecting endoplasmic reticulum stress to autophagy by unfolded protein response and calcium. Cell death and differentiation 2007;14(9):1576-1582.

20. Schonthal AH. Endoplasmic reticulum stress and autophagy as targets for cancer therapy. Cancer letters 2009;275(2):163-169.

21. Tomic T, Botton T, Cerezo M et al. Metformin inhibits melanoma development through autophagy and apoptosis mechanisms. Cell death & disease 2011;2:e199.

22. Axten JM, Medina JR, Feng Y et al. Discovery of 7-methyl-5-(1-{[3-(trifluoromethyl)phenyl]acetyl}-2,3-dihydro-1H-indol-5-yl)-7H-p yrrolo[2,3-d]pyrimidin-4-amine (GSK2606414), a potent and selective first-in-class inhibitor of protein kinase R (PKR)-like endoplasmic

reticulum kinase (PERK). Journal of medicinal chemistry 2012;55(16):7193-7207.

23. Rouschop KM, van den Beucken T, Dubois L et al. The unfolded protein response protects human tumor cells during hypoxia through regulation of the autophagy genes MAP1LC3B and ATG5. The Journal of clinical investigation 2010;120(1):127-141.

24. Maycotte P, Thorburn A. Autophagy and cancer therapy. Cancer biology & therapy 2011;11(2):127-137.

25. Zhuang Y, Miskimins WK. Metformin induces both caspase-dependent and poly(ADP-ribose) polymerase-dependent cell death in breast cancer cells. Molecular cancer research : MCR 2011;9(5):603-615.

26. Akinyeke T, Matsumura S, Wang X et al. Metformin targets c-MYC oncogene to prevent prostate cancer. Carcinogenesis 2013;34(12):2823-2832.

27. Segal ED, Yasmeen A, Beauchamp MC, Rosenblatt J, Pollak M, Gotlieb WH. Relevance of the OCT1 transporter to the antineoplastic effect of biguanides. Biochemical and biophysical research communications 2011;414(4):694-699.

28. Emami Riedmaier A, Fisel P, Nies AT, Schaeffeler E, Schwab M. Metformin and cancer: from the old medicine cabinet to pharmacological pitfalls and prospects. Trends in pharmacological sciences 2013;34(2):126-135.

29. Schaeffeler E, Hellerbrand C, Nies AT et al. DNA methylation is associated with downregulation of the organic cation transporter OCT1 (SLC22A1) in human hepatocellular carcinoma. Genome medicine 2011;3(12):82.

30. Feng Y, Ke C, Tang Q et al. Metformin promotes autophagy and apoptosis in esophageal squamous cell carcinoma by downregulating Stat3 signaling. Cell death & disease 2014;5:e1088.

31. Shi WY, Xiao D, Wang L et al. Therapeutic metformin/AMPK activation blocked lymphoma cell growth via inhibition of mTOR pathway and induction of autophagy. Cell death & disease 2012;3:e275.

32. Kouroku Y, Fujita E, Tanida I et al. ER stress (PERK/eIF2alpha phosphorylation) mediates the polyglutamine-induced LC3 conversion, an essential step for autophagy formation. Cell death and differentiation

2007;14(2):230-239.

33. Ogata M, Hino S, Saito A et al. Autophagy is activated for cell survival after endoplasmic reticulum stress. Molecular and cellular biology 2006;26(24):9220-9231.

34. Levine B. Cell biology: autophagy and cancer. Nature 2007;446(7137):745-747.

35. Ni M, Zhou H, Wey S, Baumeister P, Lee AS. Regulation of PERK signaling and leukemic cell survival by a novel cytosolic isoform of the UPR regulator GRP78/BiP. PloS one 2009;4(8):e6868.

36. Wu SH, Hang LW, Yang JS et al. Curcumin induces apoptosis in human non-small cell lung cancer NCI-H460 cells through ER stress and caspase cascade- and mitochondria-dependent pathways. Anticancer research 2010;30(6):2125-2133.

37. Xin J, Zhan Y, Liu M et al. ApoG2 induces ER stress-dependent apoptosis in gastric cancer cells in vitro and its real-time evaluation by bioluminescence imaging in vivo. Cancer letters 2013;336(2):260-269.

38. Chen S, Rehman SK, Zhang W, Wen A, Yao L, Zhang J.

Autophagy is a therapeutic target in anticancer drug resistance. Biochimica et biophysica acta 2010;1806(2):220-229.

39. Ranganathan AC, Zhang L, Adam AP, Aguirre-Ghiso JA. Functional coupling of p38-induced up-regulation of BiP and activation of RNA-dependent protein kinase-like endoplasmic reticulum kinase to drug resistance of dormant carcinoma cells. Cancer research 2006;66(3):1702-1711.

40. Yang ZJ, Chee CE, Huang S, Sinicrope FA. The role of autophagy in cancer: therapeutic implications. Molecular cancer therapeutics 2011;10(9):1533-1541.

41. Suh DH, Kim MK, Kim HS, Chung HH, Song YS. Unfolded protein response to autophagy as a promising druggable target for anticancer therapy. Annals of the New York Academy of Sciences 2012;1271:20-32.

42. Liu D, Yang Y, Liu Q, Wang J. Inhibition of autophagy by 3-MA potentiates cisplatin-induced apoptosis in esophageal squamous cell carcinoma cells. Medical oncology 2011;28(1):105-111.

43. Xi G, Hu X, Wu B et al. Autophagy inhibition promotes

paclitaxel-induced apoptosis in cancer cells. Cancer letters 2011;307(2):141-148.

44. Tang Q, Li G, Wei X et al. Resveratrol-induced apoptosis is enhanced by inhibition of autophagy in esophageal squamous cell carcinoma. Cancer letters 2013;336(2):325-337.

45. Ito H, Daido S, Kanzawa T, Kondo S, Kondo Y. Radiation-induced autophagy is associated with LC3 and its inhibition sensitizes malignant glioma cells. International journal of oncology 2005;26(5):1401-1410.

46. Kanematsu S, Uehara N, Miki H et al. Autophagy inhibition enhances sulforaphane-induced apoptosis in human breast cancer cells. Anticancer research 2010;30(9):3381-3390.

47. Cao X, Liu B, Cao W et al. Autophagy inhibition enhances apigenin-induced apoptosis in human breast cancer cells. Chinese journal of cancer research = Chung-kuo yen cheng yen chiu 2013;25(2):212-222.

국문 초록

난소암 세포주에서 Metformin에 의한 자가포식작용과 미접힘 단백질 반응의 기전 연구

Metformin은 제2형 당뇨병 치료제로써 난소암 세포주에서 항암효과를 나타내는 임상 연구 결과들이 발표되었다. Metformin은 기아상태와 같은 대사적 스트레스를 유발한다. 이에 따라 암 세포들의 생존을 위해 소포체(endoplasmic reticulum, ER) 스트레스에 의한 미접힘 단백질 반응(unfolded protein response, UPR)과 자가포식작용 (autophagy)이 활성화된다. 본 연구는 난소암 세포주에서 미접힘 단백질 반응 신호전달의 하나인 PERK/eIF2α 경로와 자가포식작용에 의해 metformin이 유도한 세포사멸 (apoptosis)이 경감된다는 것을 확인하였고, 정상 난소 세포와 말초혈 단핵세포에는 영향이 없음을 증명하였다. Metformin이 유도한 자가포식작용은 초기 단계 자식포 형성에 필요한 LC3B, ATG12–ATG5 의 발현량 증가와 후기 단계에 이루어지는 p62의 발현량 감소, 아크리딘 오렌지 염색 시약으로 산성화된 소포들의 확인을 통해 증명되었다. 흥미롭게도, metformin은 PERK/eIF2α의 경로와 자가포식작용 사이의 상호작용을 유도하였고, 이는 metformin의 세포사멸 능력을 억

제하였다. Metformin과 PERK 또는 자가포식작용의 저해제와의 병용처리는 정상 난소세포와 말초혈 단핵세포에는 영향이 없었으며, 난소암 세포주에서만 더 큰 항암효과가 나타났다. 결론적으로, 이러한 연구결과는 PERK와 자가포식작용의 억제를 통해 특이적으로 난소암 세포주에서 metformin의 항암효과를 증진시킬 수 있다고 제시한다.

주요어: 난소암, metformin, 세포사멸 (apoptosis), 자가포식작용 (autophagy), PERK

학 번: 2012-21767