Regulation of Hepatic Gluconeogenic Gene Expression by Resveratrol

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Directed by Professor Yong-Ho Ahn

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This certifies that the Master's Thesis

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‘I love you, O Lord, my strength’

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Resveratrol, a naturally occurring polyphenol found in grape and red wine, is known as an activator of silent information regulator 2 (Sir2), which is an NAD^+-dependent histone deacetylase. Sir2 induces longevity in *Saccharomyces cerevisiae* and *Caenorhabditis elegans* in response to caloric restriction. SIRT1, one of the seven mammalian homologues (sirtuins) of Sir2 family, is responsible for the regulation of diverse energy metabolism.

One of transcription factors regulating energy metabolism, the forkhead transcription factor (FOXO1), is known as a transcriptional activator of

genes involved in the gluconeogenesis. In the presence of insulin, FOXO1 is phosphorylated and exported from nucleus to cytosol, losing its ability to activate gene transcription.

This study is designed to explore the molecular mechanism of resveratol, an activator of SIRT1, with regard to insulin action on the transcriptional control of gluconeogenic genes. Resveratrol inhibited phosphorylation of PI3K/AKT and ERK 1/2. Sirtinol, an inhibitor of SIRT1, treatment restored resveratrol-induced inhibition of insulin response. Immunofluorescence microscopy revealed that FOXO1 is distributed in the nucleus by resveratrol treatment, even in the presence of insulin. Chromatin immunoprecipitation (ChIP) assay using nuclear extract showed that FOXO1 binding to the insulin responsive element (IRE) in the promoter region of gluconeogenic genes is increased by resveratrol treatment.

These results suggest that resveratrol upregulates the expression of gluconeogenic genes by either attenuating insulin signaling or increasing FOXO1 binding to the IRE.

Key words: Gluconeogenic genes, Resveratrol, Insulin, SIRT1, FOXO1

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I. INTRODUCTION

The incidence of type 2 diabetes mellitus (T2DM) is increasing throughout the world due to western style diet and sedentary lifestyle in modern life[1]. One of the characteristic features of T2DM is the increased hepatic glucose production caused by the increased gluconeogenesis[2, 3]. The rate-limiting steps of gluconeogenesis include those catalyzed by enzymes, like phosphoenolpyruvate carboxykinase (PEPCK), fructose-1, 6-bisphosphatase (F1, 6Bpase), and glucose-6-phosphatase (G6Pase)[4].

The gene expression of gluconeogenic enzymes is controlled by a variety of signaling pathways and transcription factors that respond to changes in nutrient availability. Glucagon increases mRNA level of PEPCK and G6Pase by activating cyclic AMP (cAMP) signaling[5-8]. The promoters of PEPCK and G6Pase contain insulin response element (IRE) which is regulated by glucocorticoid-stimulated transcription factors[9, 10]. In addition, insulin negatively regulates the transcription of gluconeogenic genes and inhibits cAMP- and glucocorticoid-stimulated gluconeogenesis through IRE in the promoter of gluconeogenic genes[11, 12].

Resveratrol (RSV; *trans*-3, 4′, 5-trihydroxystilbene) is a kind of natural polyphenol found in the skin of grapes, nuts and at the highest concentration in red wine. Recent studies implicated that resveratrol regulates protection of cells from lipid accumulation, chemoprevention, immunomodulation, anti-proliferation and promotion of differentiation[13-16]. Recently, resveratrol has been known to be an activator of silent information regulator 2 (Sir2), which is an NAD-dependent protein deacetylases[17, 18].

Sir2, which induces longevity in *Saccharomyces cerevisiae* and *Caenorhabditis elegans* in response to caloric restriction, has a role as a key regulator of lifespan. SIRT1 is one of the seven mammalian homologues (sirtuins) of sir2 family. SIRT1 has been known to function together with PGC-1α to regulate the transcription of gluconeogenesis in caloric restriction[19], and regulates insulin secretion by repressing directly the promoter of uncoupling protein (UCP) gene UCP2 and protein tyrosine phosphatase (PTP) 1B gene in pancreatic β-cells[20, 21]. Recently, it was

reported that SIRT1 promotes fat mobilization by repressing peroxisome proliferator-activated receptor gamma (PPARγ)[22]. Therefore, SIRT1 is known to regulate diverse cellular metabolism by regulating transcription factors.

FOXO1, a transcriptional factor which is responsible for controlling the gene expressions of diverse metabolic enzymes, is known to be regulated by SIRT1[23-27]. Furthermore, it is involved in the cell cycle regulation, stress response and longevity in *C. elegans*[28, 29]. FOXO1 is regulated by several modifications, such as ubiquitination, phosphorylation, GlcNacylation, and acetylation/deacetylation[30-37]. Of these, phosphorylation of FOXO1 by insulin is most well characterized. Once phosphorylated by insulin response, FOXO1 is extruded from nucleus to cytosol[38, 39]. In addition, FOXO1 itself is acetylated by p300/CBP, leading to reduction in the transcriptional activity. Molecular mechanisms of the reduced transcriptional activities are the altered DNA binding ability due to the acetyaltion of Lys-242, 245, 262 residues of FOXO1 by CBP/p300[36]. The positive charges conferred by these residues are shown to play a key role in the DNA binding, thus blocking of the lysine residue by acetylation hampers binding of FOXO1 to critical regions of genes. Moreover, it has been reported that acetylated FOXO1 increases the phosphorylation at Ser-256 by PI3-kinase/AKT pathway, causing FOXO1 being distributed in the cytosol[36]. Moreover, it has been reported that acetylated FOXO1 increases the phosphorylation at Ser-256 by PI3-kinase/AKT pathway, causing FOXO1 distributed in the cytosol[39].

In this study, effects of resveratrol on the gluconeogenic gene expression were studied with regard to FOXO1 binding to the IRE of gluconeogenic

genes and its subcellular distribution.

II. MATERIALS AND METHODS

Plasmids and materials

Human FOXO1 cDNA was cloned into the expression vector of myc-tagged pSG5. Expression vectors encoding mouse FOXO1 in myc-tagged pcDNA3 were kindly provided by Dr. Furuyama[40]. For transient expression of FOXO1, Lipofectamine-plus reagent (Invitrogen, Carlsbad, CA, USA) was used for transfection. All of the plasmid constructs were confirmed by DNA sequencing. Resveratrol, sirtinol, insulin, and dexamethasone were purchased from Sigma-Aldrich (St. Louis, MO. USA).

Animal and isolation of primary hepatocytes

The male Sprague–Dawley rats were purchased from Orient-Bio (Seongnam, Korea) and maintained on 12 hr dark/ 12 hr light cycles. The rats fed a chow diet (Samtako, Osan, Korea).

Primary hepatocytes were isolated from 200 g male Sprague–Dawley rats by the collagenase perfusion method[41]. Animals were anesthetized with Zoletil 50 (Virbac, Carros, France), and each liver was perfused *in situ* via the portal vein with 200 ml of Perfusion I medium (142 mM NaCl, 6.7 mM KCl, 10 mM HEPES/KOH pH 7.6, 2.5 mM EGTA). The medium was warmed to 37°C and infused at a rate of 15 ml/min. The liver was then

perfused with Perfusion II medium containing 66.7 mM NaCl, 6.7 mM KCl, 100 mM HEPES/KOH pH 7.6, 4.8 mM $CaCl_2$ supplemented with 50 mg of collagenase H (Roche, Mannheim, Germany) for 10 min at a flow rate of 15 ml/min. The liver was removed, the hepatic capsule was stripped, and the dissociated cells were dispersed by resuspension, followed by filtration through gauze into ice-cold DMEM (Dulbecco's modified Eagle's medium; Hyclone, South Logan, Utah, USA) and centrifuged at 50 *g* for 2 min. The pellets were washed twice with 40 ml of ice-cold DMEM and subjected to Percoll density-gradient centrifugation. The pellets, which were composed of viable hepatocytes, were suspended in modified DMEM supplemented with with 10% (v/v) FBS (fetal bovine serum; Hyclone), antibiotics (Hyclon), 10 nM insulin, 10 nM dexamethasone. Cell viability was measured by trypan blue exclusion. The cells were plated on 100-mm dishes in a humidified atmosphere containing 5% CO_2 at 37^{o}C.

Cell culture and transient transfection assay

Alexander cells (human hepatoma; American Type Culture Collection number CRL-8024) maintained in DMEM supplemented with 10% (v/v) FBS, 1% (v/v) antibiotics (Hyclone) and 100 μg/ml tyrosine (Serva, Heidelberg, Germany). HepG2 cells (human hepatocellular carcinoma; American Type Culture Collection number HB-8065) were maintained in DMEM supplemented with 10% (v/v) FBS and 1% (v/v) antibiotics (Hyclone) in a humidified atmosphere containing 5% CO_2 at 37^{o}C. Transient transfection

was performed using FuGENE HD following the manufacturer's protocol (Roche, Mannheim, Germany).

RNA extraction, reverse transcription, and real time PCR

Total RNA was extracted from rat primary hepatocytes, Alexander cells and HepG2 cells using TRIzol®Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Reverse transcription (RT)-PCR was performed with the ImProm-Ⅱ™ (Promega, Madison, WI, USA) using 1 μg RNA. Quantitative real time PCR was performed using ABI PRISM 7500 Sequence Detection System instrument and software (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol with a minor modification. Briefly, an appropriate amount of the reverse transcription reaction mixture was amplified with specific primers using SYBR green PCR master mix (Applied Biosystems) in a total volume of 20 μl. Data were processed by comparative CT method and expressed as fold increase relative to the basal transcription level. The amount of target mRNA was normalized by determining β-actin mRNA level. Oligonucleotides used in PCR are shown in Table 1.

Western blot analysis

The primary cultured hepatocytes were lysed by 1X Passive lysis buffer, (Promega) supplemented with protease and phosphatase inhibitors (10 mM

NaF, 10 mM Na_3VO_4, 0.1 mM PMSF, 5 μg/ml pepstatin, 5 μg/ml leupeptin and 5 μg/ml aprotinin). Supernatants were obtained by centrifugation at 12,000 *g* for 10 min. For Western-blot analysis, 45 μg of cell lysates were resuspended in the sample buffer and boiled for 5 min at 95^{O}C. Samples were separated using 8% SDS/PAGE and transferred to nitrocellulose membranes. (GE Healthcare, Buckinghamshire, UK) The membrane was incubated with antiserum against FOXO1 (H128), phospho-Ser^{253} FOXO1, HDAC1, (Santa Cruz Biotechnology, Delaware, CA, USA) Akt, phospho-Ser^{473}-Akt, p44/42 MAPK, phospho-Thr^{202}/Tyr^{204} p44/42 MAPK, (Cell Signaling, Denver, MA. USA) β–actin (Sigma) FOXOO1 (Calbiochem, Darmstadt, Germany) and then, detected by using enhanced chemiluminescence (ECL) substrate. (GE healthcare)

Preparation of nuclear extract

Nuclear extracts were prepared based on a previously described method with a minor modification[42]. The cells were lysed by buffer A containing 10 mM HEPES/KOH pH 7.6, 1.5 mM $MgCl_2$, 10 mM KCl, 5 mM EDTA, 5 mM EGTA, 250 mM sucrose, 10 mM Na_3VO_4, 10 mM NaF, 5 μg/ml pepstatin, 5 μg/ml leupeptin, 5 μg/ml aprotenin, 0.1 mM PMSF for 15 min on ice. After centrifugation, the resulting pellets were resuspended by 100 μl of buffer C containing 20 mM HEPES/KOH pH 7.6, 1.5 mM $MgCl_2$, 0.42 M NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 % glycerol, 10 mM Na_3VO_4, 10 mM NaF, 5 μg/ml pepstatin, 5 μg/ml leupeptin, 5 μg/ml aprotenin, 0.1 mM PMSF and incubated

at 4 ^{O}C for 1h with agitation. Nuclear extract was obtained by centrifugation at 55,000 rpm for 30 min.

Immunofluorescence microscopy

Alexander cells (1 X 10^5/well) were seeded in 12 well plates and transfected with the expression vector of myc-tagged FOXO1 by FuGENE HD (Roche). The cells were fixed with 3.7% formaldehyde in PBS for 20 min, washed twice with PBS. Cell membranes were permeabilized with 0.2% Triton X-100 in PBS for 20 min, washed twice with PBS. And then, cells were incubated with blocking buffer containing 1% bovine serum albumin in 0.1% PBST for 1 hr at room temperature. Cells were incubated with an anti-SIRT1 (Santa Cruz Biotechnology) and anti-myc (Cell Signaling) antibody containing 1% bovine serum albumin in 0.1% PBST for 12 hr at 4^{O}C and washed with PBST by incubating with Alexa Fluor 488-conjugated goat anti-mouse secondary antibody and Alexa Fluor 555-conjugated goat anti-rabbit secondary antibody (Invitrogen) for 1 hr under the dark. After washing with PBST, slides were mounted using Dakocytomation Fluorescent Mounting Medium (Dako, Glostrup, Denmark). Confocal microscopic images were taken on an LSM 510 META microscope (Zeiss, Jena, Germany) using 488 nm laser excitation for FITC (fluorescein isothiocyanate) and 568 nm laser excitation for rhodamine (Invitrogen). Hoechst (Invitrogen) staining was used to localize chromosomal DNA in the nucleus.

Chromatin immunoprecipitation (ChIP) Assay

The ChIP assay was performed based on a previously described method with a minor modification[43, 44]. Primary cultured hepatocytes were treated with increasing amount of resveratrol (50~100 μM) and sirtinol (50 μM) in the presence of insulin (100 nM) for 6 hr. Proteins were cross-linked to DNA by adding 1% formaldehyde directly to the culture medium for 10 min at 37^{O}C and washed twice with PBS. The cells were resuspended in 200 μl of SDS lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris-HCl, pH 8, 10 mM Na_3VO_4, 10 mM NaF, 5 μg/ml pepstatin, 5 μg/ml leupeptin, 5 μg/ml aprotenin, 0.1 mM PMSF). The lysate was sonicated on ice for 10 min with a 2.5 mm in diameter sonicator tip at 40% amplitude and a 0.5 cycle (UP400S; Dr. Hielscher GmbH, Teltow, Germany) and sheared to 200–1000 bp. To provide a positive control (1% input) for each condition, one undiluted aliquot was saved for further processing in parallel with all other samples at the reversal of the cross-linking step. To reduce nonspecific background, each chromatin sample (1 ml) was pre-cleared using 60 μl of salmon sperm DNA/protein A-agarose (Upstate, Lake placid, NY, USA) supplemented with 20 μl of protein A/G-agarose (Santa Cruz Biotechnology) for 3 hr. Chromatin complexes in the supernatnats were immunoprecipitated with 5 μg of pre-immune serum or a specific antibody for FOXO1 (Santa Cruz Biotechnology) for 12 hr at 4 ^{O}C with rotation. The complexes were collected with 70 μl of salmon sperm /protein A-agarose DNA (Upstate) and eluted from the beads and used for subsequent real time PCR analysis. Quantity of

immunoprecipitated DNA was normalized with 1% of the input. Oligonucleotides used in PCR are shown in Table 1.

Table 1. The sequences of the oligonucleotides used in this study.

Name		Species	Sequence (5' → 3')
PEPCK	For	H / M / R	TGGCCAGGATCGAAAGCAAGA
	Rev	H / M / R	GCCCCATGCTGAATGGGATG
G6Pase	For	R	GTGGGTCCTGGACACTGACT
	Rev	R	CAATGCCTGACAAGACTCCA
β-actin	For	H / M / R	CAACACCCCAGCCATGTACGT
	Rev	H / M / R	CAGCCAGGTCCAGACGCAGGA
rPEPCK_IRE	For	R	ATCCAGCAGACACCTAGTGGGG
	Rev	R	CAAGGACAGGGCTGGCCGGGAC
rG6Pase_IRE	For	R	GGAGGTCACCCCTTAGCACTGT
	Rev	R	ATATGCCCTGGATTCAGTCTGT
rLGK LXRE	For	R	TCTCATAGCCCTGTCCCTGTGG
	Rev	R	CTGCAGAATTCGCCCTTCTCTG

For, Forword; Rev, Reverse; H, human; M, mouse; R, rat

III. RESULTS

Resveratrol increases the mRNA levels of gluconeogenic genes.

Resveratrol is known to improve glucose homeostasis[15-18]. In order to know whether resveratrol affects the expression of gluconeogenic enzymes, rat primary hepatocytes were treated with resveratrol or sirtinol for 6 hr in the presence of insulin. As shown in Figure 1A and B, both PEPCK and G6Pase mRNA levels were decreased by insulin treatment. Treatment of resveratrol reversed the insulin-induced suppression of the gluconeogenic genes in a dose dependent manner. Furthermore, sirtinol counteracted the effect of resveratrol on the PEPCK as well as G6Pase gene expression. These effects were also observed in Alexander and HepG2 cells (data not shown). These results suggest that resveratrol has an opposing effect on the insulin action in the regulation of gluconeogenic gene expression. Also, up-regulation of gene expression of gluconeogenic enzymes by resveratrol is suppressed by sirtinol treatment.

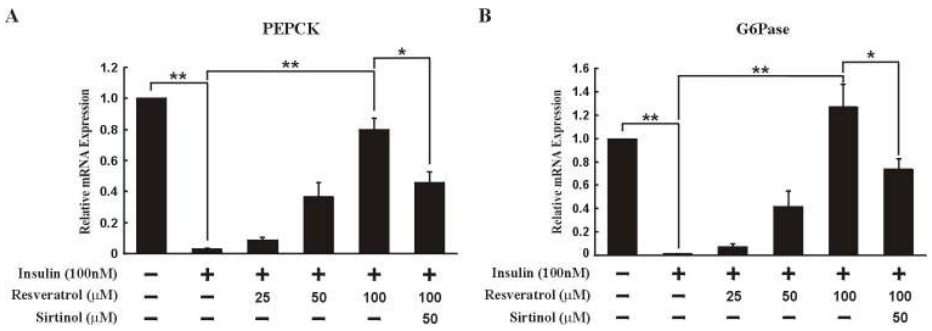

Figure 1. Resveratrol restored insulin-induced suppression of gene expression of gluconeogenic genes.

Primary cultured hepatocytes isolated from Sprague Dawley rats were incubated for 12 hr in serum-deprived DMEM which is supplemented with 200 nM dexamethasone. The cells were treated with increasing amount of resveratrol (25~100 μM) and sirtinol (50 μM) for 6 hr in the presence of insulin (100 nM). Total RNA was extracted using TRIzol® reagent as described in the Materials and Methods. Equal amount of RNA (1 μg) was used for reverse transcriptase reaction. The mRNA levels of PEPCK (A) or G6Pase (B) were measured by real time PCR. Quantity of the mRNAs was normalized with respect to β-actin mRNA. Data were processed by the comparative CT method and expressed as the fold increase relative to the basal transcription level in the absence of ligands. Data are shown as the means ± SES of nine independent experiments in triplicates. Statistical significance of differences between untreated and drug-treated hepatocytes was determined by independent-sample T test. *P<0.05, **P<0.005

Resveratrol decreases insulin-induced phosphorylation of Akt, ERK 1/2, FOXO1.

To explore the molecular role of resveratrol and sirtinol on the insulin signaling pathway, rat primary hepatocytes were pre-incubated with various concentrations of resveratrol or sirtinol for 30 min and then treated with insulin. As shown in Figure 2, phosphorylated Akt was increased by insulin and decreased by resveratrol in a dose dependent manner. Sirtinol restored the levels of phosphorylated Akt. Furthermore, insulin-mediated phosphorylation of ERK 1/2 (p44/42 MAPK) was also decreased by resveratrol.

One of the transcriptional factor responsible for the regulation of gluconegenic genes is FOXO1[45], and its transcriptional activity is known to be negatively regulated by both PI3K/Akt and ERK 1/2 signaling pathway[38, 39, 46]. The mechanism of insulin action on the suppression of gluconeogenic genes involves the phosphorylation of FOXO1, which enables shuttling of FOXO1 from nucleus to cytosol where FOXO1 is subjected to ubiquitination, destined to degradation[47]. These observations perhaps suggest that resveratrol may decrease insulin-induced PI3K/Akt and ERK 1/2 phosphorylation and sirtinol restored, indicating that resveratrol suppresses insulin signaling. The amount of phosphorylated form of FOXO1 parallels with that of PI3K/Akt and ERK 1/2. These results suggest that resveratrol decreases FOXO1 phophorylation by attenuating insulin response and then perhaps dephosphorylated FOXO1 could increase the expression of gluconeogenic genes.

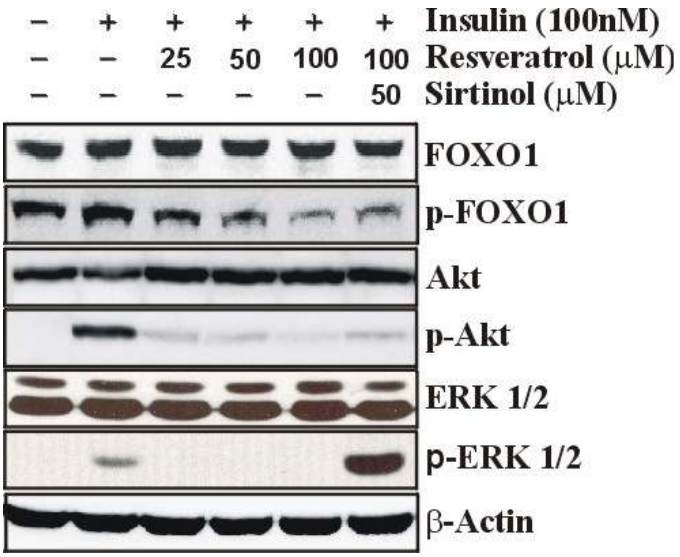

Figure 2. Resveratrol decreases phosphorylation of components of insulin signaling pathway.

Rat primary hepatocytes were cultured overnight in serum-deprived DMEM supplemented with 5.5 mM glucose and 200 nM dexamethasone. The cells were pre-treated with increasing amount of resveratrol (25~100 μM) and sirtinol (50 μM) for 30 min. And then, they were treated with insulin (100 nM) for another 10 min. The levels of phosphorylated forms of Akt, FOXO1, and ERK 1/2 were determined using Akt, phospho-Ser^{473}-Skt, p44/42 MAPK, phospho-Thr^{202}/Tyr^{204} p44/42 MAPK, FOXO1, phospho-Ser^{256} FOXO1 antibodies. β-actin was used as an internal control.

Resveratrol regulates the localization of FOXO1.

To test the effect of resveratrol on the subcellular localization of FOXO1, the expression vector for myc-tagged FOXO1 were transfected to Alexander cells, and then treated with insulin and resveratrol for 6 hr. As shown in Figure 3A, immunofluorescence microscopy showed that FOXO1 was located in the nucleus under the starvation state. But FOXO1 was excluded from nucleus to cytosol by insulin. Resveratrol induced the translocation of FOXO1 from cytosol to nucleus. FOXO1 is also located in the nucleus by treatment of resveratrol and insulin. In addition, FOXO1 is distributed in the nucleus by treatment of insulin and LY294002, known to PI3K inhibitor.

To further confirm the localization of FOXO1, Alexander cells were treated with insulin and resveratrol and FOXO1 localized in the nucleus was measured using western blot. As shown in Figure 3B, the protein level of FOXO1 in the nucleus was decreased by insulin treatment, whereas treatment of resveratrol to insulin treated group increased FOXO1 protein level in the nucleus. These results suggest that resveratrol attenuates insulin-induced phosphorylation of FOXO1 and then nuclear FOXO1 regulates the gluconeogenic genes.

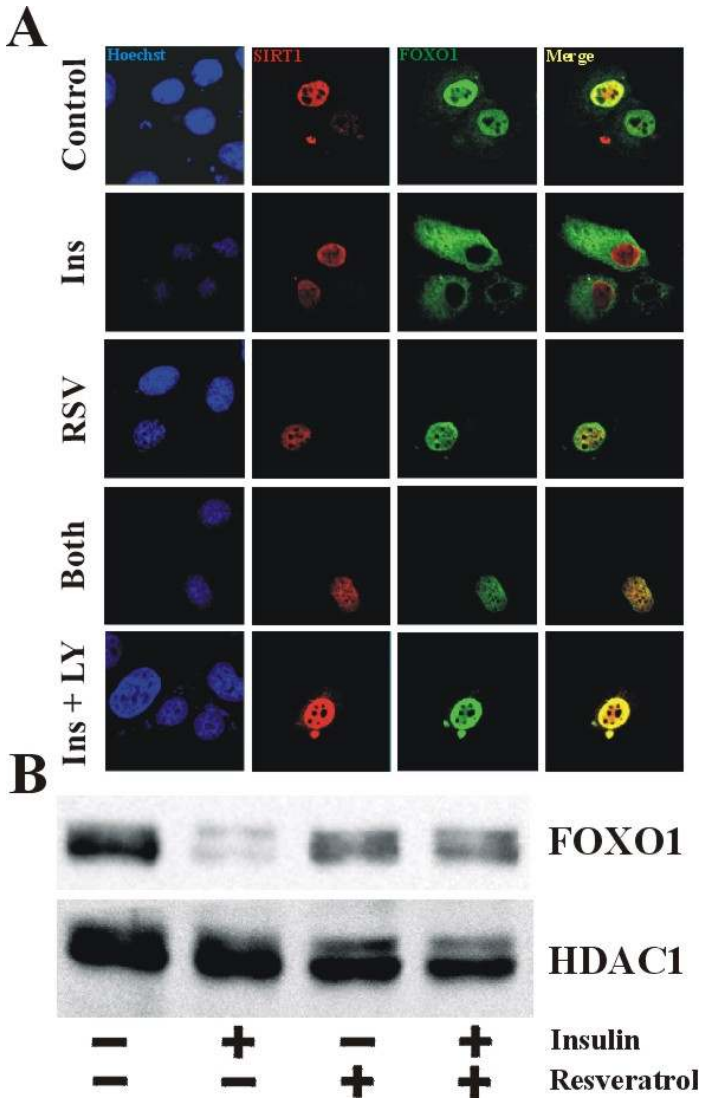

Figure 3. Resveratrol regulates FOXO1 localization.

(A) Subcellular localization of FOXO1. Alexander cells were transfected with the expression vector for myc-tagged FOXO1 (1 μg) and then the cells

were treated with resveratrol (100 μM) or insulin (100 nM) for 6 hr. Localization of endogenous SIRT1 (red fluorescence) and myc-tagged FOXO1 (green fluorescence) in the cells were observed with the immunofluorescence microscopy. LY294002 (LY, 20 μM), a PI3 Kinase inhibitor, was used as a positive control. Nucleus was identified by hoechst staining. (B) Western-blot of FOXO1. Nuclear extracts from resveratrol (100 μM) or insulin (100 nM) treated cells were prepared as described previously[42], and subjected to western bolt analysis using an anti-FOXO1antibody. The HDAC was used as an internal control.

Resveratrol increases the binding of FOXO1 to IRE in the promoters of gluconeogenic genes.

In order to know whether resveratrol regulates the promoter binding activity of FOXO1, resveratrol and sirtinol were treated to rat primary cultured hepatocytes for 6 hr and chromatin immunoprecipitation (ChIP) assay was performed. As shown in Figure 4, insulin decreased the binding activity of FOXO1 to the IRE of PEPCK and G6Pase gene whereas resveratrol treatment increased FOXO1 binding to the IRE, and the effect was reversed by sirtinol treatment. In addition, FOXO1 could not bind to LXRE of the liver type glucokinase promoter[48]. These results suggest that resveratrol plays a role in the translocation of FOXO1 from cytosol to nucleus through decreased phosphorylation of FOXO1.

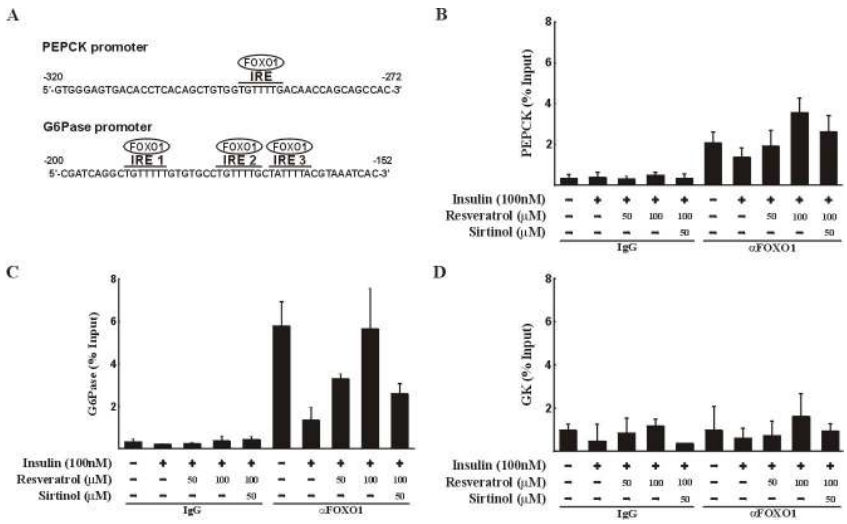

Figure 4. Resveratrol increases the binding FOXO1 to IRE of PEPCK and G6Pase promoter.

The rat PEPCK or G6Pase promoter contains the IRE binding sites (A). Primary cultured hepatocytes isolated from rats were incubated overnight in serum-deprived DMEM supplemented with 5.5 mM glucose and 200 nM dexamethasone. The cells were treated with resveratrol (50~100 μM) and sirtinol (50 μM) for 6 hr in the presence of insulin (100 nM) and then cross-linked using 1% formaldehyde. Chromatins were incubated with an anti-rabbit IgG or anti-FOXO1 antibody. The immuno-precipitated DNA fragments were amplified using sets of specific primers for the IRE binding site in the rat PEPCK (B) or G6Pase (C) promoter and for LXRE binding site in the rat GK (D) promoter by real time PCR. Quantity of immunoprecipitated DNA was normalized with 1% of the input.

IV. DISCUSSION

Resveratrol regulates a variety of metabolic pathways[13-15]. However detailed mechanism of how resveratrol affects diverse cellular metabolism is not well understood. In this study, effects of resveratrol on the expression of gluconeogenic genes were studied. Resveratrol reversed the insulin-induced suppression of gluconeogenic genes. In addition, resveratrol affected the insulin signaling pathway. These results showed that insulin induces phosphorylation of Akt and ERK 1/2, key components of insulin signaling, as well as FOXO1 in primary hepatocytes. In contrast, resveratrol decreased phosphorylation form of Akt, ERK 1/2, and FOXO1. FOXO1 is a transcriptional factor responsible for the up-regulation of gluconegenic genes[45], and its transcriptional activity has been known to be negatively regulated both by PI3K/AKt and by ERK 1/2 signaling[37-39]. It was reported that insulin response is inhibited by resveratrol in SIRT1-independent pathway[49]. However, in this study it was found that sirtinol counteracted the effect of resveratrol in terms of the gene expression of gluconeogenic enzymes. There is a possibility that SIRT1 may directly affect insulin signaling thereby increase gluconeogenesis through FOXO1 modification status. To observe the effect of resveratrol on the localization of FOXO1, immunofluorescence microscopy and immuno-blot were performed. These observations revealed that FOXO1 distributed in the nucleus by resveratrol and insulin excludes FOXO1 from nucleus to cytosol. In contrast, treatment

of insulin and resverarol induced the localization of FOXO1 in the nucleus. Also, FOXO1 and SIRT1 were colocalized in the nucleus by treatment of insulin and resveratrol. In addition, the effect of resveratrol on transcriptional binding activity of FOXO1 was observed using ChIP assay, which showed a decrease in the binding of FOXO1 to insulin response element (IRE) in the promoter regions of gluconeogenic genes by insulin. In contrast, resveratrol treatment increased the binding of FOXO1 to the IRE, and again, its effect was reversed by sirtinol treatment. These results suggest that resveratrol affected to the promoter of gluconeogenic genes though FOXO1. FOXO1 is shown to be acetylated by CBP/p300[36]. When FOXO1 was acetylated by CBP, the FOXO1-DNA interaction is destabilized and susceptible to PKB mediated phosphorylation, leading to decrease in transcriptional activities[36]. In contrast, FOXO1 which is deacetylated by SIRT1 is known to increase FOXO1-mediated gene transcription[46]. Thus, it could be speculated that FOXO1 deacetylated by resveratrol-activated SIRT1 may upregulate the expression of gluconeogenic genes. In addition, SIRT1 is known to regulate various transcription factors by deacetylation and direct interaction, such as PPARα targets and PGC-1α which are responsible for the increase of the gluconeogenesis[19, 50, 51]. However, further studies are needed to clarify the detailed mechanism of how resveratrol affects the gene expression of gluconeogenic enzymes in terms of posttranslational modification of FOXO1. This study showed that resveratrol reversed insulin-inhibition of the gluconeogenic gene expressions by increasing the bindong of FOXO1 to the IRE in the promoters of

gluconeogenic genes.

V. CONCLUSION

This study was demonstrated to know how resveratrol affects the expression of gluconeogenic enzymes. Resveratrol decreased phosphorylation of Akt and ERK 1/2 and reversed insulin-induced repression of the gluconeogenic genes. Resveratrol induced the nuclear distribution of FOXO1 and increased binding activity of FOXO1 to IRE in the promoters of gluconeogenic genes. These results may suggest that resveratrol regulates gluconeogenic genes.

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ABSTRACT (IN KOREAN)

Resveratrol에 의한 당신생합성 유전자의 발현 조절

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박 주 만

포도나 적포도주에서 천연적으로 발생되는 polyphenol 물질인 Resveratrol 은 NAD^+-dependent histone deacetylase 인 silent information regulator 2 (Sir2) 의 activator 로 알려져 있다. Sir2는 금식 시 *Saccharomyces cerevisiae*나 *Caenorhabditis elegans* 의 생명을 연장시킨다. 수명에 중요한 조절자로 알려진 Sir2 family 의 일곱 개의 포유동물의 homologues 중 하나인 SIRT1 은 다양한 물질대사의 전사조절 인자와 경로를 조절한다고 알려져 있다.

다양한 물질대사의 전사조절 인자 가운데 하나인 FOXO1 은 당신생합성 유전자들의 activator 로 알려져 있다. 인슐린이 있는 상황

에서 FOXO1 은 인산화되어 핵에서 세포질로 배출된다. 세포질 밖으로 배출되면 전사조절 인자의 활성 능력을 잃어버린다.

본 연구에서 당신생합성 유전자를 조절하는 인슐린의 action 이 있음에도 불구하고 SIRT1 의 activator로 알려진 resveratrol 의 분자적 mechanism 을 알기 위해서 연구하였다. Resveratrol 은 PI3K/AKT 와 ERK 1/2 의 인산화를 억제하였다. SIRT1 의 inhibitor 로 알려진 sirtinol 을 처리하였을 때 resveratrol 에 의해서 억제되었던 인슐린 반응이 회복되었다. 면역 현광 현미경 방법으로 resveratrol 이 처리된 것에서 FOXO1 이 핵 내에 존재한다는 것을 나타내었다. 또한 인슐린이 같이 처리된 것에서도 핵 내에 존재하는 것을 확인하였다. Nuclear extract 를 사용하여 Chromatin immunoprecipitation (Chip) assay 실험하여 FOXO1 이 당신생합성 유전자의 promoter 의 insulin responsive element (IRE) 결합하는 것이 resveratrol 을 처리하였을 때 증가가 되었다. 이것을 요약하면 이것들의 결과들은 resveratrol 에 의해서 당신생합성 유전자들을 조절한다고 제시 할 수 있을 것이다.

핵식되는 말: 당신생합성, resveratrol, 인슐린, SIRT1, FOXO1